

World Journal of *Gastroenterology*

World J Gastroenterol 2011 January 7; 17(1): 1-136





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2010-2013

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ABOUT COVER Neumann H, Neurath MF, Mudter J. New endoscopic approaches in IBD.
World J Gastroenterol 2011; 17(1): 63-68
<http://www.wjgnet.com/1007-9327/full/v17/i1/63.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
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NAME OF JOURNAL

World Journal of Gastroenterology

LAUNCH DATE

October 1, 1995

RESPONSIBLE INSTITUTION

Department of Science and Technology of Shanxi Province

SPONSOR

Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

PUBLISHING

Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

SUBSCRIPTION

Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION

RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION

One-Year Price 864.00 USD

PUBLICATION DATE

January 7, 2011

CSSN

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

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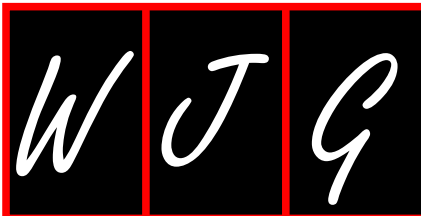
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Peroral cholangioscopy in the new millennium

Mansour A Parsi

Mansour A Parsi, Center for Endoscopy and Pancreatobiliary Disorders, Department of Gastroenterology and Hepatology, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Author contributions: Parsi MA contributed entirely to this manuscript.

Correspondence to: Mansour A Parsi, MD, Center for Endoscopy and Pancreatobiliary Disorders, Department of Gastroenterology and Hepatology, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Desk A31, Cleveland, OH 44195, United States. parsim@ccf.org

Telephone: +1-216-4454880 **Fax:** +1-216-4446284

Received: July 15, 2010 **Revised:** August 27, 2010

Accepted: September 4, 2010

Published online: January 7, 2011

Abstract

Peroral cholangioscopy was first described in 1970s and has recently gained popularity. Peroral cholangioscopy is appealing to therapeutic endoscopists because a direct intraluminal view of the biliary duct system offers possibilities for diagnosis and interventions beyond that which other imaging or endoscopic modalities can provide. As the image quality of cholangioscopies improves, so too does their diagnostic capability, and as their durability and maneuverability increases, so too does their potential use for therapeutic applications. This editorial is intended to provide a brief review of recent developments in peroral cholangioscopy and current indications for its use.

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Key words: Endoscopic retrograde cholangiopancreatography; Cholangioscopy; Peroral cholangioscopy; Cholangiocarcinoma; Biliary stricture; Pancreatic cancer; Biopsy; Brush cytology

Peer reviewer: Richard A Kozarek, MD, Executive Director, Digestive Disease Institute, Virginia Mason Medical Center 1100 Ninth Avenue, PO Box 900, Seattle, WA 98111-0900, United States

Parsi MA. Peroral cholangioscopy in the new millennium. *World J Gastroenterol* 2011; 17(1): 1-6 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/1.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.1>

INTRODUCTION

Diseases of the biliary system are frequently encountered in clinical practice^[1]. An examination of the bile ducts is often required for the appropriate diagnosis and management of patients with biliary diseases. The dramatic technical advances of flexible endoscopy during the last four decades have resulted in endoscopic retrograde cholangiopancreatography (ERCP) being used as a primary method of diagnosing and treating many biliary diseases^[1]. In the United States alone, approximately half a million ERCP procedures are performed annually. ERCP can demonstrate the anatomy of the biliary tract and reveal anatomical abnormalities, strictures and intraductal filling defects. However, this technique does not always differentiate the biological nature of bile duct lesions and can fail to determine their intraluminal extension. Furthermore, it is unable to provide information about biliary mucosal lesions that do not project into the biliary lumen. Peroral cholangioscopy as an adjunct to ERCP is a promising procedure that provides direct visualization of the biliary tree. It has been shown to have value in treating difficult-to-remove biliary stones^[2], assessing indeterminate biliary strictures^[3], and distinguishing between different intraductal lesions of the biliary tree^[4]. In recent years, cholangioscopy has gained popularity in the United States and is being performed in increasing numbers not only in academic institutions and large tertiary care referral centers, but also in smaller hospitals and private practices. In this paper, clinical applications of peroral cholangioscopy and its role in diagnosis and management of biliary disorders are reviewed.

HISTORICAL PERSPECTIVES

The first peroral cholangioscopy was performed in 1975

Table 1 Comparison of currently available single and dual operator cholangioscopies

	Endoscopists needed	Image quality	Tip deflection	Simultaneous irrigation and instrumentation	Fragility
Single operator (SpyGlass)	One	Moderate - good	4 way (up/down, left/right)	Yes	No ¹
Dual operator					
Fiberoptic cholangioscopies	Two	Moderate - good	2 way (up/down)	No	Yes
Video cholangioscopies	Two	Excellent	2 way (up/down)	No	Yes

¹All components of the spyglass system are single-use with the exception of the spyprobe (the light and image conveyor of the system) which is multi-use. The spyprobe is fragile and has to be handled with care.



Figure 1 Cholangioscopic view of normal intrahepatic biliary mucosa (image by a prototype video cholangioscope, CHF type Y0002, Olympus Corporation, Tokyo, Japan).

using a prototype cholangioscope that was thin enough to pass through the accessory channel of a duodenoscope^[5]. The concept of passing a thinner endoscope through a larger one later became known as the “mother-baby” or “mother-daughter” concept. Even today, almost all cholangioscopy systems are based on this concept. The initial prototype cholangioscope had poor image quality, no instrumentation or irrigation capability, and no tip deflection. Despite all its shortcomings, it proved that peroral cholangioscopy is feasible. In the mid-1980s second generation cholangioscopes were introduced^[5]. These cholangioscopes had added tip deflection and an accessory channel that could be used either for irrigation or instrumentation. In the late 1990s and early in the new millennium, advances in imaging technology led to the introduction of video cholangioscopies with improved image quality that enabled satisfactory views of the biliary mucosa (Figure 1). Addition of narrow band imaging (NBI) capability led to further improvements in detection of abnormal vascularization of biliary mucosa, which is of importance for diagnosis of certain biliary malignancies^[6]. The first semi-disposable single-operator cholangioscopy system was developed in 2005 and made it possible for a single endoscopist to operate both the baby and mother endoscopes.

SINGLE AND DUAL OPERATOR CHOLANGIOSCOPY SYSTEMS

In cholangioscopy, the terms “single operator” and “dual

operator” refer to the number of endoscopists required to perform the procedure. As a general rule, dual operator cholangioscopy systems require two endoscopists, while single operator cholangioscopy systems require only one endoscopist for performance. There are, however, reports of use of dual operator cholangioscopy systems by a single operator with the help of appropriate accessory equipment^[7].

Currently, most cholangioscopy systems are dual operator. Dual operator cholangioscopies of varying length, diameter and image quality are available. Most dual operator cholangioscopies have fiberoptic image quality. There is limited commercial availability of video cholangioscopies with enhanced image quality. At present, all video cholangioscopies with NBI capability are prototypes and not commercially available.

The only single operator cholangioscopy system currently available is the SpyGlass direct visualization system (Boston Scientific, Natick, MA, USA). This system is fiberoptic-based and has single and multi-use components.

Some of the advantages and disadvantages of the currently available single and dual operator cholangioscopies are summarized in Table 1.

CLINICAL APPLICATIONS

Several clinical applications for peroral cholangioscopy have been described. With expanded use, additional indications are expected to be reported. Clinical applications of cholangioscopy can be divided into common, uncommon and rare applications. Common applications include stone therapy and diagnosis of indeterminate biliary strictures. Uncommon applications include guidewire placement during ERCP, assessment of post-liver-transplantation biliary strictures, and evaluation of indeterminate intraductal filling defects or irregularities of the bile duct wall seen on imaging studies such as computed tomography (CT), magnetic resonance imaging (MRI), endoscopic ultrasound (EUS) or ERCP. Rare applications include staging and ablation of biliary neoplasms, investigation of recurrent pancreatitis, and evaluation of hemobilia.

Common applications

Currently, most peroral cholangioscopy procedures are performed for two indications: biliary stones and indeterminate biliary strictures.

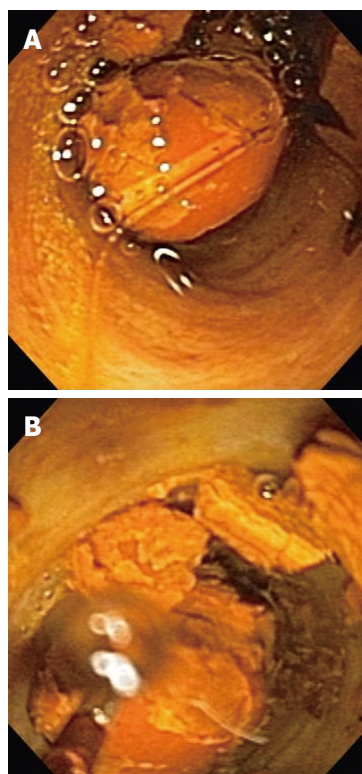
Table 2 Common factors associated with failed biliary stone removal during endoscopic retrograde cholangiopancreatography

Patient factors
Abnormal anatomy
Prior surgery
Extremely J-shaped stomach
Large hernias
Malrotations
Unstable or difficult endoscope position
Short duodenal bulb
Abnormal anatomy
Long duodenoscope position
Bile duct abnormalities
Presence of ductal strictures
Severely dilated ducts
Stone factors
Size
Large size
Location
Intrahepatic
Cystic duct
Proximal to strictures
Impacted stones

Biliary stones

Difficult to remove stones: Gallstone disease or cholelithiasis continues to be a major health problem throughout the world, and affects 10%-20% of the Caucasian population^[8-13]. It has been estimated that 15%-20% of patients with gallstone disease also have stones in their bile ducts (choledocholithiasis)^[13]. Stones in the bile ducts have to be removed because of their potential to cause jaundice, cholangitis, and pancreatitis^[14-16]. This is accomplished in close to 95% of the cases during ERCP by conventional methods such as sphincterotomy with or without sphincter dilatation, use of extraction balloons or retrieval baskets, mechanical lithotripsy, or a combination of these methods^[17]. At times, however, stone extraction by standard methods is not possible. There are a number of reasons as to why some stones cannot be removed by conventional means; some of the most common of which are presented in Table 2.

A variety of methods have been devised for endoscopic extraction of stones that are not removable by conventional means during ERCP. As a general rule, these methods involve using shock waves to crush or fragment the stones inside the bile duct, with subsequent removal of the fragments (Figure 2). The shock waves for fragmentation of biliary stones are usually generated using electric spark (electrohydraulic lithotripsy) or laser light (laser lithotripsy). Probes that pass through the accessory channels of cholangioscopes for laser or electrohydraulic lithotripsy are commercially available. Although use of these probes through an extraction balloon under fluoroscopic guidance has been reported^[18,19], in our institution, we use them under direct visualization by utilizing a cholangioscope. These probes have to be precisely positioned on the stone to increase effectiveness and reduce complications. Direct visualization ensures that the shock waves

**Figure 2** Cholangioscopic views of a bile duct stone prior to (A) and after (B) electrohydraulic lithotripsy. The lithotripsy probe is visible in the left lower corner of (B).

are aimed at the stone and not the bile duct wall, because shock waves delivered to the bile duct wall can cause bleeding and perforation. Direct visualization by cholangioscopy also allows distinction between stone fragments, air bubbles or blood clots, which can be indistinguishable on contrast cholangiography^[20].

Laser or electrohydraulic lithotripsy has been used for fragmentation and subsequent extraction of difficult to remove stones for many years, and both techniques have been shown to be safe and effective^[21,22]. In a recent multicenter study, cholangioscopy-guided laser or electrohydraulic lithotripsy were effective in > 90% of the cases^[2].

There are currently no randomized studies that have compared the effectiveness of laser and electrohydraulic lithotripsy for fragmentation and subsequent extraction of difficult-to-remove biliary stones.

Missed stones: Cholangioscopy allows detection of stones that might have been missed during cholangiography. Small stones can be “drowned” in contrast and be missed, and larger stones can block a duct, thus preventing passage of contrast, and evade detection during ERCP (Figure 3). In a study of patients with primary sclerosing cholangitis, stones were not detectable on cholangiography in seven of 23 patients (30%)^[23]. In a more recent multicenter study, stones were missed in 29% of patients who presented for ERCP for different indications. In that study, ERCP was immediately followed by peroral cholangioscopy, which led to detection of the stones^[24].

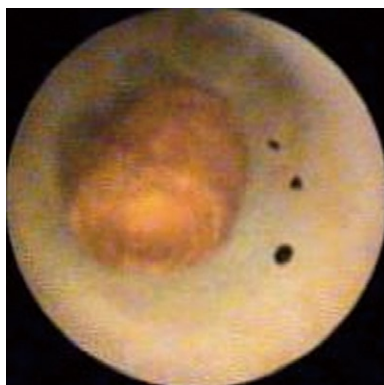


Figure 3 Cholangioscopic view of a small stone surrounded by contrast in an intrahepatic duct. The stone was missed during endoscopic retrograde cholangiopancreatography (image by Spyglass Direct Visualization System, Boston Scientific, Natick, USA).

Indeterminate biliary strictures

Biliary strictures can be benign or malignant. Accurate diagnosis of biliary strictures is essential for treatment planning and the correct choice of treatment, such as surgical resection or endoscopic stenting. However, differentiation of malignant from benign ductal lesions remains a challenge^[25]. Brush cytology during ERCP or fine needle aspiration by EUS has become the preferred initial method of pursuing a diagnosis in many patients with pancreatobiliary malignancies^[25,27]. These techniques allow easy and convenient sampling and have a low complication rate^[25,27,28]. The diagnostic specificity of biliary brush cytology or fine needle aspiration is very high and few false-positive diagnoses have been reported^[25,29]. The major limitation of these techniques has been the relatively modest diagnostic sensitivity, ranging from 10% to 50% in most series^[25,29].

There have been attempts to improve the sensitivity of brush cytology obtained during ERCP. Physical changes to the brushing device itself, such as use of longer and stiffer brushes, have not been shown to improve sensitivity^[30]. Balloon dilatation of strictures, to expose underlying tissue, prior to obtaining brush samples has been tried but not shown to be of any benefit^[31]. Mutation analysis of the cells obtained by brushing does not seem to improve diagnostic accuracy^[32], and DNA methylation analysis of ERCP brush specimens has shown only small benefit^[25].

It has been suggested that peroral cholangioscopy can improve diagnosis of indeterminate biliary strictures by visualization of the mucosa at the site of the stricture, and by targeted biopsy.

Visualization of the mucosa at the site of the stricture: It is well known that the presence of irregularly dilated and tortuous blood vessels (so-called tumor vessels) due to neovascularization at the site of pancreatic or biliary strictures is indicative of malignancy^[33]. Tumor vessels can be detected by direct visualization using a cholangioscope. Intraductal nodules or masses can also be indicative of malignancy and be easily detected by cholangioscopy. However, tumor vessels and intraductal masses can be ap-

preciated only in a fraction of malignant strictures; probably those with more advanced disease. Certain types of cholangiocarcinoma involve submucosal layers of the bile duct wall and cannot be detected by cholangioscopy, which visualizes the superficial layers. Biliary strictures caused by extraluminal compression, such as those associated with pancreatic cancer, cannot be detected by cholangioscopy, unless at later stages when the tumor has infiltrated and penetrated the bile duct wall.

Studies to assess the value of stricture visualization by cholangioscopy have reported high sensitivity for detection of malignant lesions^[4,34]. The reported sensitivity in some of these studies has approached 100%^[4]. In these studies, however, the criteria used for labeling a stricture as malignant have been somewhat lax. As an example, irregular biliary mucosa has been used to label a stricture as malignant. It is well known that irregular biliary mucosa on cholangioscopy can also be seen in benign lesions such as primary sclerosing cholangitis, or chronic inflammation associated with choledocholithiasis or recurrent cholangitis^[35]. Therefore, the high sensitivity in such studies is often achieved at the cost of lower specificity. This is alarming, because false-positive results can have a devastating impact on the affected patients' lives.

Although, undoubtedly, direct visualization of indeterminate biliary strictures can aid in their diagnosis, the true value of peroral cholangioscopy for this purpose has not been vigorously studied.

Targeted biopsy: Targeted biopsy is defined as biopsy of the sites that are clearly affected by disease under direct visualization. Theoretically, targeted biopsy should improve cancer detection rate in malignant biliary strictures by allowing sampling of the sites that appear suspicious. In a recent multicenter study that assessed the role of cholangioscopy-guided targeted biopsy for diagnosis of indeterminate biliary strictures, initial observations suggested a large improvement in sensitivity^[3]. However, later observations at conclusion of the study have indicated a somewhat more modest benefit^[24]. Well-designed studies are needed to assess better the value of cholangioscopy-guided targeted biopsy for evaluation of indeterminate biliary strictures.

Uncommon applications: In our institution, 10%-20% of peroral cholangioscopy procedures are performed for indications other than stone disease and stricture diagnosis. Some of these indications are discussed below.

Characterization of indeterminate intraductal lesions or filling defects

Increased use of imaging studies such as CT, MRI and EUS has led to an increase in incidental findings such as intraductal biliary lesions or filling defects. Although, most often these findings are real, they can also be due to artifacts.

Direct visualization of the intraluminal biliary tree is the most appropriate way to investigate further the nature of these findings. Cholangioscopy has been shown to be effective for this purpose^[4,36].

Assessing post-liver-transplantation anastomotic strictures

Various refinements in surgical techniques and postoperative and immunosuppressive management have reduced the incidence of complications after liver transplantation. Biliary complications, however, continue to be a significant cause of morbidity after liver transplantation^[37,38].

In selected cases, cholangioscopy can prove beneficial in diagnosis and treatment of biliary complications after liver transplantation. In a study of 20 liver transplant patients, cholangioscopy helped diagnose ischemia, ulcerations, scar tissue, intraductal clots, and retained suture material, which otherwise might have been missed by ERCP alone^[39]. The role of cholangioscopy in assessment of anastomotic strictures after liver transplantation is evolving.

Assistance in guidewire placement

ERCP has attained a primary role in the treatment of biliary strictures and biliary stones. Success of ERCP in these cases, however, depends on the ability to traverse the stricture or the stone with a guidewire that is then used to direct instruments such as dilating balloons or lithotripsy baskets^[40]. In the vast majority of cases, this is accomplished easily. With severe strictures or impacted stones, however, it can represent a time-consuming challenge, and in some studies, a failure rate of up to 20% has been reported^[41]. In such cases, cholangioscopy can facilitate guidewire placement and prevent more invasive procedures such as transhepatic access or surgery. Several studies have highlighted the value of cholangioscopy in such instances^[40,42].

Rare applications: We define rare applications as those responsible for $\leq 1\%$ of our peroral cholangioscopy volume. For obvious reasons, these indications have been reported only in one or two case reports and no studies have assessed the true value of peroral cholangioscopy in these settings.

Evaluation of recurrent pancreatitis

Peroral cholangioscopy was used in a 62-year-old post-cholecystectomy patient with recurrent acute pancreatitis of undetermined etiology. It revealed a T-tube remnant in the cystic duct stump, which served as a nidus for biliary sludge and stone formation. The T-tube remnant had evaded detection by ERCP, CT and magnetic resonance cholangiopancreatography. Removal of the T-tube remnant prevented further episodes of pancreatitis^[43].

Determination of source of bleeding in hemobilia

A 54-year-old man was reported to have bleeding from arteriovenous malformations of the bile duct, which was detected by peroral cholangioscopy, with subsequent successful treatment by endovascular intervention^[44]. In another study, the cause of hemobilia in a 57-year-old man could not be identified by ERCP, CT or angiography. Peroral cholangioscopy revealed multiple biliary ulcers. Biopsies were consistent with cytomegalovirus cholangiopathy that responded to antiviral therapy, with subsequent cessation of bleeding^[45].

Staging and ablation of biliary neoplasms

Peroral cholangioscopy was used in a 78-year-old man to determine the extent of a biliary neoplasm. Use of a video cholangioscope with NBI capability allowed precise determination of the margins of the lesion. Successful ablation of the neoplasm with brachytherapy was confirmed by repeat peroral cholangioscopy at 1 mo follow-up^[46].

CONCLUSION

Recent advances such as introduction of a single operator cholangioscopy system or video cholangioscopies with high image quality have led to renewed interest in cholangioscopy, with subsequent expanded use. Currently, the most common indications for cholangioscopy are stone therapy and evaluation of indeterminate biliary strictures. Several other clinical applications have been described. As this technology is gaining more popularity and use, other indications are certain to be described.

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S- Editor Sun H L- Editor Kerr C E- Editor Lin YP

Imaging techniques used for the real-time assessment of angiogenesis in digestive cancers

Adrian Săftoiu, Peter Vilmann

Adrian Săftoiu, Research Center of Gastroenterology and Hepatology, Gastroenterology Department, University of Medicine and Pharmacy Craiova, Craiova, Dolj, 200349, Romania
 Peter Vilmann, Department of Surgical Gastroenterology, Copenhagen University Hospital Herlev, Endoscopic Unit at Gentofte Hospital, 2900 Hellerup, Denmark

Author contributions: Săftoiu A and Vilmann P contributed equally to this paper.

Correspondence to: Adrian Săftoiu, Professor, MD, PhD, MSc, Research Center of Gastroenterology and Hepatology, Gastroenterology Department, University of Medicine and Pharmacy Craiova, 2-4 Petru Rares, Craiova, Dolj, 200349, Romania. adrian.saftoiu@webmail.umfcv.ro

Telephone: +40-744-823355 Fax: +40-251-310287

Received: July 2, 2010 Revised: August 29, 2010

Accepted: October 6, 2010

Published online: January 7, 2011

Peer reviewer: Tsuneo Kitamura, Associate Professor, Department of Gastroenterology, Juntendo University Urayasu Hospital, Juntendo University School of Medicine, 2-1-1 Tomioka, Urayasu-shi, Chiba 279-0021, Japan

Săftoiu A, Vilmann P. Imaging techniques used for the real-time assessment of angiogenesis in digestive cancers. *World J Gastroenterol* 2011; 17(1): 7-8 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/7.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.7>

FROM THE EDITOR

Angiogenesis plays a critical role in tumor growth and metastasis. Most of the digestive cancers depend strictly on the development of an adequate blood supply in the form of neovascularization, which has a pivotal role in primary tumor growth and the development of metastasis. Thus, new blood vessels formed inside the tumor are usually highly permeable and provide a route for cancer cells to enter blood circulation^[1]. Tumor vascularization has been attracting a lot of attention in recent years due to possible implications in semi-quantitative diagnosis, as well as in prognosis stratification and targeted treatment^[2]. There are several ways traditionally proposed for investigation of tumor vascularization, but all these methods have several methodological flaws, including artifacts induced by air or fat [contrast-enhanced transabdominal ultrasound (US)], reduced resolution [contrast-enhanced computer tomography (CT)] or invasiveness (angiography or surgery)^[3].

Categorizing the patients through mini-invasive procedures, before chemotherapy or surgery, is of crucial importance. This has implications for the appropriate design of clinical trials, but also for the improvement of the decision making process, by selecting the tumors that are most likely to respond to treatment. Recently developed angiogenesis inhibitors represent a highly attractive target for tumor therapy, since they theoretically offer the hope of long-term control of tumor progression^[4]. Several anti-angiogenic inhibitors were recently developed

Abstract

Angiogenesis has a critical role in primary tumor growth and the development of metastases. Several angiogenesis inhibitors were recently developed, being a very attractive target for digestive tumor therapy. However, individualized therapy should not only be based on the pre-treatment imaging evaluation, but also on sensitive monitoring of microvascular changes during treatment. State-of-the-art imaging techniques have the potential to visualize and characterize angiogenesis, although the technology and methodologies employed are recent and need further validation. The aim of this series of reviews was to analyze and enhance current knowledge and future perspectives about the real-time assessment of angiogenesis in digestive cancers, used for the longitudinal monitoring of the effects of chemo-radiotherapy (including anti-angiogenic therapies), as well as for the precise targeting of drugs through molecular-based drug-delivery systems.

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Key words: Angiogenesis; Digestive cancers; Chemo-radiotherapy

and have already been proven to be effective in clinical trials: bevacizumab (recombinant humanized anti-VEGF monoclonal antibody), cetuximab (anti-EGFR monoclonal antibody), erlotinib (tyrosine-kinase inhibitor), *etc*^[5]. Even though anti-angiogenic treatments are an established anti-cancer therapy, several mechanisms of tumor evasion and refractoriness have been described after inhibition of a single pro-angiogenic pathway, due to compensatory upregulation of different angiogenic pathways^[6]. Tailoring the anti-angiogenesis therapy as a function of the pre-operative imaging evaluation would be the next step, with a consequent decrease of toxicity, as well as an increase of median progression-free survival. Furthermore, these procedures would possibly allow a sensitive monitoring of microvascular changes caused by chemoradiotherapy or other ablative treatments. The ultimate goal would be to achieve an early diagnosis where the “angiogenic switch” could be delayed and tumor evasion mechanisms could be prevented, in order to induce a dormant state, while transforming cancer into a chronic disease^[7].

The main objectives of our series of reviews include the discussion of several advanced imaging techniques, complementary used for the real-time assessment of angiogenesis in digestive cancers. The advanced imaging techniques discussed will include several “red-flag” endoscopic techniques used for the depiction of minute changes in the vessel pattern of preneoplastic and neoplastic lesions (tri-modal imaging including autofluorescence imaging, zoom endoscopy and narrow band imaging)^[8]. Utility of Doppler-optical coherence tomography (D-OCT) for the depiction and quantification of low-velocity, low-volume blood flow will also be reviewed^[9]. These techniques will be supplemented by the presentation of confocal laser endomicroscopy and the potential applications of this breakthrough technique for the in-vivo assessment of vascularization based on the use of specific vascular contrast agents^[10]. Targeted contrast-enhanced ultrasound and its role in the depiction of angiogenesis, as well as ultrasound-directed drug delivery and the physics behind sonoporation will also be discussed^[11]. A comprehensive discussion will focus also on the use of contrast-enhanced endoscopic ultrasound (including specific harmonic imaging)^[12]. Current and future hybrid imaging techniques like real-time virtual sonography (a combination of US/EUS with CT/magnetic resonance imaging) will also be briefly described^[13]. Lastly, molecular imaging perspectives and new developments of targeted

contrast agents will be critically analyzed^[14].

The aim of this series of reviews will be to analyze and enhance the current knowledge and future perspectives of the *in-vivo*, real-time assessment of angiogenesis in digestive cancers, used for the longitudinal monitoring of the effects of chemo-radiotherapy (including anti-angiogenic therapies), as well as for the precise targeting of drugs through molecular based drug-delivery systems.

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S- Editor Sun H L- Editor Rutherford A E- Editor Ma WH



Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Autofluorescence imaging and magnification endoscopy

Monalisa Filip, Sevastița Iordache, Adrian Săftoiu, Tudorel Ciurea

Monalisa Filip, Sevastița Iordache, Adrian Săftoiu, Tudorel Ciurea, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy, Craiova, Dolj, 200349, Romania

Author contributions: Filip M, Iordache S, Săftoiu A and Ciurea T performed the research; Filip M, Iordache S, Săftoiu A and Ciurea T analyzed the data; Filip M and Săftoiu A wrote the paper.

Correspondence to: Monalisa Filip, MD, Gastroenterology Department, University of Medicine and Pharmacy, 2-4 Petru Rares Street, Craiova, Dolj, 200349, Romania. monalisafilip@yahoo.com

Telephone: +40-72-7972250 Fax: +40-25-1310287

Received: July 2, 2010 Revised: August 26, 2010

Accepted: September 2, 2010

Published online: January 7, 2011

are warranted before their endorsement in the routine practice of GI endoscopy.

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Key words: Angiogenesis; Autofluorescence imaging; Multiband imaging; Narrow band imaging; Zoom endoscopy

Peer reviewer: Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryō-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Filip M, Iordache S, Săftoiu A, Ciurea T. Autofluorescence imaging and magnification endoscopy. *World J Gastroenterol* 2011; 17(1): 9-14 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/9.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.9>

Abstract

It is well known that angiogenesis is critical in the transition from premalignant to malignant lesions. Consequently, early detection and diagnosis based on morphological changes to the microvessels are crucial. In the last few years, new imaging techniques which utilize the properties of light-tissue interaction have been developed to increase early diagnosis of gastrointestinal (GI) tract neoplasia. We analyzed several "red-flag" endoscopic techniques used to enhance visualization of the vascular pattern of preneoplastic and neoplastic lesions (e.g. trimodal imaging including autofluorescence imaging, magnifying endoscopy and narrow band imaging). These new endoscopic techniques provide better visualization of mucosal microsurface structure and microvascular architecture and may enhance the diagnosis and characterization of mucosal lesions in the GI tract. In the near future, it is expected that trimodal imaging endoscopy will be practiced as a standard endoscopy technique as it is quick, safe and accurate for making a precise diagnosis of gastrointestinal pathology, with an emphasis on the diagnosis of early GI tract cancers. Further large-scale randomized controlled trials comparing these modalities in different patient subpopulations

INTRODUCTION

It is well known that angiogenesis is critical in the transition from premalignant to malignant lesions. Consequently, early detection and diagnosis based on morphologic changes to the microvessels are crucial. Conventional endoscopic diagnosis using white light is based on subtle morphological changes such as superficially elevated, flat, or depressed lesions and minimal changes in color. However, these findings are difficult to recognize, especially for inexperienced endoscopists. As a result, the diagnosis may be inaccurate or a superficial cancer in the gastrointestinal (GI) tract may be overlooked.

New imaging techniques which utilize the properties of light-tissue interaction have recently been developed to enhance early diagnosis of GI tract neoplasia. Endoscopic autofluorescence imaging (AFI) produces real-time pseudocolor images based on the detection of natural tissue fluorescence generated from endogenous fluorophores (collagen, nicotinamide, adenine dinucleotide, flavin and

porphyrins) through emission induced by excitation light. The system can visualize lesions, including malignancies, by differences in tissue fluorescence properties and can reveal early stage cancers not detectable by conventional white light endoscopy (WLE)^[1,2]. Magnifying endoscopy with narrow band imaging (ME-NBI) represents a real-time endoscopic imaging technique which enhances visualization of the surface texture and the vascular network of the mucosa with the aim of improving tissue characterization and differentiation^[3].

AUTOFLUORESCENCE IMAGING

The principle of autofluorescence diagnosis is based on the interaction between light with a specific wavelength and tissue fluorophores. When tissues are exposed to short wavelength light, endogenous fluorophores (collagen, nicotinamide, adenine dinucleotide, flavin and porphyrins) are excited, leading to the emission of fluorescent light of a longer wavelength (i.e. autofluorescence)^[4]. Normal, inflamed and neoplastic tissue have different autofluorescence characteristics that may thus enable their differentiation. Thus, normal tissue is pseudocolored as green, blood vessels as dark green, while hypertrophic fundic mucosa of the stomach and dysplastic/neoplastic areas appear as magenta. During AFI, a suspected neoplasia (AFI-positive lesion) is defined as any area that is different in color from the surrounding mucosa, and which has a defined circumferential margin^[5].

Autofluorescence is abnormal in neoplastic tissues due to several mechanisms: (1) increase in the nuclear-cytoplasmic ratio, which consequently determines decreased autofluorescence as nuclei show no autofluorescence as compared with cytoplasm; (2) loss of collagen, submucosal collagen is the strongest fluorophore which disappears due to thickening of the mucosa; and (3) neovascularization, inducing increased hemoglobin concentration which absorbs autofluorescence light^[6].

Several published studies showed an increased sensitivity for the detection of high-grade dysplasia and early cancer in the GI tract when autofluorescence techniques were used^[1,2,5-9]. Kara *et al.*^[7] showed the effectiveness of AFI in identifying high-grade dysplasia and early cancer in patients with Barrett's esophagus. Compared with WLE and random 4-quadrant biopsies, AFI increased the detection of high-grade dysplasia and esophageal adenocarcinoma by an additional 6 out of 60 patients, representing an increase of 10%, from 23% to 33%. False-positive lesions were determined by the presence of acute inflammation. On the other hand, Kara *et al.*^[8] showed that fluorescence imaging with light-induced fluorescence endoscopy (LIFE) using a fiber-optic endoscope was no better than standard WLE for the detection of high-grade dysplasia and early cancer in a randomized crossover study of patients with Barrett's esophagus. Another study tested the diagnostic performance of AFI for early gastric neoplasms, thus Ohkawa *et al.*^[9] concluded that LIFE is highly sensitive (sensitivity 96.4%) but not very specific (specificity 49.1%), since 50.9% of benign lesions were also identified

as having abnormal fluorescence images. Using the latest technology incorporated in AFI systems, Kato *et al.*^[5] obtained similar results, with approximately 25% of superficial elevated neoplasia diagnosed only by AFI and missed by WLE. In principle, a superficial elevated neoplasm of a similar hue to the surrounding mucosa might be overlooked during WLE, but it can be revealed by AFI if the elevated neoplasm appears as magenta within a normal looking green mucosa (Figures 1-4).

While all these studies demonstrate the vast potential of AFI to target premalignant lesions (high grade dysplasia) and early cancers, they also reveal important limitations of this technique and set-up directions for future improvement. The large number of false-positive results with a consequent low positive predictive value implies a potential benefit from adjunct methods such as ME-NBI, optical-coherence tomography (OCT) or confocal laser endomicroscopy (CLE)^[10] which would provide greater detection specificity. The use of trimodal imaging endoscopy that includes WLE, AFI and ME-NBI incorporated in one endoscopy system might improve diagnostic accuracy for high-grade dysplasia and early cancer^[11-13]. ME-NBI is currently considered the technique of choice for improvement of diagnostic accuracy because it reduces the high rate of false-positive results associated with WLE and AFI. Autofluorescence consists primarily of visible light, resulting in images limited essentially to the mucosal surface. Consequently, the development of infrared techniques may provide greater tissue penetration, obtaining images with greater contrast between lesions and their surrounding regions, and allowing the visualization of vascularization in deeper lesions, including the submucosa. Some studies reported that infrared endoscopy is capable of detecting abnormal submucosal vascularization in tumor lesions, with retention of indocyanine green being correlated with the size of the submucosal vascular bed^[14-18]. There is also a direct correlation between the presence of infrared fluorescence and the number of submucosal vessels. With the development of tumor invasion, there is a tendency for more abnormal blood vessels to be formed, further accompanied by an increase in fluorescence^[18].

NARROW BAND IMAGING

Narrow band imaging (NBI) is an optical image technology that enhances structural mucosal patterns (pit-pattern), as well as mucosal/submucosal vessels, by employing the characteristics of the light spectrum. The technology consists of placing narrow bandpass filters in front of a conventional white-light source to obtain tissue illumination at selected narrow wavelength bands. Currently available NBI systems use 2 narrow band filters that provide tissue illumination in the blue (415 nm) and green (540 nm) spectrum of light. The superficial penetrating wavelength of 415 nm corresponds to the main peak on the absorption spectrum of hemoglobin, while the deeper penetrating wavelength of 540 nm corresponds to a secondary hemoglobin absorption peak. Capillaries in the superficial mucosal layer are emphasized by the 415 nm light and are

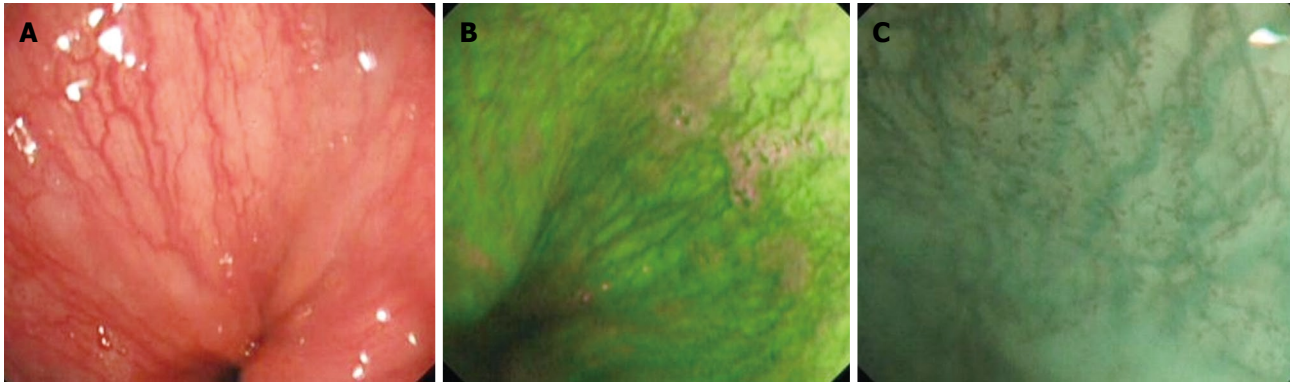


Figure 1 Normal esophageal mucosa. A: Normal vascular pattern above the gastroesophageal (GE) junction visualized in white light endoscopy; B: Autofluorescence imaging of the normal mucosa and vascular pattern above the GE junction; C: Magnifying endoscopy with narrow band imaging depicting the submucosal vessels in cyan and intrapapillary capillary loops in brown.

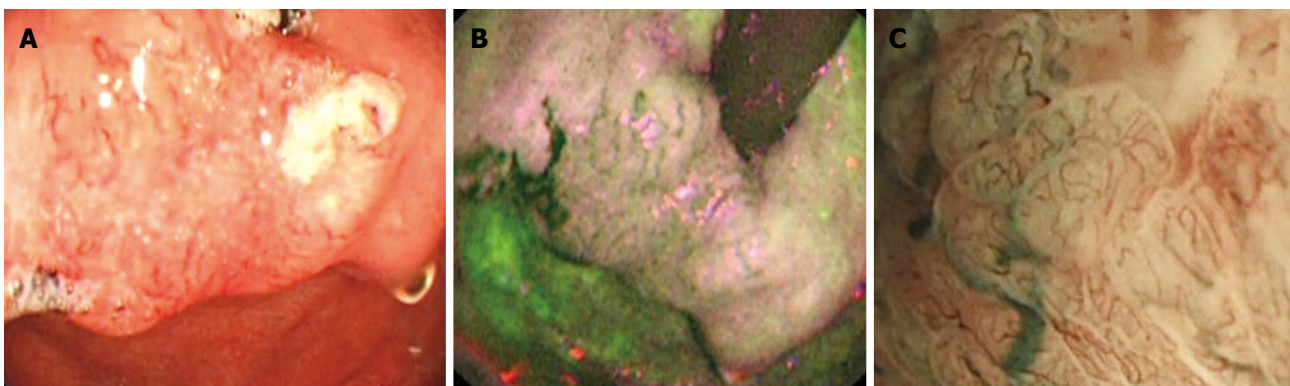


Figure 2 Esophageal squamous cell carcinoma invading the gastroesophageal junction. A: Elevated irregular mucosa with abnormal vascular pattern, difficult to see in white light endoscopy in retroflexion, immediately below the gastroesophageal junction; B: Autofluorescence imaging showing the lesion extension in magenta, with surrounding green normal mucosa; C: Magnifying endoscopy with narrow band imaging showing irregular, thick and distorted mucosal vessels characteristic for tumor angiogenesis.

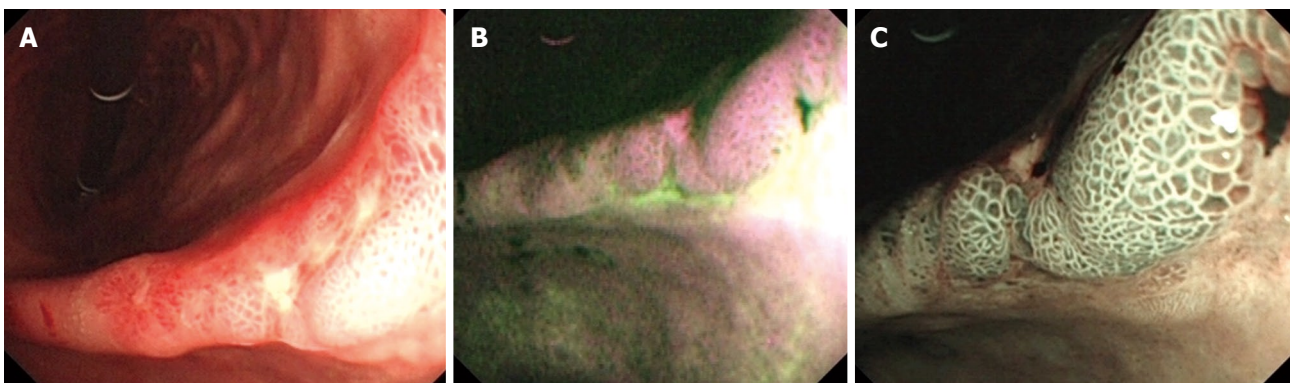


Figure 3 Early gastric adenocarcinoma at the level of the gastric angle. A: Irregular ulcer visualized in white light endoscopy (WLE); B: Autofluorescence imaging showing in magenta the neoplastic margins and a larger lesion extension, as compared with WLE; C: Magnifying endoscopy with narrow band imaging showing a modified pit pattern, with irregular and distorted vascular pattern in the center suggesting high-grade dysplasia/early cancer, and with villous pits and light blue crest sign in the margins suggesting intestinal metaplasia.

displayed in brown, whereas deeper mucosal and submucosal vessels are made visible by the 540 nm light and are displayed in cyan (Figures 1-4). NBI performance is certainly maximized when it is combined with magnification (ME-NBI)^[3,19]. This technique improves the morphological analysis of epithelial crests of the mucosa and enables

a more precise analysis of the abnormal surface architecture (pit-pattern) of neoplastic lesions. However, the most important contribution is represented by the clear visualization of the vascular network in the mucosa, being especially useful in evaluation of the abnormal neoangiogenesis process in high-grade dysplasia/early cancer^[20].



Figure 4 Gastric polyp with moderate dysplasia. A: White light endoscopy showing a 10 mm gastric polyp; B: Autofluorescence imaging with magenta areas on the surface of the polyp, surrounded by green normal mucosa; C: Magnifying endoscopy with narrow band imaging showing a modified pit pattern of the mucosa with an increased number of capillaries.

It was previously recognized that the morphological changes of an intrapapillary capillary loop (IPCL) might represent a new option for early diagnosis of squamous cell carcinoma in the esophagus^[21,22]. However, evaluation of IPCLs under white light observation requires high levels of proficiency and is usually not possible during the usual clinical workup. By using the magnifying scope, the normal appearance of the IPCL is identified as red dots. NBI enables a more vivid observation of the IPCLs, increasing diagnostic accuracy, especially for inexperienced endoscopists (Figure 1C)^[23]. Branching vessels which are located relatively deeper in the wall layers are observed in cyan, while IPCLs which are located in a more superficial layer, are observed as brown loops (brown dots).

Changes in the IPCL pattern include dilatation, tortuosity and/or caliber change of individual IPCL or multiple IPCLs of various shapes. According to the degree of change, these are classified into five types^[23,24]: type I is associated with normal epithelium and IPCLs are observed as smooth running small-diameter capillary vessels; type II involves minimal dilatation and elongation of IPCLs, and is often equivalent to regenerative tissue or inflammation; type III assumes minimal changes in IPCLs and corresponds to borderline lesions which potentially include esophagitis and low-grade intraepithelial neoplasia; in type IV, 3 of the 4 abnormal IPCLs patterns are present and correspond to high-grade intraepithelial neoplasia; finally, type V includes all 4 abnormal IPCLs characteristics and signifies the presence of cancer. Type V is subdivided in four types, type V-1, V-2, V-3 to V_N which reflect cancer infiltration depth. In type V-1, IPCLs demonstrate characteristic changes, dilatation, meandering, irregular caliber and variable form, and corresponds to m1 lesions (carcinoma *in situ*). As it advances to m2 and m3, destruction of IPCL advances gradually and these changes are further extended into the submucosa. Thus, in type V_N, which is characteristic of sm deep invasive carcinoma, new tumor vessels appear, around 10 times larger than the irregular vessels which appear in IPCLs type V-3 (Figure 2C)^[23,24].

ME-NBI is very useful for identifying superficial squamous cell carcinoma in the head and neck region. Muto *et al*^[25,26] reported that visualization of abnormal

microvessel architecture in cancerous lesions is significantly improved by NBI as compared with WLE. This finding is clinically significant because no cases of superficial cancer in the oropharynx or hypopharynx were previously reported before the advent of NBI.

Most of the studies using NBI were designed to evaluate the mucosal pattern and capillary network of patients with Barrett's esophagus, knowing that during WLE it is difficult to identify dysplastic and early neoplastic changes. In all these studies, the accuracy of the diagnosis was higher for NBI as compared with WLE^[27-34]. NBI with magnifying endoscopy thus enables visualization of the details of the mucosal surface and capillary networks without using dyes. Regular villous/gyrus-forming mucosal patterns, as well as flat mucosa with long branching blood vessels are highly predictive for specialized intestinal metaplasia without dysplasia. Irregular/disrupted mucosal pattern, an irregular vascular pattern and abnormal blood vessels are associated with high-grade intraepithelial neoplasia or early cancer. Abnormal vascularity was defined as dilated, corkscrew vessels with increased vascularity and an abnormal, nonuniform branching pattern (Figure 2C)^[27,28]. All high-grade intraepithelial neoplasia have at least one abnormality, and 85% have two or more abnormalities^[27]. Goda *et al*^[29] reported that the addition of capillary pattern to fine mucosal patterns improved the diagnostic value of ME-NBI for detecting specialized intestinal metaplasia and superficial adenocarcinoma.

In a recent study, Singh *et al*^[30] validated a simplified classification of the various morphologic patterns visualized in Barrett's esophagus in four easily distinguishable types: A, round pits with regular microvasculature (columnar mucosa without intestinal mucosa); B, villous/ridge pits with regular microvasculature (intestinal metaplasia); C, absent pits with regular microvasculature (intestinal metaplasia); D, distorted pits with irregular microvasculature (high-grade intraepithelial neoplasia). This classification showed reproducibility and repeatability, both by experienced endoscopists and for those unfamiliar with NBI, suggesting a rapid learning curve. Therefore, ME-NBI allows all endoscopists to perform targeted biopsies for specialized intestinal metaplasia and high-grade intraepi-

thelial neoplasia with a high rate of success^[30-34].

There is no evidence to prove the clinical usefulness of NBI during non-magnifying endoscopic observation for detecting abnormal pathology within the stomach and the duodenum. From a technical point of view, the mucosal image by non-magnification observation with NBI is too dark and noisy for meaningful investigation, because the lumen of the stomach is large^[35]. Feasibility studies showed the potential of NBI with magnification to identify gastric intestinal metaplasia^[36], predict the histologic subtypes of early gastric cancer^[37], and improve margin delineation of gastric cancer for endoscopic mucosal resection^[38]. Uedo *et al.*^[36] reported that a distinctive finding called light blue crests is a good indicator of histological intestinal metaplasia, which is a well-known risk factor for the development of differentiated-type gastric cancer. NBI observation of a light blue crest, defined as a fine blue-white line on the crests of the epithelial surface or gyri, correlated with the histologic diagnosis of intestinal metaplasia with 89% sensitivity and 93% specificity. The light blue crest was frequently observed in the mucosa surrounding differentiated-type early gastric cancers, and it demarcated the extent of the tumors (Figure 3C).

In a study involving 165 patients with depressed-type early gastric cancers, Nakayoshi *et al.*^[37] reported that ME-NBI is not sufficient to replace conventional histology, but is capable of predicting the histological characteristics of gastric cancer. They classified the abnormal microvascular pattern into two types. In the case of differentiated-type depressed early gastric cancer, a relatively regular fine network pattern was more likely to be observed, while for the undifferentiated-type, a irregular, twisting, or corkscrew pattern was more likely to be observed, representing a relatively low density of microvessels. However, ME-NBI may not be sufficient to replace conventional histology, but it may allow improved differentiation between benign and malignant minute lesions and may be useful for diagnosing the extent of cancerous infiltration.

MULTIBAND IMAGING

Multiband imaging (MBI) represents a digital image processing technique that enhances the appearance of mucosal surface structures by using selected wavelengths of light in reconstructed virtual images. MBI technology uses a software-driven image-processing algorithm that is based on spectral estimation methods. A standard image captured by a color charge-coupled device video endoscope is sent to a spectral estimation matrix processing circuit contained in the video processor. Here, reflectance spectra of corresponding pixels that make up the conventional image are mathematically estimated. From these spectra, it is feasible to reconstruct a virtual image of a single wavelength. Three such single-wavelength images can be selected and assigned to the red, green and blue monitor inputs, respectively, to display a composite color-enhanced MBI image in real-time^[9]. There are very few published data thus far on the efficacy of MBI for detection or differentiation of GI tract lesions, although the technique

seems to be superior to WLE, noninvasive and may more easily detect lesions without dye, during both routine and detailed examinations^[39,40].

In conclusion, these new endoscopic techniques provide better visualization of mucosal surface microstructure and microvascular architecture and may enhance the diagnosis and characterization of mucosal lesions in the GI tract. In the near future, trimodal imaging endoscopy, which combines WLE, AFI and ME-NBI, is expected to be practiced as a standard endoscopy technique as it is quick, safe and accurate for making a precise diagnosis of GI pathology. Although there is compelling evidence that these new techniques are superior to conventional endoscopy, current clinical guidelines are still limited. Further large-scale randomized controlled trials comparing these modalities in different patient subpopulations are, of course, warranted before their endorsement in the routine practice of GI endoscopy.

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S- Editor Sun H L- Editor Webster JR E- Editor Ma WH



Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Optical coherence tomography and Doppler optical coherence tomography in the gastrointestinal tract

Eugen Osiac, Adrian Săftoiu, Dan Ionut Gheonea, Ion Mandrila, Radu Angelescu

Eugen Osiac, Adrian Săftoiu, Dan Ionut Gheonea, Ion Mandrila, Radu Angelescu, Centre for Research in Gastroenterology and Hepatology, University of Medicine and Pharmacy, 200349 Craiova, Romania

Author contributions: Osiac E and Gheonea DI wrote this paper; Osiac E, Săftoiu A and Gheonea DI designed research, collected tissue biopsies and performed the imaging procedures; Mandrila I and Angelescu R produced experimental models; Săftoiu A assisted the scientific writing of the paper.

Supported by Project number 99CP/I//2007 ANCS and Project number 12-106/2008 CNMP, Romania

Correspondence to: Dr. Eugen Osiac, Centre for Research in Gastroenterology and Hepatology, University of Medicine and Pharmacy, Petru Rares Street, Number 2, 200349 Craiova, Romania. e_osiac@yahoo.com

Telephone: +40-351-405683 Fax: +40-351-405683

Received: July 2, 2010 Revised: July 28, 2010

Accepted: August 4, 2010

Published online: January 7, 2011

Peer reviewer: Xiao-Peng Zhang, Professor, Department of Radiology, Peking University School of Oncology, Beijing Cancer Hospital and Institute, No. 52 Haidian District, Beijing 100142, China

Osiac E, Săftoiu A, Gheonea DI, Mandrila I, Angelescu R. Optical coherence tomography and Doppler optical coherence tomography in the gastrointestinal tract. *World J Gastroenterol* 2011; 17(1): 15-20 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/15.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.15>

INTRODUCTION

Optical coherence tomography (OCT) is a relatively new method in medical imaging, with first attempts being recorded at the beginning of the last decade^[1]. Based on the analyses of light interference properties generated by a low-coherence source (low-coherence interference), OCT is a noninvasive, high-resolution method. With a resolution in the range of 10-15 μm for standard OCT and up to 1-5 μm if special sources are used^[2,3], this method offers cross-section imaging of the sample with a penetration depth of 2-3 mm. Depending on the OCT configuration, Doppler capabilities are also available in order to provide both structural and functional analyses. With the first application in the field of ophthalmology^[4], promising OCT systems have recently been developed for other fields of medicine including cardiology^[5-7], dermatology^[8] and gastroenterology^[9].

Angiogenesis describes the formation of new vessels from preexisting vasculature under physiological and pathological conditions. Tumor neo-angiogenesis is a fundamental step for solid tumor growth and for metastasis, because it results in spread and development of the network of capillaries from existing blood vessels that are capable of providing the necessary oxygen and nutrients. The process is mediated by various stimulatory and inhibi-

Abstract

Optical coherence tomography (OCT) is a noninvasive, high-resolution, high-potential imaging method that has recently been introduced into medical investigations. A growing number of studies have used this technique in the field of gastroenterology in order to assist classical analyses. Lately, 3D-imaging and Doppler capabilities have been developed in different configurations, which make this type of investigation more attractive. This paper reviews the principles and characteristics of OCT and Doppler-OCT in connection with analyses of the detection of normal and pathological structures, and with the possibility to investigate angiogenesis in the gastrointestinal tract.

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Key words: Optical coherence tomography; Doppler; Gastrointestinal tract; Gastrointestinal cancer; Angiogenesis

tory factors^[10], including cytokines (hormone-like peptides and low-molecular-weight albumins) and growth factors. These factors activate different enzymes (proteases, collagenases, gelatinases and heparinases) that degrade the extracellular matrix and proteins of the basal membrane, thus generating the proliferation and migration of endothelial cells. One of the main triggers of neo-angiogenesis is the secretion of the vascular endothelial growth factor (VEGF)/vascular permeability family of factors^[11,12]. VEGFs are cytokines that have many effects on vascular endothelium, and these multifunctional effects can contribute to angiogenic responses^[11].

The influence of these angiogenic factors is included in different mathematical models, using different living tissue or artificial tissue models such as chick embryo chorioallantoic membrane (CAM) or porous biomaterials^[13-15], in order to describe both the vascular network growth and flow modeling. The purpose of these studies was either to develop understanding of the angiogenesis mechanism or to describe the possibility of direct drug delivery and monitoring of the effects at the level of tumor blood vessels.

Evaluation of angiogenesis thus represents an important step for tumor assessment, especially because of the advent of angiogenesis inhibitors that are already established as anticancer agents. Consequently, to study such a complex process requires multiple correlated investigations that can assess and quantify low-velocity, low-volume blood flow. High-resolution functional imaging methods like 3D-OCT and/or Doppler-OCT could prove to be of considerable importance, especially for the real-time assessment of tumor microvascular flow.

OCT

For the sake of clarity and to associate the physics of the method with its medical applications, a short description of the OCT and Doppler-OCT principles, together with specific characteristics of the experimental system, are provided. Detailed description of the physical principles and mathematical equations that govern the method have been provided by several other papers^[16-20].

Basically, an OCT device provides an analysis similar to B-mode ultrasound investigation^[18]. The main difference is that, instead of using an ultrasound beam, OCT uses low-coherence light, which is provided by a source with emission in the infrared range, typically between 700 and 1500 nm, depending on the optical properties of the target^[21]. In the case of OCT measurements, direct analyses of the reflected intensity is not possible, because of using light, contrary to the case of ultrasound investigation, for which the image obtained is a direct mapping process of the reflected intensity from the target. This is due to the large difference between the velocity of the ultrasound beam and that of the light. Instead, in a standard OCT experiment (Figure 1), the infrared radiation is directed towards an interferometric set-up, where it is split into two beams: one used as reference and directed to a moving mirror, and the other is sent towards the

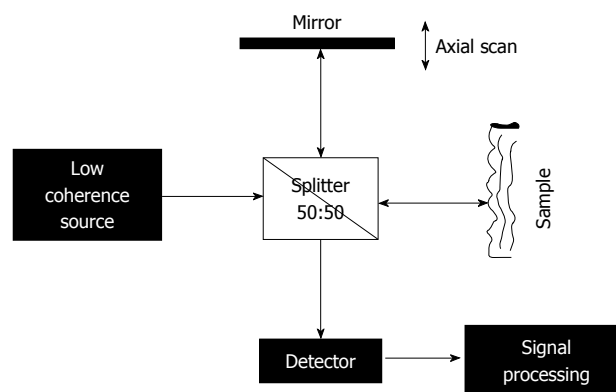


Figure 1 Scheme of a standard optical coherence tomography set-up based on a Michelson interferometer.

sample. After reflection, the sample and reference beams are mixed together, which produces the physical process of interference. Interference occurs only as long as they remain coherent, and the properties of the interference pattern are used as input signal for the imaging process. It should be emphasized that the characteristics of the OCT method are directly related to the properties of the light source used, with the optical characteristics of the sample and with the set-up type.

Based on the above-mentioned factors, advantages and disadvantages of OCT analysis in comparison with ultrasound investigation can be summarized as follows. (1) The main advantage of OCT analysis is higher resolution; 3-15 μm is possible^[2,3]. This is explained by the conditions for the interference process; to produce interference, two beams should be coherent. The light source is partially coherent, which means that coherence of the source is finite, and consequently, the two waves emitted by the source are coherent as long as they are not apart by more than a coherence length of the source. This means that the (optical) length of the reference and sample arm should not differ by more than the coherence length of the source; otherwise the two waves travelling back and forth along these arms do not interfere with each other. It also means that, by controlling the moving mirror in the reference arm (i.e. controlling the length of the reference arm), one has the possibility to control the depth in the sample, from where the reflected signal is received and analyzed (axial resolution); (2) Special mention should be made about the fact that, in an OCT experiment, no connection between the lateral and axial resolution of the measurements exists (in lateral resolution, both control and shape of the beam are playing a major role)^[18,20]. Axial resolution can be estimated using the formula^[16,18]: $\xi_z = [(2 \ln 2)/\pi][\lambda_0^2/\Delta\lambda]$ (eq. 1), where λ_0 is the central wavelength and $\Delta\lambda$ is the bandwidth, with the assumption that the incoming beam has a Gaussian shape and it travels through the air; nevertheless, in many practical set-ups, the radiation is travelling through optical fibers; (3) Infrared radiation (low-energy photons) is used and the source also has low power (mW range), therefore, this method is completely noninvasive; and (4) A major disadvantage compared to ultrasound as-

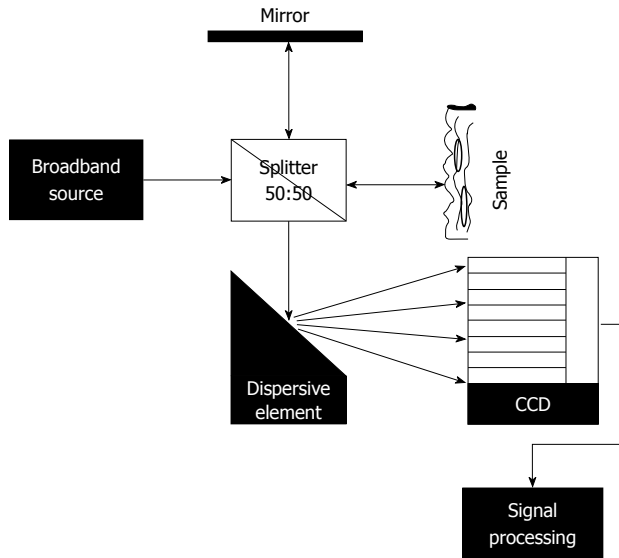


Figure 2 Scheme of Fourier domain-optical coherence tomography set-up.

assessment is the low penetration depth (2–3 mm, depending on the wavelength and optical properties of the analyzed sample^[21,22]), due to the high dispersion and absorption coefficient for the visible-infrared light in biological tissues. Nevertheless, in order to perform analyses that require only millimeter-range penetration depth (different types of mucosa investigation, surface blood microvessels), OCT investigations provide a valuable tool.

As mentioned above, a few parameters (properties of the light source, optical characteristics of the sample, and set-up type) determine the OCT experiments. There are several different set-up configurations for an OCT device used for medical investigations^[16–20]. These include time domain OCT (TD-OCT), Fourier domain OCT (FD-OCT), and swept source OCT (SS-OCT). FD-OCT and SS-OCT are usually grouped together under the name of spectral domain OCT.

TD-OCT was one of the first methods used and it involves a set-up similar to the well-known Michelson interferometer (Figure 1), which has the moving mirror in one arm and the sample in the other. It uses a broad-band light source^[23] and it works similar to the above description. The produced interferogram is measured by a single detector as a function of time delay between the light travelling back and forth along the two arms^[17]. The axial resolution depends on the coherence length of the source^[16,17], and can be given by the equation above (eq. 1). The main disadvantage of this OCT set-up is that it involves mechanical moving parts (moving mirror) that make it difficult to achieve the high scanning rate necessary for *in vivo* 2D and 3D investigations.

FD-OCT uses a charge coupled device or an array photodetector for registering the signal from the interfering light, instead of using a moving mirror and a single detector^[17,18]. A dispersive element (grating or spectrometer) is introduced in the set-up (Figure 2), which projects on to the detector a distribution of the intensity as a function of wavelength. A Fourier transform of the registered

signal provides the back-scattering signal from the sample as a function of time (practically as a function of penetration depth^[16–18]). The main disadvantage in this case is the presence of motion artefacts^[16,19,24].

SS-OCT is similar to FD-OCT but, instead of using a dispersive element to select different wavelengths and an array detector for analyses of the signal, it has a swept source (tunable laser) and a single detector^[17,18,25].

Both FD-OCT and SS-OCT allow direct access to the whole spectrum within one measurement, which offers high sensitivity and imaging resolution, together with high scan rates, which make possible 2D and 3D investigations or fast Doppler measurements^[18,22].

Of these three basic schemes, polarization sensitive detection or phase-sensitive detection can be added in order to improve the imaging process^[16,17].

Doppler-OCT basically estimates the shift in frequency of the laser beam (laser Doppler velocimetry) when the process of the scattering takes place on a moving element^[18,26,27]. As a result of the fact that OCT does not measure directly the reflected intensity, but an interference signal, special mathematical methods are required in order to analyze the received signal; methods which depend on the OCT set-up type^[18]. An important detail is represented by the fact that, if laminar flow is assumed^[20,28], then transversal measurements (in the range of $\pm 15^\circ$) are possible in order to visualize the blood vessels under the sample surface.

The system we have used in our measurements is an SS-OCT from THORLABS (OCS1300SS; Munich, Germany) (Figure 3). The source is a swept laser (55 kHz) with a central wavelength of 1325 nm and an average power of 12 mW. The system is capable of 2D and 3D scans (with an A-scan rate of 55 KHz), with an axial resolution of 12 μm and a lateral resolution of 15 μm . Optical power on the sample is 5 mW. A Doppler module is also available. The system has an image acquisition rate of 50 frames/s.

CLINICAL APPLICATIONS

The possibility offered by the OCT system to deliver near histopathological resolution images makes this method a valuable tool for assessing the gastrointestinal tract. The possibility to investigate tumor structures with high resolution, without performing a classical biopsy, has become very attractive, especially in situations when harvesting biopsy is difficult to realize, or is very hazardous^[9]. Several authors have investigated gastrointestinal cancers by OCT but, due to the late development of Doppler capabilities, few have performed investigations of the angiogenesis process. Generally, to date, most of the studies have evaluated the structural (architectural) changes in the digestive mucosa under pathological conditions, and have identified the correct OCT layer structure inside normal/pathological tissue.

Early studies using OCT techniques have focused on the structure and characteristics of the gastrointestinal tract layers, in order to distinguish between different conditions^[29–32] and patterns, in an attempt to develop computer-assisted diagnostic methods. Also, a lot of interest

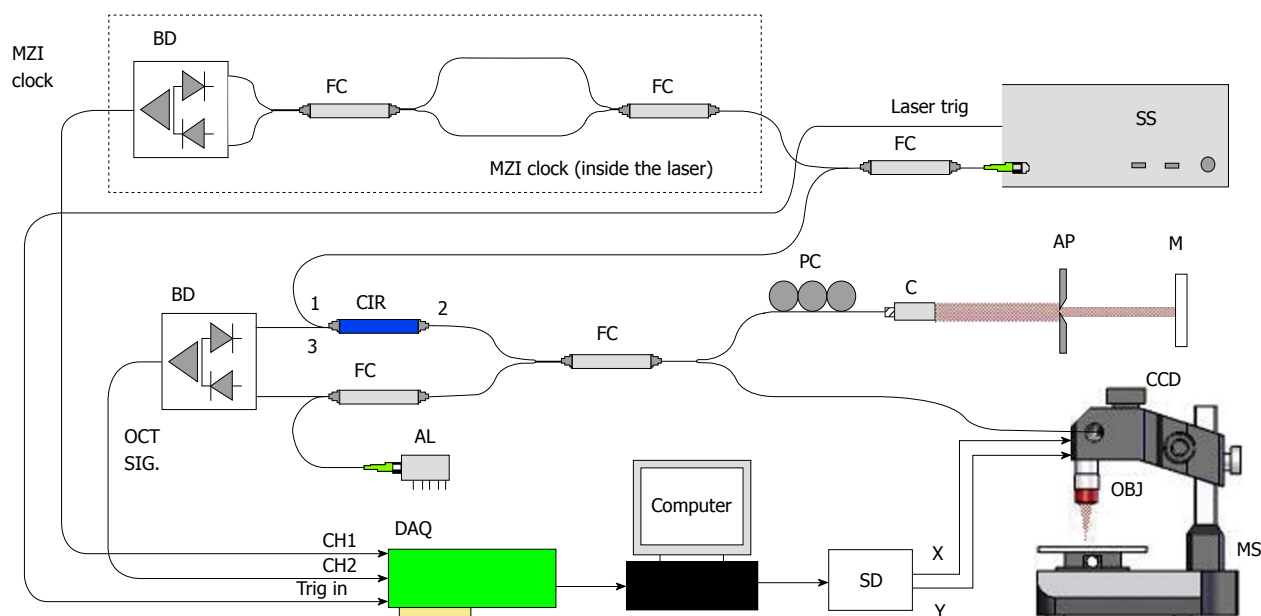


Figure 3 Set-up for OCS1300SS (courtesy of Thorlabs). OCT: Optical coherence tomography; SS: Swept source.

has been directed towards the development of the OCT systems (e.g. new sources, fast acquisition rates, different types of catheters, and modeling of the involved physical processes) for the purpose of management during *in vivo* investigations^[29,31,33]. For example, it has been shown that the structure of the esophageal wall (i.e. squamous epithelium, lamina propria, muscularis mucosa, submucosa, and muscularis propria), as well as the stomach layers (i.e. glandular epithelium, muscularis mucosa, submucosa, and muscularis propria) can be assessed by OCT in order to diagnose pathological aspects^[32].

Recently^[34], ultra-high resolution investigations have been performed in gastric tissues. Based on a higher resolution than standard OCT (10 μm), these studies have demonstrated that computer-aided image analysis is possible, even for detection of different grades of dysplasia^[34,35]. Furthermore, it has been shown that OCT imaging using a standard resolution OCT system with a catheter, which acquires a linear longitudinal plane at a rate of 2/s, can identify specialized intestinal metaplasia at the squamo-columnar junction, with an accuracy close to that of endoscopy^[36]. A pertinent review of OCT in the gastrointestinal tract (esophagus, stomach, small intestine and colon) has been carried out^[37] for normal and pathological (pre-neoplastic tissue, dysplasia and neoplasia) tissues, specifying the detected characteristics. For bile and pancreatic ducts, OCT has also been shown to be a valid tool^[38,39] that allows analysis of near histopathological resolution. The lower gastrointestinal tract has been subjected to OCT investigations^[40] in correlation with histology. Colon cancer was initially studied on mouse models, which have shown that OCT is capable of distinguishing between characteristics of the mucosa (i.e. thickening of the mucosal layer and loss of visibility of tissue boundary lines^[41]), even before adenoma arises. Recently, 3D-OCT investigation of the colon has been performed^[42], in order

to illustrate the differences in pathology. A pilot study on six volunteers was performed, which made possible the observation of clear differences between normal glandular epithelium, normal squamous epithelium and chronic inflammation from ulcerative colitis.

From the point of view of gastrointestinal tract mapping, most of the studies have involved the upper tract (esophagus, with emphasis on Barrett's esophagus), where studies and imaging analysis are the most advanced^[9,20,43,44] and can assist classic endoscopy. A more challenging task is the stomach, where low resolution and penetration depths are observed^[9,20]. In contrast, the colon is a region where images with good investigative potential have been recorded^[9,18,31].

The Doppler-OCT technique was developed later than standard OCT, and only a few studies have been published^[27,45-48]. Most of the expectations for this method were initially to detect and analyze mucosal and submucosal microvascularization. Position (e.g. lamina propria and submucosa) and size (small vessel $\leq 100 \mu\text{m}$, medium vessels $\leq 400 \mu\text{m}$, and large vessels $\geq 400 \mu\text{m}$) of the blood vessels could be detected^[48] in different parts of the gastrointestinal tract. Nevertheless, for the clinical translation of Doppler-OCT into a standard medical investigation, patterns and different characteristics of the microvascularization should be identified and used to produce computer-aided diagnostic procedures. There are not many sources that provide values for physical parameters of biological tissues, e.g. (refractive index, dispersion coefficient); values which are necessary for physical and mathematical modelling, and research should be directed towards such measurements.

We have conducted our early experiments on biopsies (Figure 4A and B) and in a chick embryo CAM model (Figures 5 and 6), where human biopsy implants (xenografts) were placed. The images clearly showed the pos-

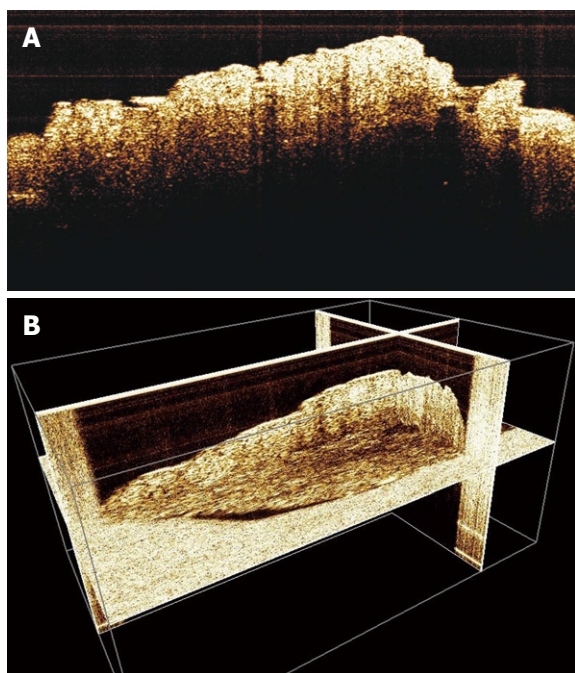


Figure 4 2-D (A) and 3-D (B) optical coherence tomography images of gastric tissue biopsy with visualization of normal components of the parietal layers.

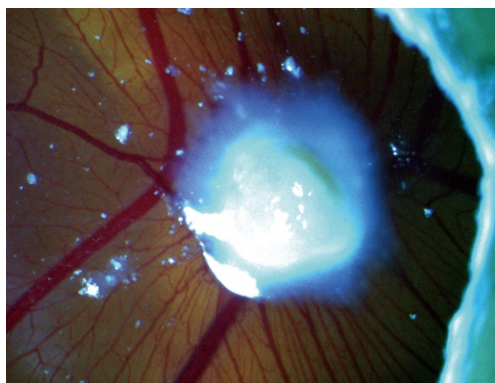


Figure 5 Macroscopic view of a chick embryo chorioallantoic membrane with human gastric tumor implant.

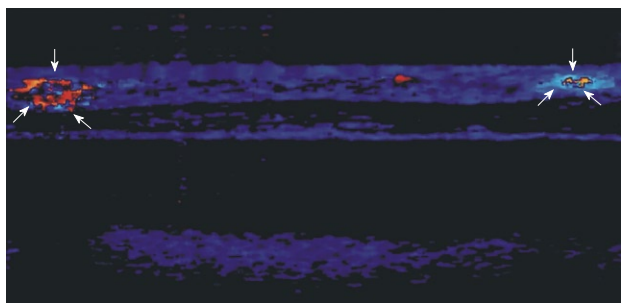


Figure 6 Doppler-optical coherence tomography imaging of a chick embryo chorioallantoic membrane (2 mm × 2 mm) showing capillary activity (arrows) near a human gastric tumor implant.

sibility to identify the microvascularization around the implant zone and the detection of blood flow. It seems that

parameters that characterize the capillary network, such as density, flux and diameter, are accessible to the imaging process, and in addition with other characteristics provided by OCT (i.e. architectural distortion, presence of submucosal glands, and alteration of the layer structure^[9,20]), they can be useful to describe the angiogenesis process.

CONCLUSION

Even though, at present, OCT investigations in gastroenterology are not able alone to provide adequate results (most of the published studies report accuracy and positive predictive values lower than endoscopy), it is worth developing combination of classical endoscopy and OCT techniques to harness the advantages of both. Also, given the large number of medical studies that use these types of devices, as well as recent technical improvements to the systems, it is clear that OCT methods are very promising. At the same time, acquisition of Doppler 3-D images could prove very valuable for the quantitative description of the angiogenesis process, due to the high resolution of this method and the fact that the results can be correlated with structural and functional properties of the samples.

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Confocal laser endomicroscopy and immunoendoscopy for real-time assessment of vascularization in gastrointestinal malignancies

Dan Ionuț Gheonea, Tatiana Cârțână, Tudorel Ciurea, Carmen Popescu, Anca Bădăraș, Adrian Săftoiu

Dan Ionuț Gheonea, Tatiana Cârțână, Tudorel Ciurea, Adrian Săftoiu, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Craiova 200349, Romania

Dan Ionuț Gheonea, Physiology Department, University of Medicine and Pharmacy Carol Davila Bucharest, Bucharest 050474, Romania

Carmen Popescu, Cytology Laboratory, Emergency Hospital Craiova, Craiova 200642, Romania

Anca Bădăraș, Physiology Department, University of Medicine and Pharmacy Carol Davila Bucharest, Bucharest 050474, Romania

Author contributions: Gheonea DI and Cârțână T wrote this paper; Ciurea T, Săftoiu A and Gheonea DI designed the research and performed the imaging procedures; Popescu C interpreted the histology images; Bădăraș A assisted in the scientific writing of the paper.

Supported by Research Grant, No. 239/2007, entitled OCTEUS, financed by the CNCSIS Romania and by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/89/1.5/S/64109

Correspondence to: Dan Ionuț Gheonea, MD, PhD, MSc, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, 1 Mai, 66, Craiova, 200639, Romania. digheonea@gmail.com

Telephone: +40-751-268731 Fax: +40-251-310287

Received: September 12, 2010 Revised: October 19, 2010

Accepted: October 26, 2010

Published online: January 7, 2011

voted capillary network. Confocal laser endomicroscopy is a new technology which allows *in vivo* microscopic analysis of the gastrointestinal mucosa and its microvascularization during ongoing endoscopy by using topically or systemically administered contrast agents. Targeting markers of angiogenesis in association with confocal laser endomicroscopic examination (immunoendoscopy), as a future challenge, will add functional analysis to the morphological aspect of the neoplastic process. This review describes previous experience in endomicroscopic examination of the upper and lower digestive tract with emphasis on vascularization, resulting in a broad spectrum of potential clinical applications, and also preclinical research that could be translated to human studies.

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Key words: Confocal laser endomicroscopy; Immunoendoscopy; Fluoresceine; Acriflavine; Cancer

Peer reviewer: Yuji Naito, Professor, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

Gheonea DI, Cârțână T, Ciurea T, Popescu C, Bădăraș A, Săftoiu A. Confocal laser endomicroscopy and immunoendoscopy for real-time assessment of vascularization in gastrointestinal malignancies. *World J Gastroenterol* 2011; 17(1): 21-27 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/21.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.21>

Abstract

Gastrointestinal cancers represent a major cause of morbidity and mortality, with incomplete response to chemotherapy in the advanced stages and poor prognosis. Angiogenesis plays a crucial part in tumor growth and metastasis, with most gastrointestinal cancers depending strictly on the development of a new and de-

INTRODUCTION

Confocal laser endomicroscopy (CLE) is an emerging technology which allows *in vivo* imaging of cellular and subcellular details of the gut mucosa and vessels during ongoing endoscopy. With real-time microscopic analysis of the mucosal layer at high resolution, an immediate di-

agnosis is possible for different diseases and types of tissues. A magnification of about $1000\times$ enables evaluation of the epithelial cells, connective tissue and changes in vascular patterns. By using intravenously administered fluorescein sodium, images of vessel architecture are readily available, and early recognition of vascular changes in the gastrointestinal (GI) neoplasia is possible^[1]. Furthermore, if real-time diagnosis cannot be obtained, the same technique allows targeted sampling of relevant areas ("smart biopsies") with fewer specimens sent to the pathologist but with significantly higher diagnostic yield compared to random biopsies^[2].

The potential role of CLE has been explored in different pathologic conditions of the GI tract, the possibility of diagnosing premalignant and malignant lesions of the GI tract being particularly important considering the prognostic implications. Gastroenterologists have shown significant interest in this technique, looking for further potential applications in molecular imaging.

Cancers of the digestive tract nowadays represent a major cause of morbidity and mortality. Tumor growth and metastatic potential are strictly dependent on the development of a new and devoted capillary network, which will supply oxygen and nutrients to the newly formed tumor and will allow malignant cells to access the systemic circulation^[3]. Following intravenous administration of contrast agent, CLE allows *in vivo* real-time visualization of the tumor vasculature which is structurally and functionally altered by comparison with the pre-existing vessel network. Furthermore, pre-clinical trials have shown that quantitative characterization of microvessels *in vivo* is possible with confocal mini probes and the additional image processing software^[4]. Such analysis of the microvascular architecture is fundamental for early detection of neoplastic transformation and assessment of the tumor's metastatic potential. The complex, multistep process of neovascularization is the consequence of a dynamic balance between pro- and anti-angiogenic factors^[5]. Targeting markers of angiogenesis, such as vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), in association with endomicroscopic examination, as a future challenge, will add to the morphological aspect functional analysis of the neoplastic process. First steps towards such molecular endomicroscopic imaging have been made by recent studies on animal models.

PRINCIPLES AND TECHNIQUE

The newly developed endomicroscope integrates a miniature confocal microscope into the distal tip of a conventional endoscope enabling simultaneous standard video imaging and confocal microscopy of the mucosal layer. During CLE, an argon ion laser delivers an excitation beam of 488 nm wavelength at the surface of the tissue allowing targeted endomicroscopic images to be captured^[6]. By placing the distal tip of the endomicroscope in intimate contact with the mucosa, multiple grayscale optical sections are recorded at different depths within a range of 0-250 μm . The optical slices are parallel with

the mucosal surface with a 7 μm thickness and a lateral resolution of 0.7 μm , the field of view being $475\ \mu\text{m} \times 475\ \mu\text{m}$ ^[7,8].

Apart from this dedicated endomicroscope, CLE can be performed with a miniprobe. This stand-alone confocal probe can be passed through the working channel of most endoscopes for examination of both the upper and the lower digestive tract. The entire probe-based endomicroscopy system consists of a flexible catheter probe representing a bundle of optical fibers linked to a micro-objective, a laser scanning unit (excitation wavelength 488 nm) and control and acquisition software. A range of mini probes were designed with various optical parameters for different endoscopic procedures^[8,9]. The advanced image processing software allows real-time sequence display for an immediate morphologic diagnosis and also post-procedural analysis and editing. It enables vessel detection and quantitative microvascular measurements, features that make the system suitable for angiogenesis studies^[4].

Contrast agents

High-resolution confocal imaging is achieved by using an exogenous fluorescence technique. Of the potentially suitable contrast agents in humans, the most commonly used are intravenous fluorescein sodium (10%) and topically applied acriflavine (0.2%)^[10]. Acriflavine hydrochloride passes the cell membrane and strongly labels acidic constituents, providing clear visualization of the nuclei and cytoplasm, but only in the superficial layers of the mucosa (0-100 μm). Thus, it is particularly important in depicting intraepithelial neoplasia and cancer of the GI tract^[11]. Fluorescein is a slightly acidic, hydrophilic dye with nonspecific staining properties. Within seconds of intravenous administration it strongly binds to serum albumin and makes the vascular pattern easily detectable. The remaining unbound dye diffuses across the capillaries and, by entering the tissue, it highlights the extracellular matrix^[10,11].

With increasing interest by gastroenterologists in molecular imaging, novel fluorescent contrast agents have been developed targeting disease-specific biomarkers. These include labeled peptides which are easy to deliver to the target structure due to their low molecular weight, but they have variable affinity^[12]. A heptapeptide conjugated with fluorescein has been topically administered for specific *in vivo* imaging of human colorectal neoplasia^[13]. Fluorescently labeled antibodies, on the other hand, are highly selective, binding to their defined target, but may induce immune reactions. Already approved therapeutic antibodies could be labeled for imaging tumors, thus deciding the choice of targeted chemotherapy and potentially predicting the response to treatment^[12]. CLE was able to accurately classify human xenograft tumors in mice and human tissue specimens based on their EGFR expression using fluorescently labeled antibodies against EGFR^[14]. Anti-VEGF antibodies were also used as fluorescent contrast agents for *in vivo* molecular imaging in animal models^[15]. Extensive pharmacokinetic and safety studies are needed to define clinical applications for these molecular probes.

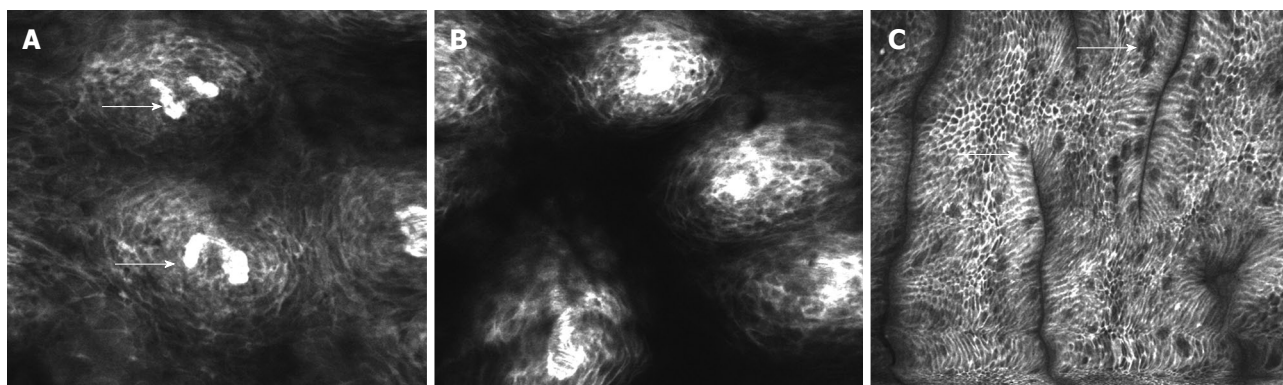


Figure 1 Esophageal surface epithelium visualized using intravenous fluorescein. A: Capillary loops (arrows) of the esophageal papillae; B: Dilatation of intercellular spaces and increased vasculature off the papillae in reflux esophagitis; C: Presence of cylindrical epithelial cells and goblet cells (arrows) in the distal esophagus suggesting Barrett's epithelium.

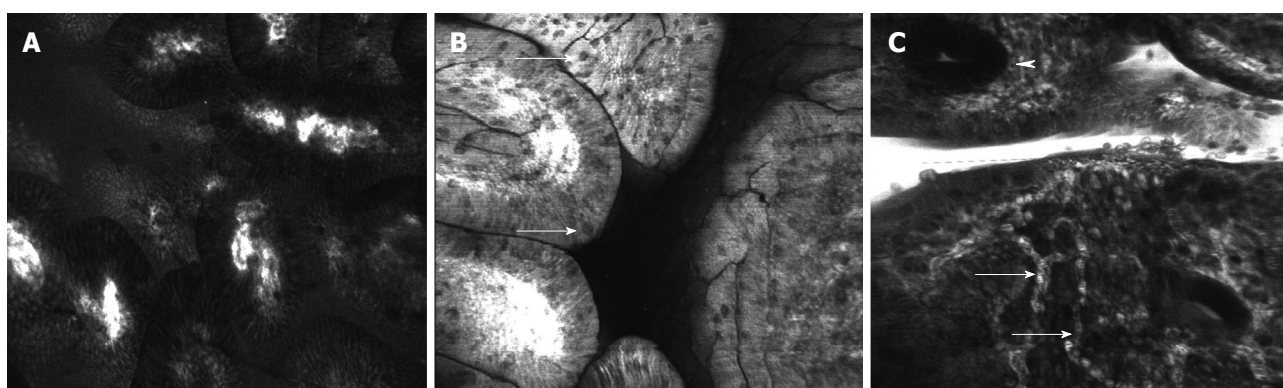


Figure 2 Confocal laser endomicroscopy of the stomach using intravenous fluorescein. A: Columnar epithelium of the antral gastric mucosa and regulated microvascular matrix; B: Atrophic gastritis with intestinal metaplasia and presence of goblet cells (arrows); C: Early gastric cancer with disorganized tissue architecture, few regular crypts (arrowhead) and very tortuous, dilated, irregular vessels (arrows).

CLINICAL APPLICATIONS IN GI CANCERS

The spectrum of potential indications for CLE is broad and includes pathology of both the upper and lower GI tract, most importantly screening and surveillance for cancer based on the cellular and vascular changes. Microscopic imaging of the mucosa is performed after previous detection by standard or optically enhanced endoscopy (autofluorescence imaging, narrow band imaging, i-scan, *etc.*) of “areas of interest”^[10].

Esophagus

The normal endomicroscopic view of the esophageal surface includes polygonal cells within the non-keratinized squamous epithelium and the regular capillary loops of the esophageal papillae directed towards the luminal surface (Figure 1A)^[7,11].

In patients with gastroesophageal reflux disease (Figure 1B), Barrett's epithelium is a well defined premalignant condition associated with adenocarcinoma of the lower esophagus. Although the main diagnostic feature of Barrett's esophagus is the presence of goblet cells, easily detectable with CLE, the vascular pattern, highlighted by fluorescein staining, can also be considered. The subepithelial capillaries are still regular in shape beneath a

specialized columnar epithelium (Figure 1C)^[7]. Significant alterations of the capillary loops are notable in neoplastic tissue with irregular, dilated vessels of increased permeability recognized in the lamina propria due to the brighter signal intensity. Thus CLE is able to detect early neoplastic transformation based on changes in the vascular architecture^[1,16]. In the progression of cancers associated with Barrett's esophagus, angiogenesis represents an essential step. Confocal endomicroscopy can detect this newly developed vascular network and also it enables quantitative measurements such as microvessel density (MVD). Becker *et al.*^[17] evaluated confocal microscopy images acquired from 20 patients, which showed that, in neoplastic Barrett's esophagus, MVD was significantly higher compared to benign conditions.

Stomach

In imaging the stomach, confocal diagnostic criteria have been established in clinical studies for normal gastric mucosa (Figure 2A) as well as for chronic gastritis with intestinal metaplasia (Figure 2B) and cancer by correlation with histopathological examination as the gold standard^[18-22]. While most of these studies classified lesions based mainly on the changes in cells and pit patterns, Liu *et al.*^[23] described endomicroscopic aspects of vascular architecture

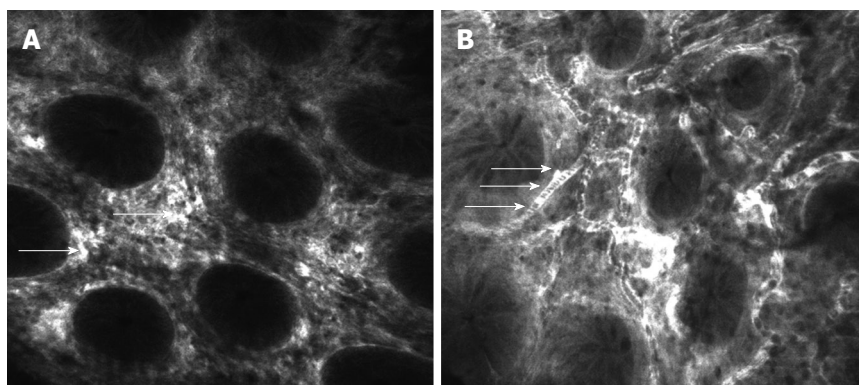


Figure 3 Confocal laser endomicroscopy of the normal colon using intravenous fluorescein. A: Normal aspect of colonic mucosa showing regular architecture of crypts and capillaries of lamina propria (arrows); B: Dark shadows in the lumen of the vessels representing the red blood cells (arrows).

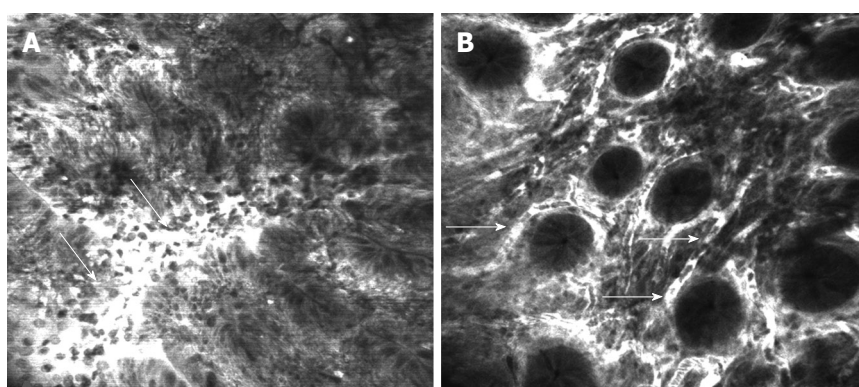


Figure 4 Confocal laser endomicroscopy of the colon using intravenous fluorescein. A: Colon carcinoma with total disorganization of cell architecture, invasion and destruction of the vessels with leakage of fluorescein (arrows); B: Severe inflammatory changes in ulcerative colitis with cellular infiltrate causing an increase in the distance between crypts and excessive vascularity (arrows).

in both normal and malignant mucosa of the upper GI tract. Contrast enhanced CLE (fluorescein sodium 10%, 5 mL) highlights the vascular network of the normal gastric mucosa revealing different aspects between parts of the stomach. The subepithelial capillaries of the gastric body show a honeycomb-like pattern surrounding the gastric pits while in the antrum they are typically coil-shaped. Differentiated early gastric cancer appears to be hypervascular with tortuous, dilated vessels, irregular in shape and size (Figure 2C). In contrast, a hypovascular aspect was described for undifferentiated cancer with isolated, short branch vessels that did not interconnect. In one case report, probe-based endomicroscopy made it possible to diagnose angiodysplasia as a cause of chronic anemia by real-time imaging of a dilated blood vessel with moving red blood cells^[24], adding proof to CLE's feasibility for vascular imaging.

Colon

Clinical trials have delineated potential applications for endomicroscopic examination of the colon. Following intravenous administration of fluorescein the capillaries of the normal colonic mucosa are clearly visualized in the deeper layers of the lamina propria as bright structures (Figure 3A) with moving dark shadows in the lumen representing

the red blood cells which are not labeled by fluorescein (Figure 3B). This network of capillaries circumscribing the regular round mucosal glands results in a typical honeycomb pattern in CLE sequences^[6,11]. In the proximal colon the vasculature appears more dense than in the distal colon, an aspect which correlates with the physiological profile of water absorption along the large intestine^[25].

Confocal patterns based on changes in vessel and crypt architecture have been defined to differentiate among neoplastic and non-neoplastic tissue^[26,27]. In contrast to the regular vascular pattern of the normal mucosa, in colonic cancer the vessels are dilated, distorted with little or no orientation to the adjacent tissue. Increased permeability of the new tumoral vessels is proved by fluorescein passing from the lumen into the interstice which appears intensely bright (Figure 4A)^[11,26]. Colon cancer, with 655 000 deaths worldwide per year, is the second leading cause of cancer-related death in the Western world^[28]. In order to improve the prognosis, colorectal cancer should be detected in the early stages or even precursor stages, when it is possible to cure the selected cases by immediate endoscopic resection. Flat lesions are difficult to detect using standard endoscopic methods and conventional colonoscopy has a significant false-negative rate for intraepithelial neoplasia. CLE is able to predict intraepithelial neoplasia with high

accuracy (99.1%) based on the morphological changes in vascularization mentioned above and the irregular cell architecture^[27].

Precursor neoplastic lesions are also detected by endomicroscopic examination, such as adenomatous polyps, the most important risk factor for colon cancer^[29]. They can be diagnosed microscopically *in situ* with high accuracy, taking into account the altered vascular pattern and also the lack of epithelial surface maturation, crypt budding, and loss of cell polarity^[30].

Patients with inflammatory bowel diseases (IBD), both ulcerative colitis (UC) and Crohn's disease (CD) have a higher risk of developing colorectal cancer as a result of persistent inflammation of the colon. In long-standing ulcerative colitis, CLE is a feasible solution for detecting neoplastic changes in suspect areas. When preceded by a wide-field examination technique, such as chromoendoscopy, CLE has been shown to increase the diagnostic yield of intraepithelial neoplasia while reducing the number of biopsy samples^[31,32]. Inflammatory changes are also depicted by endomicroscopic examination. Increased vasculature of the mucosa (Figure 4B) together with the chronic inflammatory infiltrate result in an enlarged distance between crypts, which show different shapes and sizes^[11].

A recent published study aimed to evaluate the role of CLE in the assessment of inflammatory activity in UC^[33]. On CLE images microvascular alterations and fluorescein leakage as well as crypt architecture were analyzed, and showed good correlation with the histological findings.

While the reported studies have considered mainly descriptive features of the vascular architecture for diagnosis purposes, quantitative measurements of the normal and tumoral vessels could be possible by translating results from preclinical research on small animal models to clinical trials. Laemmel *et al.*^[4] demonstrated in a murine model that is possible to make *in vivo* microvascular observation with a miniprobe confocal fluorescence microscope. They used fluorescein-isothiocyanate-labeled dextran (FITC-dextran) or FITC-albumin injected intra-arterially for clear visualization of the microvascular network. Confocal microscopy enabled observations and measurements usually provided by intravital microscopy: functional capillary density, capillary permeability, vasoconstriction and dilation effects in a minimally invasive procedure. Thus, confocal microscopy has shown potential in the field of microcirculation that should be explored for clinical translation.

IMMUNOENDOSCOPY

The association of endomicroscopic examination with tagged markers of inflammation and proliferation represents an evolutionary leap in GI endoscopy. Thus "immunoendoscopy" aims at the detection and characterization of lesions based on the molecular changes found in inflammation and neoplasia, with potential major impact on current diagnostic and therapeutic algorithms. The exogenous contrast agents for molecular imaging include

fluorescently labeled antibodies and peptides which directly bind to their targets, and "smart" probes with tumor-specific activation^[12].

Goetz *et al.*^[34] approached *in vivo* molecular imaging in a study of human inflammatory and neoplastic diseases in rodent models. They aimed to evaluate a newly developed, handheld confocal miniprobe for *in vivo* subsurface morphological, functional and molecular imaging. Octreotate was labeled with 5-carboxyfluorescein for targeted imaging of somatostatin receptors. After systemic application of developed tracer, neuroendocrine tumors and somatostatin-receptor-expressing pancreatic islet cells were visualized with excellent correlation with immunohistochemistry.

In a pilot *in vivo* human study, a phage library was screened to isolate a peptide that specifically bound colonic dysplasia. The identified heptapeptide sequence was then synthesized and conjugated with fluorescein for use in patients undergoing colonoscopy. After its topical administration, endomicroscopic examination visualized preferential binding of the labeled peptide to dysplastic colonocytes over normal mucosa with high sensitivity and specificity (81% and 82%, respectively), although the molecular target of the sequence was unknown^[13].

With constant interest in understanding angiogenesis and recent advances in antiangiogenic therapies for oncologic patients came the need for imaging this complex process *in vivo*. Targeting angiogenic regulators has been already performed on animal models with promising results for future clinical trials.

One trial evaluated the feasibility of CLE for real-time molecular imaging of EGFR expression, an already established therapeutic target for colorectal cancer^[14]. EGFR is overexpressed in many tumors, playing a central role in proliferation, angiogenesis, invasion, and metastasis^[35,36]. In this trial it was possible to visualize and differentiate EGFR expression patterns in human xenograft tumors in mice with a handheld CLE probe after injection of fluorescently labeled antibodies. Additionally, topical application of fluorescently labeled antibodies provided adequate contrast, enabling distinction of neoplastic from non-neoplastic human colorectal tissue samples based on their EGFR expression^[14]. The same team specifically targeted VEGF for imaging its expression in murine tumors, human xenografts and human tissue samples of colorectal cancer^[15]. VEGF has been intensely studied in basic and clinical research and is already a therapeutic target in metastatic colorectal cancer^[5]. CLE images showed a VEGF-specific signal which correlated well with immunohistochemistry. These studies provide evidence that "immunoendoscopy" is feasible, and results from basic research could be technically translated into clinical practice. *In vivo* molecular imaging could enable selection of patients who would benefit from targeted therapies and could monitor the response to treatment.

Chick embryo chorioallantoic membrane (CAM) is a very convenient experimental model for studying angiogenesis of grafted tumors^[37]. The feasibility of gastric and

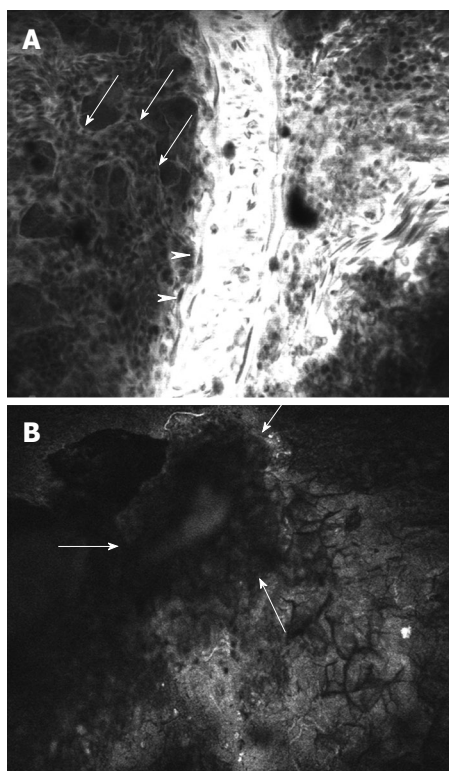


Figure 5 Confocal laser microscopy of the chick embryo chorioallantoic membrane. A: Chick normal chorioallantoic membrane with visualization of large vessels (arrowheads), medium vessels (arrows) and circulating nucleated erythrocytes; B: Fragment of viable human colon cancer tissue (arrows) implanted on chick embryo chorioallantoic membrane.

colonic biopsy implantation, both normal and neoplastic, on the chick CAM for the study of angiogenesis was recently demonstrated^[38]. Fragments of human gastric and colonic mucosa were obtained through endoscopic biopsy, immersed in saline and implanted within 60 min on the chick CAM. Next, the implanted tissue fragments were examined using the confocal laser microscope after previous intravascular administration of 10% fluorescein. Confocal microscopic examination managed to identify both the initial vascularization (Figure 5A) and the newly formed vessels of the grafted tissue (Figure 5B), including intravascular blood flow, representing a starting point for future immunoendoscopy studies in humans, with great therapeutic implications for angiogenic inhibitors.

In conclusion, CLE has led GI endoscopy into a new era. CLE is certainly no longer considered just another endoscopic technique, but a crucial and revolutionary imaging method for real-time assessment of changes in the vascularization pattern of GI structures. Furthermore, *in vivo* molecular imaging, combining CLE with targeted staining, will have a significant impact on both basic research and clinical practice.

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Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Contrast-enhanced and targeted ultrasound

Michiel Postema, Odd Helge Gilja

Michiel Postema, Emmy Noether Research Group, Institute of Medical Engineering, Department of Electrical Engineering and Information Sciences, Ruhr-Universität Bochum, 44780 Bochum, Germany

Michiel Postema, Department of Physics and Technology, University of Bergen, 5007 Bergen, Norway

Michiel Postema, Department of Engineering, The University of Hull, Kingston upon Hull HU6 7RX, United Kingdom

Michiel Postema, Centre de Biophysique Moléculaire, UPR 4301 CNRS affiliated to the University of Orléans, 45071 Orléans, France

Odd Helge Gilja, National Center for Ultrasound in Gastroenterology, Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway

Odd Helge Gilja, Institute of Medicine, University of Bergen, 5007 Bergen, Norway

Author contributions: Postema M and Gilja OH wrote the paper. Correspondence to: Dr. Michiel Postema, Professor, Department of Physics and Technology, University of Bergen, Allégaten 55, 5007 Bergen, Norway. michiel.postema@rub.de
Telephone: +47-555-82880 Fax: +47-555-89440

Received: July 2, 2010 Revised: September 3, 2010

Accepted: September 10, 2010

Published online: January 7, 2011

Abstract

Ultrasonic imaging is becoming the most popular medical imaging modality, owing to the low price per examination and its safety. However, blood is a poor scatterer of ultrasound waves at clinical diagnostic transmit frequencies. For perfusion imaging, markers have been designed to enhance the contrast in B-mode imaging. These so-called ultrasound contrast agents consist of microscopically small gas bubbles encapsulated in biodegradable shells. In this review, the physical principles of ultrasound contrast agent microbubble behavior and their adjustment for drug delivery including sonoporation are described. Furthermore, an outline of clinical imaging applications of contrast-enhanced ultrasound is given. It is a challenging task to quantify and predict which bubble phenomenon occurs under which acoustic condition, and how these phenomena may be utilized in

ultrasonic imaging. Aided by high-speed photography, our improved understanding of encapsulated microbubble behavior will lead to more sophisticated detection and delivery techniques. More sophisticated methods use quantitative approaches to measure the amount and the time course of bolus or reperfusion curves, and have shown great promise in revealing effective tumor responses to anti-angiogenic drugs in humans before tumor shrinkage occurs. These are beginning to be accepted into clinical practice. In the long term, targeted microbubbles for molecular imaging and eventually for directed anti-tumor therapy are expected to be tested.

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Key words: Ultrasound; Drug delivery systems; Drug targeting; Sonoporation; Contrast media; Liver; Pancreas; Gastrointestinal tract

Peer reviewer: Dr. Mirella Fraquelli, Postgraduate School of Gastroenterology, IRCCS Ospedale Maggiore, Milano, 20122, Italy

Postema M, Gilja OH. Contrast-enhanced and targeted ultrasound. *World J Gastroenterol* 2011; 17(1): 28-41 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/28.htm>
DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.28>

INTRODUCTION

Advanced medical imaging has a strong impact on research and clinical decision-making in real-time assessment of angiogenesis in digestive cancers. Ultrasonic imaging is becoming the most popular medical imaging modality, owing to the low price per examination^[1] and its safety^[2]. A B-mode ultrasound scan shows contrasted regions from transitions in acoustic impedance, i.e. transitions in tissue type, in the form of brighter pixels. However, blood is a poor scatterer of ultrasound waves at clinical diagnostic transmit frequencies, which lie between 1 and 40 MHz. For perfusion imaging, markers have been designed to en-

hance the contrast in B-mode imaging. These so-called ultrasound contrast agents consist of microscopically small gas bubbles encapsulated in biodegradable shells.

Contrast-enhanced ultrasound (CEUS) represents a significant advancement in the evaluation of angiogenesis in digestive cancers. In particular, in the study of focal liver lesions, CEUS has been widely used for detection and characterization of malignancy. The unique feature of CEUS of non-invasive assessment in real-time liver perfusion throughout the vascular phases has led to a great improvement in diagnostic accuracy of ultrasound, but also in guidance and evaluation of responses to therapy. Currently, CEUS is part of the state-of-the-art diagnostic work-up of focal liver lesions, resulting in safe and cost-effective patient management.

In this review, the physical principles of ultrasound contrast agent microbubble behavior and adjustments for drug delivery, including sonoporation, are described. Furthermore, an outline of clinical imaging applications of CEUS is given.

Ultrasound

The sound that humans can perceive lies within the frequency range 20 Hz–20 kHz. Ultrasound is by definition all sound higher than 20 kHz. The ultrasound frequencies utilized in medical imaging are mainly in the range 1–40 MHz. Such high frequencies cannot be transmitted through air but can be transmitted satisfactorily through solid or fluid materials. An ultrasonic transducer serves a dual function as both transmitter and receiver of ultrasound. A signal generated by an ultrasonic transducer typically consists of a pulse of a few μ s with a certain center frequency. Part of this signal propagates through target tissue, part is reflected by macroscopic tissue structures, part is absorbed by tissue, and part is scattered by structures in the tissue smaller than the acoustic wavelength. Only a small portion of the transmitted acoustic energy is received by the transducer, but this portion is used to build an ultrasonic image. The received signal is the superposition of specular reflections at tissue boundaries and echoes from tissue backscattering^[3]. Current real-time 2-dimensional imaging capabilities are in excess of 30 frames per second^[4]. Contemporary imaging techniques have been summarized by Wells^[5].

The quality of a B-mode scan is expressed by the contrast-to-noise ratio, which is defined as the absolute difference of the signal-to-noise ratio in the target tissue and the signal-to-noise ratio in the surrounding tissue^[4].

On clinical ultrasound devices, the intensity of the ultrasonic field is generally adjusted with a switch for the mechanical index (MI) instead of the acoustic amplitude. The MI depends on the maximum value of peak negative pressure and the centre frequency of the ultrasound field^[6]. For $MI < 0.3$, the acoustic amplitude is considered low. For $0.3 < MI < 0.7$, there is a possibility of minor damage to neonatal lung or intestine^[6]. These are considered moderate acoustic amplitudes. For $MI > 0.7$, there is a risk of cavitation if an ultrasound contrast agent

containing gas microspheres is being used, and there is a theoretical risk of cavitation without the presence of ultrasound contrast agents^[6]. The risk increases with MI values above this threshold^[6]. These are considered high acoustic amplitudes^[7]. In commercial scanners, the MI has been limited to 1.9 for medical imaging^[8]. Figure 1 shows examples of B-mode scans recorded at different MI. At higher MI, the contrast-to-noise ratio increases.

Microbubble physics

The density and compressibility parameters of blood cells hardly differ from those of plasma. Therefore, blood cells are poor scatterers in the clinical diagnostic frequency range^[9]. Since imaging blood flow and measuring organ perfusion are desirable for diagnostic purposes, markers should be added to the blood to differentiate between blood and other tissue types. Such markers must have resonance frequencies in the medical ultrasonic range. Figure 2 shows the resonance frequencies of free and encapsulated gas microbubbles as a function of their equilibrium radius. The resonance frequencies of encapsulated microbubbles lie slightly higher than those of free gas bubbles^[10,11], but clearly well within the clinical diagnostic range, too. Based on their acoustic properties, microbubbles are well suited as an ultrasound contrast agent.

The pressure inside a bubble must be higher than the ambient pressure^[12]. This difference is generally referred to as the surface pressure. The smaller the bubble, the higher is the surface pressure. Since fluids are forced to flow from a location with a higher pressure to a location with a lower pressure, a bubble cannot exist in true equilibrium. For example, a free air bubble with a 6 μ m diameter dissolves within 100 ms^[13]. To prevent quick dissolution, ultrasound contrast agent microbubbles contain low-solubility gas, such as SF₆ or C₃F₈^[14]. The encapsulating shells are made of biodegradable materials, such as phospholipids or albumin^[15]. With mean diameters below 6 μ m, these microbubbles are small enough to pass through the lung capillaries. Detailed overviews of the compositions of the ultrasound contrast agents used most in imaging research have been given by Postema *et al.*^[3], Sboros^[16] and Tinkov *et al.*^[17]. In this section, we classify ultrasound contrast agents into only 4 categories, based on the presence of an encapsulating shell and its thickness, similar to Tinkov *et al.*^[17].

A bubble in a low-amplitude sound field can be considered a forced damped harmonic oscillator^[18,19] and its oscillating behavior can, as a result, be modeled as a mass-spring-dashpot system^[20]. The spherically symmetric oscillating behavior of ultrasound contrast agent microbubbles has been described with models based on the Rayleigh-Plesset equation^[21], modified for the presence of an encapsulating shell^[22–32]. Generally, the presence of blood has a relatively small effect on bubble dynamics^[33]. To give an indication of the vast amount of existing models: Qin *et al.*^[34] defined 16 separate dynamic bubble model classes. The reason for the high number of existing models is the fact that most physical properties of en-

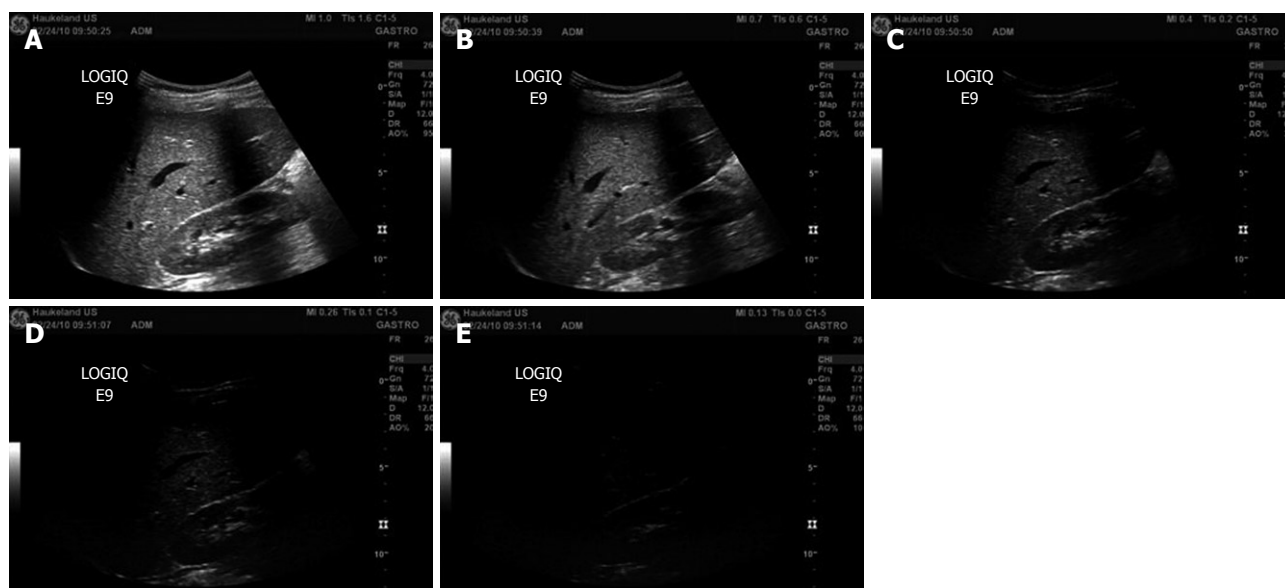


Figure 1 B-mode images of the liver recorded at decreasing mechanical index values (A-E). A: Mechanical index (MI) = 1.0; B: MI = 0.7; C: MI = 0.4; D: MI = 0.26; E: MI = 0.13.

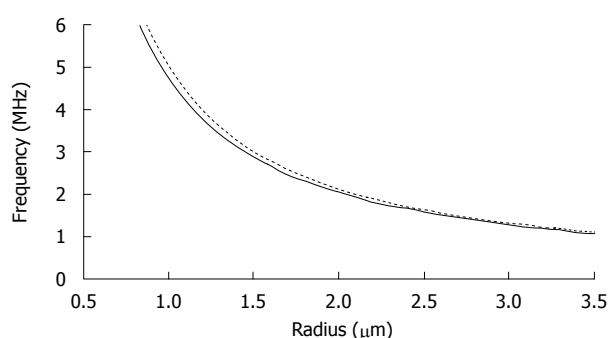


Figure 2 Resonance frequencies of free (unencapsulated) (solid line) and lipid-encapsulated (dotted line) microbubbles as a function of equilibrium radius.

capsulated microbubbles cannot actually be measured, so that pseudo-material properties have to be chosen when predicting ultrasound contrast agent microbubble behavior. Examples of such pseudo-material properties are shell elasticity parameters and shell friction parameters. At low-amplitude driving pressures, an ultrasound contrast agent microbubble oscillates linearly, i.e. the bubble excursion is proportional to the instantaneous pressure. However, at high-amplitude driving pressures, it oscillates nonlinearly. Figure 3 demonstrates the oscillation behavior of 2 contrast microbubbles subjected to continuous sine pressure waves with low, moderate, and high amplitudes. Both bubbles oscillate linearly at $MI = 0.01$. With increasing driving amplitude, asymmetries in radial excursion and expansion time rise, especially for the bigger bubble, which is closer to the resonance size. At $MI = 0.8$, both bubbles expand to a factor of the initial size, followed by a rapid collapse of the smaller bubble. The bigger bubble demonstrates collapse at $MI = 0.18$ and higher.

A dynamic bubble generates an acoustic signal that depends on the fluid displacement by the bubble as a func-

tion of time. Detection strategies have been developed to discriminate acoustic signal-generated by ultrasound contrast agent microbubbles from other acoustic signals such as specular reflections and tissue scattering. These strategies are the reason that CEUS is suitable for the detection of blood. The 10 most common detection strategies include coded excitation, harmonic power Doppler, phase inversion and power modulation^[34,35]. All single-pulse and multi-pulse imaging detection strategies make use of the nonlinear behavior of microbubbles^[34,35].

Other types of nonlinear behavior than asymmetric oscillations are discussed below.

If a bubble with a negligible shell collapses near a free or a solid boundary, the retardation of the liquid near the boundary may cause bubble asymmetry. This asymmetry causes differences in acceleration on the bubble surface. During further collapse, a funnel-shaped jet may protrude through the bubble, shooting liquid to the boundary^[36]. Such jets have been observed in high-speed observations of ultrasound contrast agent microbubbles^[37-40]. Empirical relations exist between the collapsing bubble radius, the jet length, and the pressure at the tip of jets^[41-43]. It has been speculated whether microbubble jetting can be applied for ultrasound-guided drug delivery^[38,39,42].

During the collapse phase, a bubble may fragment into a number of smaller bubbles^[44]. Fragmentation has been observed with contrast agents with thin elastic shells. The number of fragments into which a contrast microbubble breaks up has been associated with asymmetric oscillations^[40,45]. Fragmentation can be predicted from the moment when the kinetic energy of the bubble surpasses its surface energy^[27]. Bubble fragmentation costs energy, but the subsequent coalescence of bubble fragments generates enough acoustic energy to be detected^[27].

Thick-shelled microbubbles have demonstrated sonic cracking during a high-amplitude ultrasonic cycle^[46,47]. The

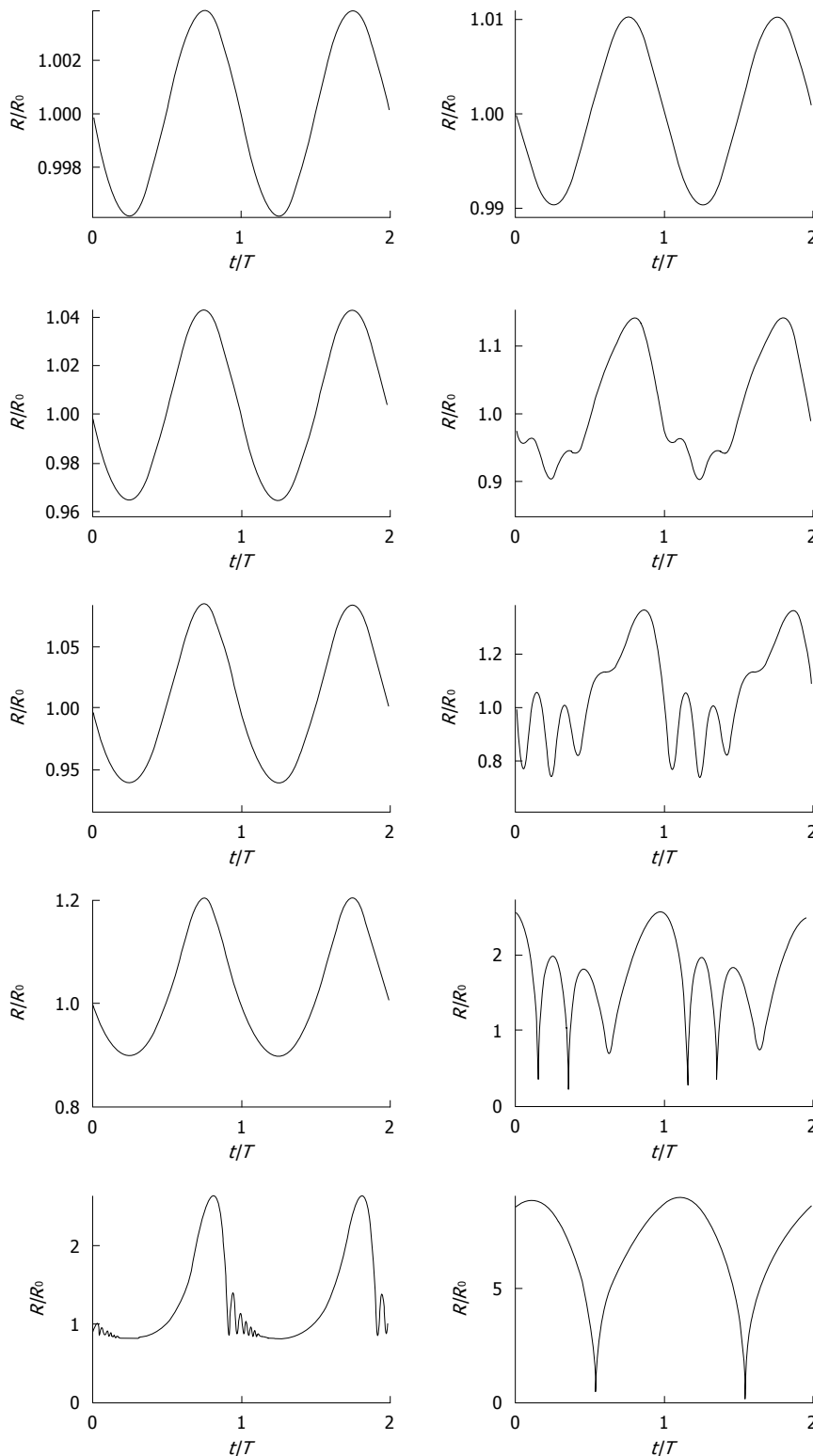


Figure 3 Simulated radius-time curves (radius R normalized with equilibrium radius R_0 , time t normalized with period T_0) of ultrasound contrast microbubbles with $0.55\ \mu\text{m}$ (left column) and $2.3\ \mu\text{m}$ (right column) equilibrium radii, respectively, modeled with a conservative Rayleigh-Plesset equation^[3], using a conservative shell stiffness parameter^[48]. The modeled ultrasound field was a continuous sine wave with a frequency of 0.5 MHz and acoustic amplitudes corresponding to (top-bottom) mechanical index = 0.01, 0.10, 0.18, 0.35, and 0.80, similar to the experiments by Karshafian *et al.*^[92].

increased pressure difference between inside and outside of the microbubble during the expansion phase of the wave^[48] causes the shell to be stretched until it surpasses a critical deformation^[49], resulting in its mechanical cracking. The

released bubble has an expansion amplitude much higher than an encapsulated bubble of identical size. Therefore, the acoustic signal from an ultrasound contrast agent after gas release differs from that of the same contrast agent be-







Phenomenon	Schematic representation	Microbubble classification	Acoustic regime
Translation		I ^[40] , II ^[34,40,149] , III ^[150] , IV ^[56]	L ^[34,56,149] , M, H ^[40]
Fragmentation		I ^[151] , II ^[40,152-155]	L ^[152] , M ^[151,152,154,155] , H ^[40,152,153]
Coalescence		I ^[151] , II ^[154,156]	L ^[154] , M ^[151] , H ^[156]
Jetting		I ^[38,39] , II ^[37,39]	H ^[38,39]
Clustering		II ^[61] , III ^[150]	L ^[61] , M ^[54] , H ^[61,150]
Cracking		II ^[157] , III ^[47,158,159] , IV ^[159]	L ^[158] , M ^[159] , H ^[47]

Figure 4 Nonlinear phenomena and the regimes for their occurrence. Microbubble shell classes: (I) free or released gas; (II) thin shells < 10 nm; (III) thick shells < 500 nm; (IV) very thick shells > 500 nm. Acoustic regimes: low (L) for mechanical index (MI) < 0.3; medium (M) for 0.3 < MI < 0.7; high (H) for MI > 0.7. The figure has been based on Postema^[12].

fore gas release, until the released gas has dissolved^[50].

After a disruptive ultrasonic burst, the disappearance of microbubble fragments or released gas can be traced with low-amplitude ultrasound, as well as the wash-in rate of fresh contrast agent^[51]. Hence, the efficiency of the disruptive burst can be measured.

Bubble translation in the direction of the sound field is caused by a primary radiation force resulting from a pressure gradient across the bubble surface^[52]. The translation is maximal in the contraction phase of the oscillating microbubble. Making use of this phenomenon, ultrasound contrast agent microbubbles can be forced to move farther away from the transducer, towards vessel walls^[53-61], increasing the success rate of targeting to a boundary.

In a standing sound wave field^[62], bubbles can aggregate to clusters ultimately a quarter of the acoustic wavelength apart^[61]. The formation of ultrasound contrast agent microbubble clusters and the ultrasonic pushing of these clusters towards a vessel wall have been recently observed using high-speed photography^[61].

The occurrence of the above-mentioned phenomena is influenced by (1) the ultrasonic parameters: transmit frequency, acoustic amplitude, pulse length, pulse repetition rate and transmit phase; (2) the ultrasound contrast agent composition: the composition of the shell, the bubble sizes, the size distribution and the gas; and (3) the physical properties of the medium: viscosity, surface tension, saturation.

Figure 4 gives an overview of the nonlinear phenomena that have been observed with ultrasound contrast agents, the type of ultrasound contrast agent in which they have occurred, and the minimum acoustic regime required.

Molecular imaging

Dayton *et al.*^[35] defined molecular imaging as the non-invasive application of an imaging modality to discern changes in physiology on a molecular level^[12]. Although ultrasound contrast agents were intended for perfusion imaging, they

have proven useful in molecular imaging as well, after modification of the microbubble shell. Dayton *et al.*^[35] discerned 2 targeting strategies: active targeting, in which a ligand specific for the molecular target, and passive targeting, in which the physiochemical properties of the agent are used to achieve retention at the target site^[12]. Molecular imaging and targeting have been reviewed elsewhere in depth^[35,63]. In summary, the main applications include the detection of angiogenesis, inflammation, plaques and thrombi^[8,12,17].

Drug delivery

It has been proven by numerous groups, that the cellular uptake of drugs and genes is increased, when the region of interest is under sonication, and even more so when a contrast agent is present^[12,64-91]. This increased uptake has been attributed to the formation of transient porosities in the cell membrane, which are big enough for the transport of drugs into the cell. The transient permeabilization and resealing of a cell membrane is called sonoporation^[64]. The sonoporation-induced cellular uptake of markers with molecular weights between 10 kDa and 3 MDa has been reported in several studies^[17,74,92]. Schlicher *et al.*^[93] showed that ultrasound-induced cavitation facilitated cellular uptake of macromolecules with diameters up to 56 nm. Even solid spheres with a 100 nm diameter have been successfully delivered with the aid of sonoporation^[82]. This implies that drug size is not a limiting factor for intracellular delivery^[92]. However, the pore opening times can be so short that, if the drug is to be effectively internalized, it should be released close to the cell membrane when poration occurs^[94].

There are 2 hypotheses for explaining the sonoporation phenomenon, the first being microbubble oscillations near a cell membrane, the second being microbubble jetting through the cell membrane. Based on modeling, high-speed photography, and recent cellular uptake measurements, we concluded that microbubble jetting behavior can be excluded as the dominant sonoporation

mechanism^[7]. The influence of microbubble disruption, i.e. fragmentation or sonic cracking, on sonoporation will have to be further investigated^[7]. Without the presence of an agent, it has been assumed that sonoporation is caused by bubbles, which have been generated in the transducer focus as a result of inertial cavitation^[95,96].

Instead of just facilitating the transient opening up of cell membranes, a microbubble might also act as the vehicle itself to carry a drug or gene load to a perfused region of interest, in which case the load has to be released with the assistance of ultrasound. Apart from mixing ultrasound contrast agent with a therapeutic agent, several schemes have been proposed to combine microbubbles with a therapeutic load^[97]. Tinkov *et al.*^[17] discriminated the following 7 microbubble structure classes for drug delivery: (1) attachment to the outer shell surface; (2) intercalation between monolayer phospholipids; (3) incorporation in a layer of oil; (4) formation of complexes with smaller particles (secondary carriers); (5) physical encapsulation in a polymer layer and coating with biocompatible material; (6) surface loading of protein-shelled microbubbles; and (7) entire volume loading of protein-shelled microbubbles. The drugs are to be released at the site of interest during insonication^[98], presumably by disrupting the microbubble shell. It has been demonstrated *in vitro*, that higher doses of DNA were delivered during ultrasound insonication when the DNA was loaded on albumin-encapsulated microbubbles than when unloaded microbubbles were mixed with plasmid DNA^[67]. Amounts of DNA loading on microbubbles have been between 0.002 (pg/ μm^3)^[99] and 2.4 (pg/ μm^3)^[17,67].

Instead of attaching a drug to the capsule, therapeutic compounds in the gas phase might be encapsulated with thick shells, to keep them from dissolving. At the region of interest, the shell should be cracked with ultrasound, releasing the gaseous content^[46,47,100,101]. However, only a few therapeutic compounds exist in the gaseous phase, e.g. nitric oxide^[48] and several gaseous anesthetics.

A therapeutic agent inside the microbubble shell may react with the shell and dampen the bubble oscillations. Therefore, it might be more suitable to have the therapeutic agent in the core of the microbubble, separated from the shell by a gaseous layer. Incorporating a liquid drop containing drugs or genes inside an ultrasound contrast agent microbubble, however, is technically challenging^[102]. As opposed to bubbles, antibubbles consist of a liquid core encapsulated by gas^[103]. Such a droplet inside a bubble may be generated with the jetting phenomenon: the collapse of a bubble near a free surface produces a liquid jet^[104], which may break up into one or several droplets^[105]. Another option would be to stabilize the liquid core by means of a biodegradable skeleton attached to the microbubble shell.

It has been noted, that, if microbubbles can create pores, it is also possible to create severe cell and tissue damage^[106]. There is an inverse correlation between cell permeability and cell viability^[92,107-109], i.e. not all cell membrane pores are temporary. This indicates that sonoporation is

just a transitory membrane damage in the surviving cell^[92]. Cell lysis results from irreversible mechanical cell membrane damage^[110], which allows the intracellular content to leak out^[64]. Only recently, ultrasound-induced apoptosis has been observed with cancer cells *in vitro*^[110,111], and also in the presence of an ultrasound contrast agent^[112]. Apart from situations where lysis is desired (sonolysis)^[113], ultrasonic settings should be chosen such that cell lysis is minimal. Side effects observed are capillary rupture, hemorrhage, and dye extravasation^[106]. These side effects, however, have been associated with relatively high microbubble concentrations, long ultrasonic pulse lengths, and high acoustic intensities^[106].

CLINICAL IMAGING APPLICATIONS

Liver

Ultrasonography is the most commonly used imaging modality worldwide for diseases of the liver. However, it has limited sensitivity in the detection of small tumor nodules. In addition, ultrasonographic findings are often nonspecific, as images of benign and malignant liver lesions overlap considerably. The introduction of microbubble contrast agents and the development of contrast-specific techniques have opened new prospects in liver ultrasonography. The advent of second-generation agents that enable continuous real-time contrast-enhanced imaging has been instrumental in improving the acceptance and reproducibility of the examination. With the publication of guidelines for the use of contrast agents in liver ultrasonography by the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB)^[114,115], CEUS is now routinely used in clinical practice.

As opposed to contrast media used with computed tomography (CT) and magnetic resonance (MR) imaging, ultrasound contrast agents can visualize the capillary net of the examined tissue, because CEUS is considerably more sensitive to very small amounts of contrast agent, even to single bubbles. Furthermore, because sonography is a dynamic method that is performed in real time, additional information about tissue perfusion can be deduced from the influx and washout of the contrast media, thus facilitating the differential diagnosis of tumors. In addition, signals from the microbubbles enable the visualization of slow flow in microscopic vessels without Doppler-related artifacts. Various software packages have been developed to enable quantification of changes in contrast intensity and to provide additional objective information over the entire course of the contrast examination.

Microbubbles enable dynamic imaging of tumor angiogenesis. This approach is now routinely used for diagnosis, particularly for the detection and characterization of various liver tumors.

The most common malignancy of the liver is metastases. Hepatic metastasis is a sign of advanced tumor stage, and curative treatment is only possible in a very small number of patients. When the objective is cure, liver resection is the most effective therapy, but several ablation

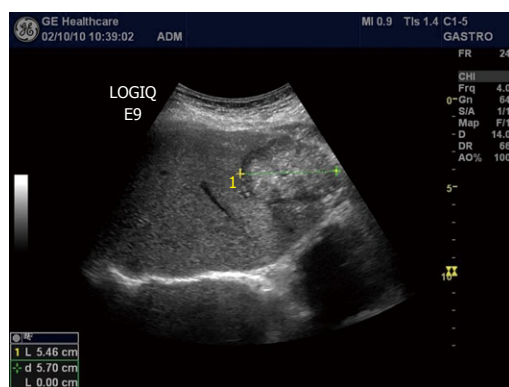


Figure 5 B-mode image of a metastasis from a colon cancer to the liver appearing hyperechoic with a dark halo.

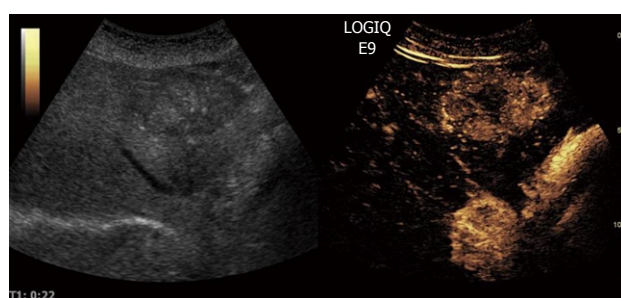


Figure 6 Contrast-enhanced ultrasound B-mode image of a colon cancer metastasis (same as in Figure 5) in the arterial phase showing marked hyperenhancement in the right panel. Note also the dark centre of the tumor, indicating a necrotic portion of the metastasis.

techniques have evolved. For directed tumor therapy, accurate imaging of the number and distribution of the metastases is required. On grey-scale ultrasound images, metastases may appear as hypo-, iso- or hyperechoic lesions, and some of them have a halo (Figure 5). Unenhanced ultrasonography achieves a sensitivity between 45% and 80% in detecting liver metastases^[116,117]. Not surprisingly, this compares unfavorably with the results of studies with contrast-enhanced CT and MR. However, the application of an intravascular ultrasound contrast agent during transcutaneous ultrasonography of the liver improves detection of metastases significantly^[118-120].

After injection, 3 phases of contrast enhancement can be differentiated: the arterial phase, in which the contrast agent reaches the liver first *via* the hepatic artery; the portal phase, where the contrast agent has passed circulation and spreads through the liver in the portal branches; and the late or parenchymal phase, in which the agent slowly distributes within the entire liver parenchyma. Metastases show characteristic features in all 3 phases after contrast agent injection. Differentiation of hypervascular from hypovascular metastases is achieved perfectly by real-time imaging during the arterial phase: hypervascular metastases, e.g. from malignant melanoma, thyroid carcinoma, or neuroendocrine carcinoma, appear as hyperenhancing, usually with a typical rim enhancement of varying size (Figure 6). In contrast, hypovascular metastases le-

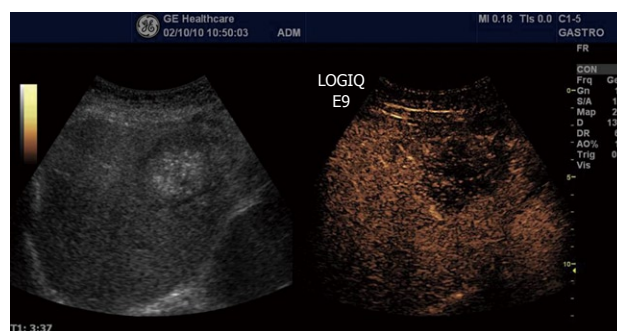


Figure 7 Contrast-enhanced ultrasound B-mode image of a colon cancer metastasis (same as in Figure 5) in the sinusoidal (late) phase, showing marked hypoenhancement in the right panel.

sions, e.g. from colorectal carcinoma (great variability) or bronchogenic carcinoma, may appear as hypoenhancing lesions in the arterial phase. Large metastases may have inhomogeneous enhancement because of necrosis, as shown in Figure 6. At the beginning of the portal phase, the enhancement fades and the entire lesion becomes increasingly hypoechoic. In the late phase, both hypovascular and hypervascular metastases invariably appear as dark defects, whereas the enhancement persists in the normal liver parenchyma (Figure 7). During this phase, the lesions are usually particularly well defined, often with sharp punched-out borders. Both portal venous and late-phase imaging markedly increase the contrast between the enhancing normal liver and the nonenhancing metastases and thus improve detection, particularly of small lesions, i.e. < 1 cm in diameter. The improved detection obtained by the use of ultrasound contrast agents allows for the implementation of CEUS for the follow-up of patients undergoing surgery and chemotherapy, to assess the efficacy of antineoplastic treatment^[121-124]. To determine the utility of CEUS as a prognostic tool for metastatic renal cell carcinoma patients receiving sunitinib, Lassau and co-workers studied 38 patients receiving 50 mg/d sunitinib^[125]. They found that time to peak intensity and slope of the wash-in curve were significantly associated with disease-free survival; time to peak intensity was also significantly associated with overall survival^[125]. Furthermore, they concluded that CEUS is a useful tool for predicting early efficacy of sunitinib in metastatic renal cell carcinoma patients^[125].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the second common malignant liver tumor and the most common primary liver cancer, usually occurring as a complication of chronic liver disease and most often arising in a cirrhotic liver. The accurate and early diagnosis of HCC is essential for treatment of the affected patients. Surgical resection, liver transplantation, percutaneous alcohol ablation and radio-frequency ablation are potentially curative therapies. On grey-scale sonography, HCCs may be hypoechoic (26%), hyperechoic (13%) or have mixed (61%) echogenicity depending on the size of the tumor, the fat content, the degree of differentiation and the scarring of necrosis^[126].

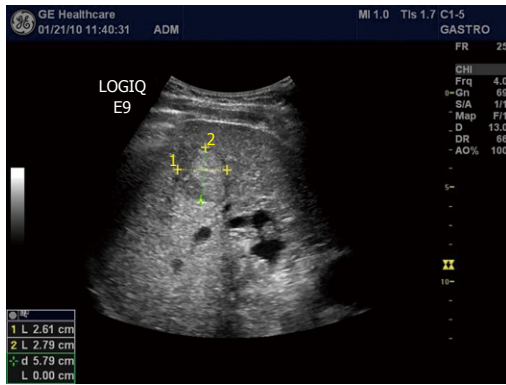


Figure 8 B-mode image of hepatocellular carcinoma with well-demarcated margins and a perilesional halo.

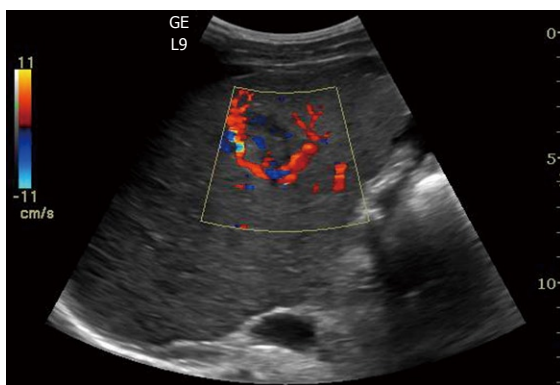


Figure 9 Color Doppler in hepatocellular carcinoma reveals a basket pattern around the tumor, illustrating the anatomy of the arterial tumor supply.

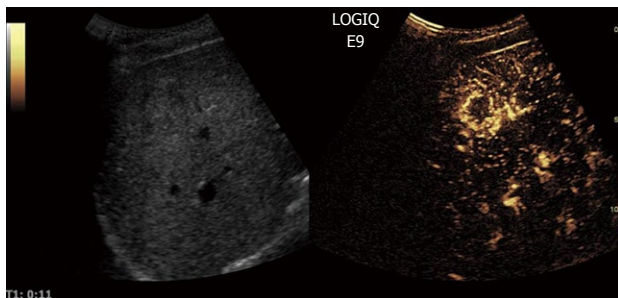


Figure 10 Contrast-enhanced ultrasound allows for visualization of the arteriogram of hepatocellular carcinoma in the early arterial phase. The feeding vessel is visible on the tumor right side. Typically there is initial peripheral enhancement before the centripetal influx to the center of the tumor.

HCCs with well-demarcated margins, perilesional halos or a hypoechoic pattern have a greater rate of detection by ultrasonography (Figure 8). Controversially, infiltrative or iso-hyperechoic HCCs without peripheral halos, as well as HCCs with internal septa or posterior echo enhancement, are harder to detect, with lower reported sensitivities. The use of Doppler in HCC can sometimes reveal a basket pattern around the tumor, depicting the anatomy of the arterial tumor supply (Figure 9).

When CEUS is applied, HCCs are typically characterized by hypervascularity in the arterial phase. Using real-

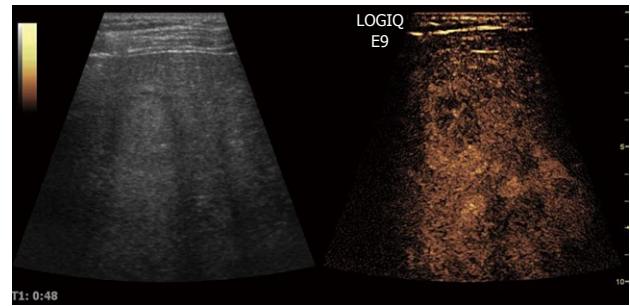


Figure 11 The portal phase of hepatocellular carcinoma. Because of high circulation velocity within hepatocellular carcinoma, there is relatively rapid washout, often starting in the portal phase.

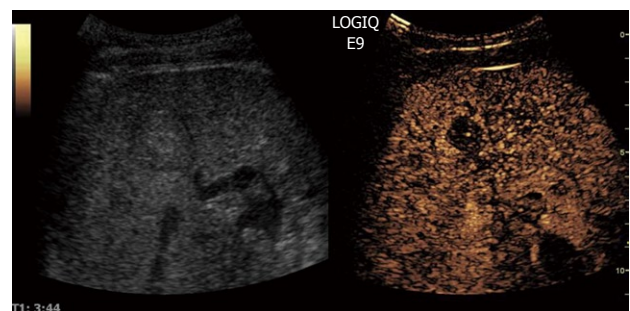


Figure 12 The sinusoidal (late) phase of hepatocellular carcinoma is shown. Typically, hepatocellular carcinoma is hypovascular (hypoechoic) during the late phase of perfusion confirming the malignant nature of the tumor.

time evaluation with low MI, early and usually intense arterial enhancement is identified and in most cases a feeding artery is clearly visible. Tumor vessels, often appearing with a basket-like pattern, tend to enhance in a centripetal fashion extending from the periphery to the centre of the tumor (Figure 10). Arterial enhancement may be inhomogeneous, because the tumor contains septa, regions of different tissue differentiation and shunting among the neo-formed vessels, and sometimes necrosis^[127]. Because of the high circulation velocity within HCC, there is relatively rapid nodular washout, often starting in the portal phase (Figure 11). Typically, HCC is hypovascular (hypoechoic) during the late phase of perfusion (Figure 12). At the same time, normal liver parenchyma increases the echogenicity and homogeneity because of portal venous enhancement.

Surveillance of patients at risk of developing HCC is based on ultrasound examinations performed at either 6 or 12 mo intervals. Early detection of HCC in patients with cirrhosis is a clinical challenge, since the different entities that are involved in the multi-step process of hepatocarcinogenesis, such as low-grade and high-grade dysplastic nodule, share common ultrasonic features. However, CEUS allows for reliable detection of arterial angiogenesis associated with a malignant transformation. When whole lesion enhancement or mosaic enhancement in the arterial phase with an enhancement defect in the portal phase was regarded as a positive finding of HCC, a sensitivity of 92% and a specificity of 87% were found^[128]. It has been shown that the ability of CEUS to diagnose HCC cur-

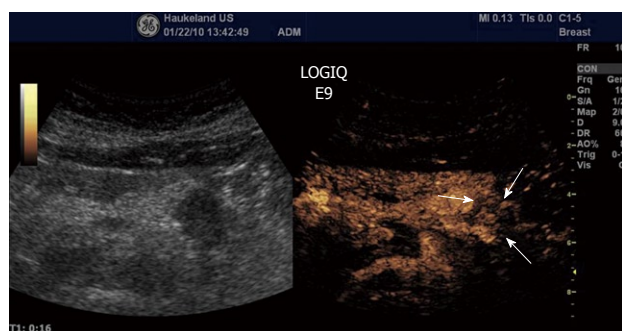


Figure 13 Ductal adenocarcinoma (between arrows) of the pancreas showing poor enhancement in the arterial phase. The same is true for the late venous phase.

rently approaches that of optimized multi-detector CT or dynamic MR imaging protocols^[129-136]. The use of CEUS to characterize nodular lesions in cirrhosis have been recommended by the clinical practice guidelines issued by the European Federation of Societies for Ultrasound in Medicine and Biology and the American Association for the Study of Liver Diseases^[115].

Pancreas

The pancreas, lying deep to the stomach and duodenum, is among the most inaccessible organs in the body for visualization with ultrasonography. Hence, confirmation of pancreatic disease has remained a great challenge in clinical imaging. However, transabdominal ultrasonography has developed to be a useful tool in the differential diagnosis of pancreatic tumors because the technique is inexpensive, easy to perform, and widely available. Nevertheless, only after the introduction of second-generation contrast media^[3], has transabdominal sonography yielded results comparable to those of other diagnostic modalities. CEUS can be used to improve detection of pancreatic lesions or to characterize pancreatic lesions already visible with ultrasonography. Furthermore, the staging of some pancreatic lesions can be improved by the use of contrast media. However, there is an important difference between a pancreatic CEUS study and the well-established liver CEUS study: the blood supply of the pancreas is entirely arterial and the enhancement of the gland begins almost together with the aortic enhancement. With CEUS the enhancement reaches its peak between 15 and 20 s after injection of the ultrasound contrast agent. Accordingly, pancreatic tissue enhancement is earlier and shorter than that of the liver because of the absence of a venous blood supply such as the portal vein in the liver. After a marked parenchymal enhancement in the early contrast-enhanced arterial phase, there is a progressive washout of contrast medium with gradual loss of echogenicity.

Ductal adenocarcinoma is the most frequent tumor of the pancreas, comprising between 80% and 90% of all tumors of the exocrine pancreas. Ultrasonographic findings typically are a hypoechoic lesion with ill-defined margins, often with spicules and tending to alter the gland contour^[137-139]. Characteristically, ductal adenocarcinoma

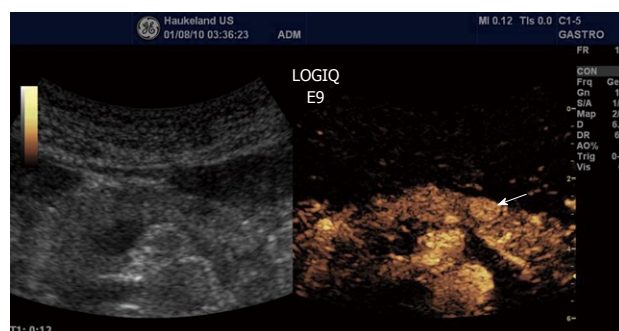


Figure 14 Neuroendocrine tumour (arrow) shows a rapid intense enhancement in the early arterial phase of contrast-enhanced ultrasound examination.

shows poor enhancement in all CEUS phases (Figure 13). On the contrary, neuroendocrine tumors (NETs) appear hypervascular in CEUS imaging. Imaging is important for the differentiation between NETs and ductal adenocarcinoma in selecting the correct therapeutic strategy and determining prognosis. With color- and power-Doppler ultrasonography a spotted pattern can sometimes be observed inside endocrine tumors^[140]. However, Doppler signals are not always detected because of the small size of the lesion or of the tumor vascular network. Typically, NETs show a rapid intense enhancement in the early contrast-enhanced phases (Figure 14), with the exception of possible necrotic intralesional areas.

Gastrointestinal tract

Colon cancer is one of the world's most common malignancies. The main therapy is surgical resection. To diagnose colon cancer, endoscopy is the preferred method, but in many places around the world, X-ray is still used. Using ultrasonography, the normal gastrointestinal (GI) wall is visualized as a layered structure consisting of 5 to 9 layers, depending on transmitted frequency^[141-143]. When digestive cancers develop, the wall layers become blurred, wall thickness is increased, and the ultrasound appearance of the GI wall resembles a kidney, i.e. pseudo-kidney sign or target lesion. However, CEUS does not yet have a place in the work-up of patients with suspected colonic cancer.

In oncology, early evaluation of targeted treatment response with functional imaging is of major importance. Dynamic CEUS is now recognized as a functional imaging technique able to evaluate new antiangiogenic drugs targeting cancers in the abdomen. This therapy evaluation is based on analysis of the curve of signal intensity over time after injection of ultrasound contrast agents (Figure 15). Novel quantification software allows for objective quantification of tumor perfusion parameters including maximum intensity of enhancement, mean transit time, time to peak, and wash-in slope coefficient. CEUS allows for early prediction of tumor response to treatment based on changes in vascularity, before morphological changes become apparent^[144]. Lassau and co-workers evaluated CEUS with perfusion software as a predictor of early tumor response to imatinib (Glivec) in c-kit-positive gas-

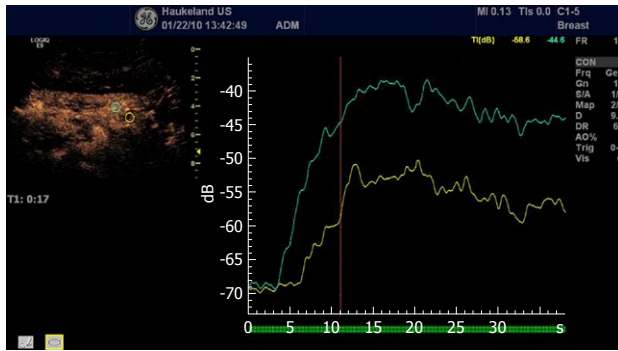


Figure 15 Analysis of time intensity curves after injection of contrast agents depicting a pancreatic carcinoma (same as Figure 13). The green curve depicts the normal pancreatic perfusion whereas the red curve illustrates the hypoenhancing malignancy.

trointestinal stromal tumors (GISTs)^[145]. They studied 59 tumors with metastases or a recurrence from a GIST prospectively and found that initial contrast uptake at day 1 was predictive of the future response^[145]. A strong correlation was found between the decline in tumor contrast uptake at days 7 and 14 and tumor response^[145]. They concluded that CEUS is a non-invasive imaging technique that allows the early prediction of tumor response in c-kit-positive GISTs treated with Glivec^[145].

Tumor growth is dependent on both endothelial and tumor cells. One question is whether changes in tumor vasculature are implicated in tumor tissue degeneration during antiangiogenic therapies. In a study using CEUS, it was shown that tumor cells abruptly became necrotic following antivascular therapy, whereas untreated tumors were protected from degeneration by a significant blood supply^[146]. Because antiangiogenic therapies inhibit the growth of new tumor-associated blood vessels, as well as prune newly formed vasculature, they would be expected to reduce the supply of oxygen and thus increase tumor hypoxia. Franco and co-workers used DC101, an anti-vascular endothelial growth factor receptor 2 antibody to study tumor hypoxia^[147]. Using ultrasonography, they observed consistent reductions in microvascular density, blood flow, and perfusion^[147]. The increase in tumor hypoxia was evident within 5 d and remained so throughout the entire course of treatment^[147]. These results suggest that sustained hypoxia and impairment of vascular function can be 2 consistent consequences of antiangiogenic drug treatment.

Concluding remarks

It is a challenging task to quantify and predict which bubble phenomenon occurs under which acoustic condition, and how these may be utilized in ultrasonic imaging. Aided by high-speed photography, our improved understanding of encapsulated microbubble behavior will lead to more sophisticated detection and delivery techniques.

More sophisticated methods use quantitative approaches to measure the amount and the time course of bolus or reperfusion curves and have shown great promise in revealing an effective tumor response to anti-angiogenic

drugs in humans before tumor shrinkage occurs. These are beginning to be accepted into clinical practice. In the long term, targeted microbubbles for molecular imaging and eventually for directed anti-tumor therapy are expected to be developed.

In principle, in any perfused region that can be reached by ultrasound, ultrasound-directed drug delivery could be performed. However, since the ultrasonic fields used with diagnostic ultrasound scanners differ greatly per organ targeted, some regions will be far from ideal. The ultrasonic frequencies transmitted in endoscopy are much higher than the resonance frequencies of conventional ultrasound contrast agents. Therefore, for such applications, smaller carriers will have to be developed for ultrasound-directed drug delivery.

In conclusion, combining ultrasound contrast agents with therapeutic substances may lead to simple and economic methods of treatment with fewer side effects, using conventional ultrasound scanners. Ultrasound-directed drug delivery has great potential in the treatment of malignancies in the digestive system.

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S- Editor Sun H L- Editor Cant MR E- Editor Zheng XM

Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Contrast-enhanced endoscopic ultrasonography

Nischita K Reddy, Ana Maria Ionică, Adrian Săftoiu, Peter Vilmann, Manoop S Bhutani

Nischita K Reddy, Department of Medicine, University of Texas Medical Branch, Galveston, TX 77555-0144, United States
 Ana Maria Ionică, Adrian Săftoiu, Department of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Craiova 200349, Romania

Peter Vilmann, Department of Surgical Gastroenterology, Gentofte and Herlev University Hospital, 2730 Herlev, Copenhagen, Denmark

Manoop S Bhutani, Department of Gastroenterology, Hepatology and Nutrition-Unit 1466, University of Texas, MD Anderson Cancer Center, Houston, TX 77030-4009, United States

Author contributions: All the authors contributed equally to this review.

Correspondence to: Manoop S Bhutani, MD, FASGE, FACP, AGAF, Professor of Medicine, Experimental Diagnostic Imaging and Biomedical Engineering, Director of Endoscopic Research and Development, Department of Gastroenterology, Hepatology and Nutrition-Unit 1466, UT MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030-4009, United States. manoop.bhutani@mdanderson.org

Telephone: +1-713-7945073 Fax: +1-713-5634398

Received: July 2, 2010 Revised: October 27, 2010

Accepted: November 3, 2010

Published online: January 7, 2011

various pancreatic malignancies. The sensitivity of power Doppler sonography to depict tumor neovascularization can be increased by contrast agents. Contrast-enhanced harmonic imaging is a useful aid in identifying the tumor vasculature and studying pancreatic microperfusion. In the future, these techniques could potentially be used to quantify tumor perfusion, to assess and monitor the efficacy of antiangiogenic agents, to assist targeted drug delivery and allow molecular imaging.

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Key words: Contrast media; Endoscopic ultrasonography; Gastrointestinal neoplasms; Doppler ultrasonography; Pancreatic cancer

Peer reviewer: Richard Hu, MD, MSc, Division of Gastroenterology, Department of Medicine, Olive view-UCLA Medical Center, 14445 Olive View Drive, Los Angeles, CA 91342, United States

Reddy NK, Ionică AM, Săftoiu A, Vilmann P, Bhutani MS. Contrast-enhanced endoscopic ultrasonography. *World J Gastroenterol* 2011; 17(1): 42-48 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/42.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.42>

Abstract

Contrast agents are increasingly being used to characterize the vasculature in an organ of interest, to better delineate benign from malignant pathology and to aid in staging and directing therapeutic procedures. We review the mechanisms of action of first, second and third generation contrast agents and their use in various endoscopic procedures in the gastrointestinal tract. Various applications of contrast-enhanced endoscopic ultrasonography include differentiating benign from malignant mediastinal lymphadenopathy, assessment of depth of invasion of esophageal, gastric and gall bladder cancers and visualization of the portal venous system and esophageal varices. In addition, contrast agents can be used to differentiate pancreatic lesions. The use of color Doppler further increases the ability to diagnose and differentiate

CONTRAST-ENHANCED ENDOSCOPIC ULTRASONOGRAPHY

The use of intravenous contrast agents in ultrasonography was first utilized in echocardiography to enhance imaging of cardiac chambers and great vessels^[1]. Since then, they have been used in transabdominal ultrasonography and more recently in endoscopic ultrasonography (EUS). Use of contrast agents in EUS has been shown to improve the characterization of the vasculature inside the organ of interest, to better delineate benign from malignant pathology^[2-8], to aid in staging and directing therapeutic procedures and thereby to determine prognosis.

CONTRAST AGENTS

Contrast agents are made of gas-filled microbubbles encapsulated by a phospholipid or albumin shell. They are categorized into first, second and third generation based on their capability for transpulmonary passage and their half-life in the human body. Commonly used first generation agents include Albunex, Levovist and Echovist. Second generation agents include SonoVue, Sonazoid, and Optison among others^[9,10]. The only third generation agent currently available is Echogen, capable of phase-shift from liquid to gas form once it attains body temperature^[11]. Contrast agents in use today are relatively safe and have demonstrated no severe, long-lasting adverse effects in humans (Table 1)^[12,13].

MECHANISMS OF ACTION

Contrast agents were specifically developed to image vascularity and vessel patterns, especially for small volume and slow velocity blood flow. This is highly important in tumors, where angiogenesis completely alters the vascular structure. The principle of ultrasound contrast agents is that they create multiple small interfaces with high echogenicity, a process best achieved by gaseous microbubbles, surrounded by a shell used to increase stability^[14]. Microbubbles are very good backscatters, effectively reflecting the ultrasound waves. However, the microbubbles respond and oscillate to sound pressure in a non-linear fashion, with an asymmetrical diameter induced by ultrasound pressure. The diameter is variable between 2 and 10 μm , about the size of red blood cells. Consequently, they do not leave the vascular system (blood pool contrast agents). The ultrasound contrast agents are administered through intravenous bolus injection, in a large arm vein. Second-generation contrast agents are those passing through the lungs, allowing contrast enhancement of the entire vascular system.

Contrast agents were used initially as Doppler signal enhancers, including in contrast-enhanced EUS examinations (CE-EUS). Both color Doppler and power Doppler can be used, especially for regions with very low flow volumes, where the unenhanced signal is too weak or the signal-noise ratio is too poor (Figures 1 and 2). Although the contrast agent selectively enhances the useful signal to the detriment of the noise, the main disadvantage of these techniques is the presence of artifacts. Both tissue motion (flash) artifacts and blooming artifacts appear and impede the examinations. Flash artifacts are specific to the Doppler mode, appearing as color signals caused by tissue motion, being most commonly seen in hypoechoic areas, induced by cardiac or respiratory motion^[15]. Blooming artifacts appear as a consequence of the high amplification of the backscattered signal, which saturates the receiver and causes smearing of the color signal. They appear immediately after the wash-in phase and disappear when the concentration of contrast is lower^[16]. The introduction of second generation contrast agents made it possible to

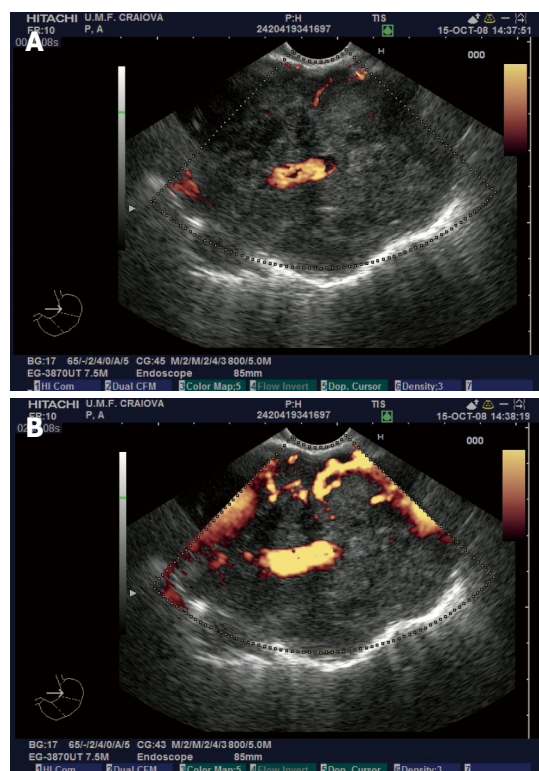


Figure 1 Contrast enhanced endoscopic ultrasonography exam of lung adenocarcinoma. A: Non-enhanced power Doppler image of a lung adenocarcinoma visualized in the aorto-pulmonary window from the mid-esophagus, with discrete Doppler signals in the periphery of the mass and embedding of a large branch of the left pulmonary artery; B: Same tumor visualized after contrast-enhancement with SonoVue, with a better depiction of the vascular peripheral signals and the possibility of quantification of the vascular index. The relationship to the aorta and pulmonary artery is clearly depicted.

enhance B-mode and contrast-harmonic imaging for improved visualization (Figure 3)^[17,18].

CLINICAL USES OF CE-EUS

Differentiating benign from malignant mediastinal lymphadenopathy

EUS-guided fine-needle aspiration (FNA) represents the current “gold-standard” for the diagnosis of malignant mediastinal lymphadenopathy^[19,20], and is further improved by localized cytopathologic assessment of the specimens^[21]. Nonetheless, EUS-FNA carries the risk of mediastinitis if inflammatory nodes are aspirated, contamination and tumor seeding. However, studies have not been consistent in their findings^[6,22]. CE-EUS may offer a non-invasive method to increase the specificity of diagnosis of benign lymph nodes, and aid in targeting aspiration of only high-yield lymph nodes.

Differentiating benign from malignant lymph nodes using appearance and type (arterial, venous) of vascularity may not be reliable since non-lymphomatous cancer cells invade lymph nodes heterogeneously^[23]. Currently, CE-EUS cannot replace EUS-guided FNA in confirming malignant mediastinal lymph nodes. The combination of CE-EUS and EUS-FNA will possibly improve diagnostic accuracy.

Table 1 Intravenous contrast agents, their composition and manufacturers^[10,13]

Contrast agent	Composition	Manufacturer
First generation		
Albunex	5% human albumin with stabilized microbubbles	Mallinckrodt
Echovist (SHU 454)	Standardized microbubbles with galactose shell	Schering
Levovist (SHU 508)	Stabilized, standardized microbubbles with galactose, 0.1% palmitic acid shell	Schering
Myomap	Albumin shell	Quadrant
Quantison	Albumin shell	Quadrant
Sonavist	Cyanoacrylate shell	Schering
Sonazoid	C ₄ F ₁₀ with lipid stabilizer shell	GE healthcare
Second generation		
Definity/luminity	C ₃ F ₈ with lipid stabilizer shell	Bristol-myers squibb medical imaging
Imagent-imavist	C ₆ H ₁₄ with lipid stabilizer shell	Alliance
Optison	C ₃ F ₈ with denatured human albumin shell	GE healthcare
Bisphere/cardiophere	Poly lactide-coglycolide shell with albumin overcoat	-
SonoVue (BR1, Bracco, Italy)	SF ₆ gas with lipid stabilizer shell	Bracco
AI700/imagify	C ₄ F ₁₀ gas core stabilized with polymer shell	Acusphere
Third generation		
Echogen	Dodecafluoropentane (DDEFP) liquid in phase shift colloid emulsion	Sonus pharmaceuticals

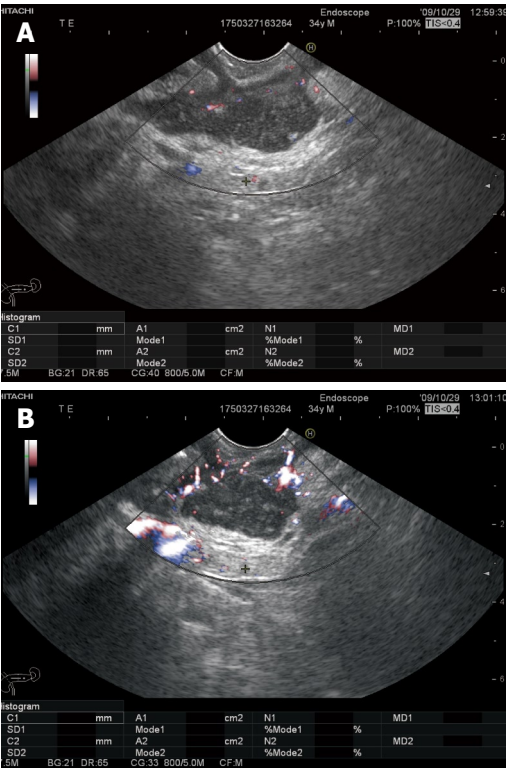


Figure 2 Contrast enhanced endoscopic ultrasonography of pancreatic cancer. A: Pancreatic head adenocarcinoma visualized in bidirectional non-enhanced power Doppler mode; B: Contrast-enhancement with SonoVue indicates a hypovascular mass with increased collateral circulation.

Esophageal and gastric cancer

EUS can provide cross-sectional imaging of the wall of the gastrointestinal tract, and determine the depth of invasion of cancers. The normal esophageal and gastric walls consist of five layers on EUS images with enhancement of the third and fifth layers (submucosa and subserosa, respectively). Esophageal cancers are not enhanced with CE-EUS because of their relative avascularity^[8]. In gastric cancer, assessment of the depth of invasion can be im-

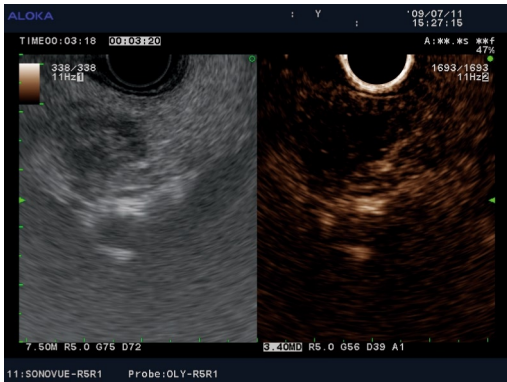


Figure 3 Contrast-enhanced (SonoVue) harmonic endoscopic ultrasound imaging showing a small (12 mm) hypovascular adenocarcinoma in the head of the pancreas. The tumor tissue did not enhance in the early arterial phase, nor in the late venous phase, as compared to the surrounding pancreatic parenchyma.

proved with CE-EUS, especially for depressed, endophytic cancers. Use of CE-EUS improves the overall accuracy of assessment of depth of invasion of gastric carcinoma from 70% to 90%. Active ulcers and scars in both malignant and non-malignant lesions are not enhanced post-contrast. This has been attributed to the nature of vascularity (linear convergence) and foci of fibrosis. Gastric myogenic tumors appear as hypoechoic masses linked to the fourth layer on EUS^[8]. Hirooka *et al*^[24] demonstrated that poorly differentiated gastric carcinomas were enhanced by infusion of Albunex whereas well differentiated ones were not. However, prediction of histologic type of gastric carcinoma by the nature of the enhancement has been inconsistent.

Gallbladder diseases

CE-EUS has the potential to differentiate gallbladder lesions and assess the depth of tumor infiltration in gallbladder carcinomas^[5]. It can differentiate chronic cholecystitis and cholesterol polyps from infiltrating and exophytic gallbladder cancer, respectively, since the three-layer structure

remains intact in the benign conditions. Majority of gallbladder adenocarcinomas enhance with EUS on administration of Alburnex^[5], unlike other gallbladder diseases including adenosquamous carcinoma cholesterol polyps and chronic cholecystitis^[5,24]. CE-EUS is also able to clearly differentiate the depth of invasion in the gallbladder wall from T1b from T1a, improving accuracy over standard EUS^[5].

Pancreatic diseases

First generation: CE-EUS has been used in pancreatic cancer to demarcate vascular landmarks, detect vascular obliteration by a thrombus or tumor, and examine microvascular blood flow to organs and lesions.

Alburnex was the earliest contrast agent used to enhance EUS images. Hirooka *et al.*^[24] demonstrated that peripheral injection of Alburnex can enhance B-mode images of pancreatic pathology during high-frequency EUS. Enhancement was marked in cases of pancreatic islet cell tumors. Pancreatic ductal cell carcinomas remained unenhanced compared to the surrounding normal parenchyma and fibrosis thereby making boundaries clearer^[24]. Administration of contrast also improved the capability for differential diagnosis of lesions identified by B-mode EUS^[25].

A study by the same authors in 1998 demonstrated similar findings with 100% image enhancement (at 12 Hz) using Alburnex in islet cell carcinomas and serous cystadenomas, 80% enhancement in mucin-producing tumors, and 75% in chronic pancreatitis. Also, no enhancement was noted with ductal cell carcinomas and pancreatic pseudocysts, consistent with hypovascularity. Differences in vascularity as demonstrated on angiography paralleled the enhancement patterns during CE-EUS, except in 20% and 25% of cases with mucin-producing tumors and chronic pancreatitis, respectively. In addition, they demonstrated that areas of normal parenchyma and fibrosis around a lesion can be enhanced, demarcating the boundaries. This could lead to accurate pre-operative staging and planning of surgical resection lines, in the case of mucinous tumors involving the main pancreatic duct^[4].

The authors propose that enhancement after Alburnex may be related, at least in part, to the nature of microcirculation and vascular permeability of the lesion, which determine the concentration of contrast agent within the lesion. This could account for the less predictive pattern of angiography with sonographic enhancement^[4]. Concentric bile duct wall thickening on intraductal ultrasonography with strong enhancement after administration of Levovist was seen in patients with autoimmune pancreatitis with a reduction in enhancement after steroid therapy likely indicating resolution of inflammation^[26].

Second generation: Using an experimental second generation microbubble contrast agent, Wong *et al.*^[27] demonstrated hypoechogenicity in normal pancreatic tissue after a bolus but not with continuous infusion. This could be related to a greater intravascular density of contrast material with bolus injection compared to a continuous infu-

sion. A decreased echo signal from the pancreatic parenchyma after contrast injection may be due to an increased signal from the pancreatic interface with adjacent structures^[27]. Whether this will have a clinically useful application remains to be determined. Certainly, lesions that are enhanced with contrast will be more easily distinguished from the surrounding normal pancreas.

CE-EUS reveals the characteristic vascularity and can diagnose and follow up intraductal papillary mucinous neoplasms (IPMN) of the pancreas. Enlargement or enhancement of a mural nodule accurately indicated the presence of atypical malignant epithelium and determined the need for surgical resection^[28]. Mural nodules were classified into four types based on morphology during EUS, before and after contrast: Type 1 (low papillary type), Type 2 (polypoid type), Type 3 (villous type) and Type 4 (invasive type with a blurred hypoechoic area between lesion and parenchyma). When IPMNs with Type 3/4 were diagnosed as malignant, accuracy was higher^[29].

The use of CE-EUS has also been used to differentiate between mass-forming chronic pancreatitis and pancreatic cancer. Focal lesions can be seen in alcohol and autoimmune pancreatitis, with presentation similar to that of pancreatic cancer. CE-EUS produces a “parenchymographic” enhancement (i.e. isovascular to pancreatic parenchyma) in inflammatory benign masses and shows an inverse correlation with the degree of fibrosis within the mass, the duration of the inflammatory process and the enhancement with contrast infusion. Ductal carcinomas exhibit complete absence or a low level of enhancement due to greater fibrosis within the tumor^[30,31].

Color Doppler

Combining B-mode EUS with CE-Doppler ultrasound improves the visualization of the vascularity of a pancreatic lesion, with malignant ductal adenocarcinoma demonstrating low flow and a relatively avascular pattern. Bhutani *et al.*^[32] described enhancement of color Doppler signals from the celiac artery, superior mesenteric artery, and portal vein during EUS in a swine model after administration of Levovist. This effect was easily appreciated without the need for complex quantitative measurements. No visually obvious enhancement was evident in vessels such as the aorta that already had a pronounced unenhanced color Doppler signal^[32]. Using Optison (FS069), Becker *et al.*^[33] demonstrated that the sensitivity and specificity of echo-enhanced color-Doppler EUS are comparable with the cytopathology results. Ueno *et al.*^[34] differentiated islet cell tumors and ductal cell cancer with color-Doppler EUS. Islet cell tumors had marked hypervascularization whereas patients with adenocarcinoma had vascularity only around the tumor. These results have been confirmed by several other investigators. Hypovascularity as a sign of malignancy in CE-EUS can provide 92% sensitivity and 100% specificity (89%-100%)^[2]. Using CE-Doppler EUS, hypovascularized malignant ductal adenocarcinoma and hypervascularized benign tumor entities, mostly neuroendocrine tumors, and serous microcystic adenomas of the pancreas

can be easily differentiated. This is of pivotal importance since serous microcystic adenomas can be observed due to low growth potential, and neuroendocrine tumors may be enucleated or otherwise and less radically resected compared to ductal adenocarcinoma.

Power Doppler

Unenhanced power Doppler ultrasonography is unable to provide tumor differentiation, as a previous study showed a very low specificity (77%) of unenhanced power Doppler EUS^[35]. Although other factors like the presence of peripancreatic collaterals, might improve the specificity, this was not confirmed in larger studies. It is possible to misdiagnose necrotic pancreatitis as ductal adenocarcinoma and also to find inflammation surrounding ductal adenocarcinomas. Indeed, the presence of power Doppler inside the inflammatory masses is variable as a function of inflammation and necrosis, thus complicating the differential diagnosis^[35].

The sensitivity of power Doppler sonography to depict tumor neovascularization can be increased by contrast agents. In an animal model of pancreatic vascular disruption using 50% ethanol plus purified carbon particle solution, standard EUS demonstrated hypoechogenicity in the ethanol treated area. With injection of Definity, power Doppler EUS revealed marked contrast enhancement of normal pancreatic parenchyma from the ethanol-treated area^[36]. Several studies using CE-EUS with power Doppler scanning also demonstrated an improvement in discrimination of pancreatic cancer from chronic pancreatitis^[33,37,38] and may also help to localize small benign tumors such as insulinomas^[7].

Hocke *et al.*^[38], using pulsed Doppler analysis with CE-Doppler EUS, demonstrated an improvement in the differentiation between chronic pancreatitis and malignancy. They used specific criteria to define malignancy: lack of vascularization before injection of SonoVue, irregular appearance of arterial vessels over a short distance post-injection, and absence of detection of venous vessels in the lesion^[38]. In contrast to the technique described by Becker *et al.*^[33], Hocke *et al.*^[38] combined the analysis of the detected vessels with pulsed wave Doppler analysis. They concluded that the use of second generation contrast agents with low mechanical index techniques will possibly allow real-time imaging with or without three-dimensional reconstructions in EUS imaging.

With CE power Doppler sonography, the signal intensity from flowing blood is lower compared to that of moving solid tissue structures. Harmonic imaging was specifically developed to overcome these obstacles, since tissue particles have fewer harmonic waves than intravascular microbubbles, thus avoiding flash and blooming artifacts^[35].

Harmonic imaging

CE harmonic imaging techniques are currently available for EUS, as a result of the improvement in transducer technology. Thus, the use of adequate broadband transducers that can detect harmonic signals was recently

been reported^[17,25]. A pilot study previously described an experimental technique with low mechanical index, which allowed differentiation between chronic pancreatitis and pancreatic cancer, based on tissue microperfusion characteristics^[17]. Another feasibility study demonstrated both parenchymal perfusion and microvasculature in the pancreas^[25]. Both intermittent homogeneous parenchymal perfusion images and real-time continuous images of finely branching vessels of the pancreas were obtained with a mechanical index of 0.4. Although the initial study included a small number of patients with pancreatic lesions, it seemed that tumor characterization was possible based on the vascular or perfusion pattern. Thus, pancreatic carcinomas had absent or heterogeneous perfusion images in the intermittent mode, while the vessels were visualized as irregular “network like” structures in real-time mode. Both neuroendocrine tumors and chronic pseudotumoral pancreatitis were homogenous and iso- or hyper-vascular. Several other research groups are testing the feasibility of CE-EUS^[39-41]. CE-EUS with low mechanical index (0.4) was tested in 25 patients, after peripheral injection of SonoVue^[39]. The method seemed feasible for differentiating adenocarcinoma from other focal mass lesions, being proposed as the method of choice to establish the management of patients when EUS-FNA is non-contributive. Harmonic imaging has also been used with CE-EUS after peripheral injection of Sonazoid in two settings, WPI (wide-band pulse inversion harmonic) and EXPHD (extended pure harmonic detection)^[40]. The change in echo-intensity was evaluated. Ductal carcinomas, IPMTs, chronic pancreatitis and endocrine tumors demonstrate varied echo-intensities after infusion of contrast agent. CE harmonic EUS can be a useful aid in identifying tumor vasculature, especially that of pancreatic masses^[41].

Esophageal varices and portal hypertension

B-mode EUS can detect grade II varices or larger. After administration of Levovist, flow signals can become evident beneath the third echogenic layer of the esophageal wall and help visualize perforating veins and periesophageal vessels^[42]. EUS-guided portal vein angiography by using CO₂ as a contrast agent, has been evaluated in a porcine model. This is less viscous, making it easier to inject through small-caliber needles, minimizing damage to the vascular wall compared with iodinated contrast (Table 2)^[43].

FUTURE PERSPECTIVES

Tumor blood flow was previously linked in several studies with both metastasis potential and poor prognosis. A clear correlation was also proven between microvessel density, different angiogenic factors [e.g. vascular endothelial growth factor (VEGF)] and the tumors with definite vascular signals demonstrated by CE ultrasound^[44]. Quantification of tumor perfusion has been proven feasible for the early assessment and monitoring of the efficacy of antiangiogenic agents in quantitative terms based on changes in vascularity, before morphological changes become apparent^[45].

Table 2 Indications for the use of contrast agents during endoscopic ultrasonography

Study	Indication	Agent used
Hocke <i>et al</i> ^[6]	Differentiating benign from malignant lymph nodes	SonoVue
Kanamori <i>et al</i> ^[22]		Levovist
Nomura <i>et al</i> ^[8]	Assessment of depth of invasion of esophageal cancer	Air-filled Albumin
Nomura <i>et al</i> ^[8]	Assessment of depth of invasion of gastric cancer	Air-filled Albumin
Itoh <i>et al</i> ^[28]	Differentiating benign from malignant intraductal papillary mucinous tumors of the pancreas	Levovist
Hirooka <i>et al</i> ^[5]	Gallbladder diseases	Albunex
Ueno <i>et al</i> ^[34]	Diagnosing Islet cell tumors	Levovist
Sakamoto <i>et al</i> ^[40]	Determining origin of solid pancreatic masses	Levovist with suspension of monosaccharide microparticles
Hirooka <i>et al</i> ^[24]		Albunex
Dietrich <i>et al</i> ^[2]		Levovist
Sofuni <i>et al</i> ^[41]		Levovist
Becker <i>et al</i> ^[33]	Discriminating between mass forming pancreatitis and pancreatic cancer	Optison
Hocke <i>et al</i> ^[6]		SonoVue
D'Onofrio <i>et al</i> ^[31]		SonoVue
Hyodo <i>et al</i> ^[26]	Diagnosing cause of chronic pancreatitis/mass forming pancreatitis (autoimmune pancreatitis)	Levovist
D'Onofrio <i>et al</i> ^[31]		SonoVue
Zhu <i>et al</i> ^[30]		SonoVue
Kasono <i>et al</i> ^[7]	Localizing small insulinomas	Levovist

The feasibility of new technologies using CE ultrasound with microbubbles targeted to VEGF receptor type 2 are currently being tested^[46-48]. Several applications of molecular imaging and targeted ultrasound therapy can also be envisioned in the near future, including determination of the detailed physical processes behind sonoporation (increased uptake of drugs inside the cell through transient porositities in the cell membrane in the presence of contrast agents).

In this context, the development of CE-EUS will be clearly beneficial for targeted ultrasound imaging and ultrasound-assisted drug-delivery applications in gastrointestinal tract tumors, as well as other tumors accessible by EUS (pancreatic and lung tumors, *etc.*).

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S- Editor Sun H L- Editor Cant MR E- Editor Ma WH



Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Hybrid ultrasound imaging techniques (fusion imaging)

Daniela Larisa Sandulescu, Daniela Dumitrescu, Ion Rogoveanu, Adrian Saftoiu

Daniela Larisa Sandulescu, Daniela Dumitrescu, Ion Rogoveanu, Adrian Saftoiu, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Craiova 200638, Romania

Author contributions: Sandulescu DL drafted the manuscript; Rogoveanu I and Dumitrescu D gathered the data; Saftoiu A reviewed and edited the manuscript.

Supported by Research Grant No. 570/2008, Entitled "Integrative Prediction Model of Malignant Transformation of Regeneration Lesions in Cirrhotic Liver Using Noninvasive Imaging Techniques, Immunohistochemical and Molecular Genetic Methods" Financed by the Romanian Ministry of Education and Research-National Authority for Scientific Research (PN2-ID-PCE)

Correspondence to: Daniela Larisa Sandulescu, MD, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Petru Rares Street, No. 2, Craiova 200638, Dolj, Romania. larisasandulescu@yahoo.com
Telephone: +40-251-522458 Fax: +40-251-593077

Received: July 2, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: January 7, 2011

Key words: Hybrid imaging; Real time virtual sonography; Angiogenesis; Focal liver lesions

Peer reviewers: Giedrius Barauskas, Professor, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas, LT-50009, Lithuania; Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryō-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Sandulescu DL, Dumitrescu D, Rogoveanu I, Saftoiu A. Hybrid ultrasound imaging techniques (fusion imaging). *World J Gastroenterol* 2011; 17(1): 49-52 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/49.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.49>

INTRODUCTION

Conventional cross-sectional imaging techniques [ultrasound, computed tomography (CT), magnetic resonance (MR), etc.] have important roles in noninvasive diagnosis, and in tumor treatment strategies. The techniques employed have different working principles, consequently complementing each other with respect to the information obtained. The combination (fusion) of two imaging techniques was developed in recent years, defining the so-called "hybrid techniques" or "fusion imaging". Combinations of anatomical imaging techniques (ultrasound with CT or MR imaging), as well as associations between anatomical (CT or MR imaging) and molecular (SPECT or PET) imaging modalities are currently used in clinical practice.

TECHNICAL ISSUES

One example of fusion imaging is real-time virtual sonography (RVS), a technique that enables the display of an ultrasound B mode image and CT or MR images in real-time^[1]. The system includes a magnetic positioning sensor fixed on the convex-shaped probe of the ultrasound scanner, for the creation of images with identical cross-

Abstract

Visualization of tumor angiogenesis can facilitate non-invasive evaluation of tumor vascular characteristics to supplement the conventional diagnostic imaging goals of depicting tumor location, size, and morphology. Hybrid imaging techniques combine anatomic [ultrasound, computed tomography (CT), and/or magnetic resonance imaging (MRI)] and molecular (single photon emission CT and positron emission tomography) imaging modalities. One example is real-time virtual sonography, which combines ultrasound (grayscale, colour Doppler, or dynamic contrast harmonic imaging) with contrast-enhanced CT/MRI. The benefits of fusion imaging include an increased diagnostic confidence, direct comparison of the lesions using different imaging modalities, more precise monitoring of interventional procedures, and reduced radiation exposure.

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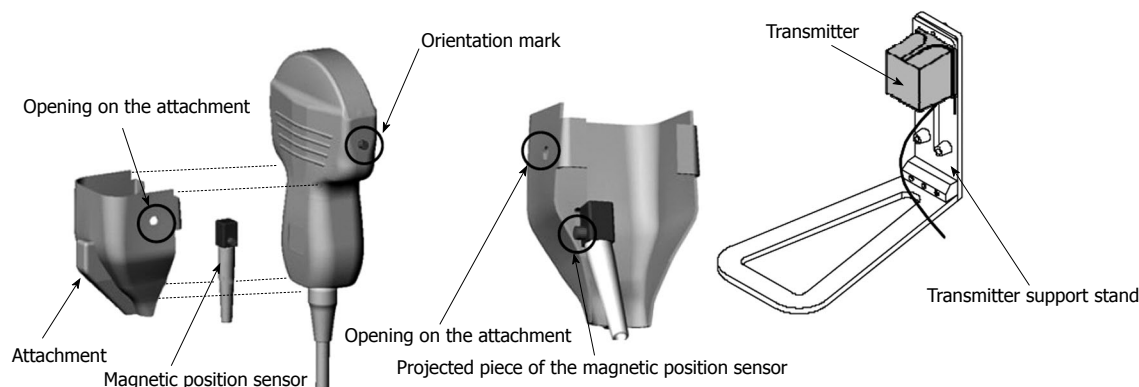


Figure 1 The probe and the magnetic sensor are assembled with careful attention to the positions of the orientation marks.

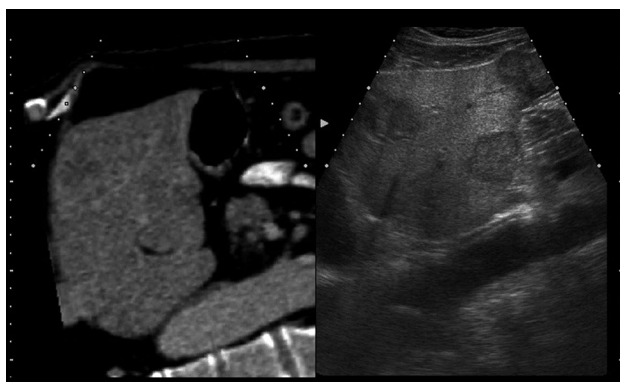


Figure 2 The left liver lobe ultrasound and computed tomography images are visualized at the same time in real-time virtual sonography.

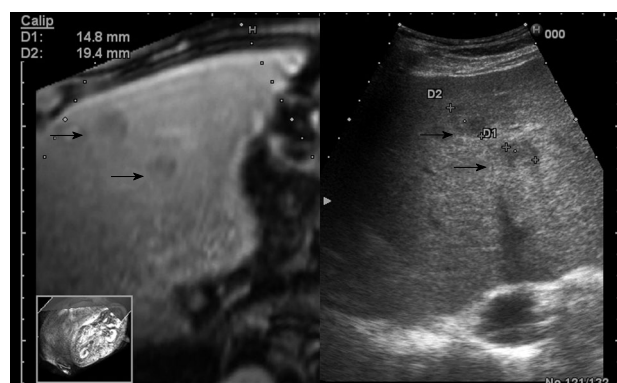


Figure 3 A 65-year-old male patient with liver metastases. The workstation monitor displays the ultrasound and computed tomography images in real-time.

sections in real-time. This is done according to the position and the angle of the probe in relation to previously acquired CT and MR volume data. To be compatible with the RVS module, CT examination must meet certain requirements: (1) the volume data must be archived in the DICOM format; (2) the slice thickness should be 3 mm or less and image reconstruction the same; and (3) the CT scan area must include the xiphoid process.

To display virtual images, it is necessary to transfer the CT or MR data to the ultrasound machine^[2]. The magnetic positioning sensor unit is carefully assembled, based on the position of orientation marks. It uses a distal attachment, which helps install the magnetic positioning sensor on the probe, as shown in Figure 1. The magnetic sensor detects the changes in location, direction, and rotation of the probe during normal ultrasound scanning of the patient. The transmitter (the instrument that produces the magnetic waves) for the magnetic positioning sensor unit is installed on the left flank of the patient. Using a probe equipped with the magnetic sensor, a sagittal section of the left hepatic lobe is then captured (Figure 2). The xiphoid process is usually chosen as a reference anatomical point. In case of a mismatch between the ultrasound and the virtual image (CT or MR), it is possible to readjust and correct the mismatch during the exam. The adjustment is made possible by freezing the CT/MR images on a section with clearly visible anatomical landmarks, for

example the portal vein bifurcation or the right kidney in a longitudinal view, followed by identification of a similar ultrasound image and continuation of examination^[3]. The workstation monitor displays two images: the ultrasound real-time image and the virtual reconstructed CT/MR image (Figure 3).

CLILINICAL APPLICATIONS

The founding principles of combining the ultrasound image and the CT/MR images were based on several observations. In recent years, frequent imaging investigations led to the discovery of small focal liver lesions, which can be treated locally by ultrasound-guided radiofrequency ablation (RFA) or other ablation techniques. However, ultrasound exams are cannot always identify isoechoic tumors, tumors recurring locally in areas treated with lipiodol following transarterial chemoembolization (TACE), or tumors recurring in areas treated previously by RFA or percutaneous ethanol injection (PEI) procedures^[4]. In addition, in ultrasound examinations, there are a few dead angles and it is difficult to examine the whole liver, especially in obese patients. Nodules that are poorly identified on ultrasound are clearly visible on CT/MR. However, interventional treatment is easier to perform under ultrasound-guidance, while the exposure to increased doses of radiation is also avoided. Thus, real-time virtual sonogra-

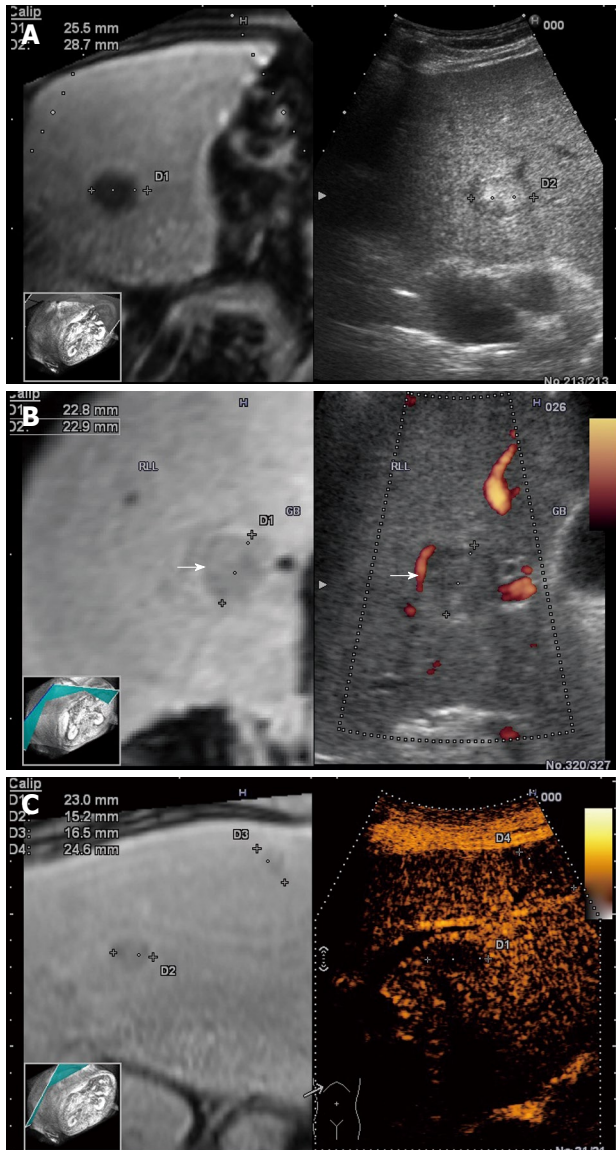


Figure 4 Real-time virtual sonography of liver nodules with simultaneous display of the contrast-enhanced magnetic resonance section and ultrasound images. 2D (A), Power Doppler (B), and contrast enhanced ultrasound (C) vs the reconstructed corresponding image.

phy combines the imaging advantages of both techniques. Several studies have already proved the feasibility of the RVS module, especially for percutaneous RFA of poorly visible or unidentifiable focal liver lesions during B-mode sonography^[5-7].

The RVS module is compatible with B-mode (Figure 4A), color Doppler imaging (CDI) mode (Figure 4B), and dynamic contrast harmonic imaging (D-CHI) mode (Figure 4C)^[8]. Therefore, RVS might have important clinical applications in the assessment of tumor angiogenesis. Hepatocellular carcinoma, a hypervascular tumor mainly supplied by hepatic arteries, shows a typical pattern in contrast-enhanced ultrasound: arterial hypervascularization (Figure 5A) with washout in the portal venous during the late phase (Figure 5B)^[9]. Real-time virtual ultrasound can reveal the contrast-enhanced ultrasound image in a similar way to contrast-enhanced CT/MR, although the

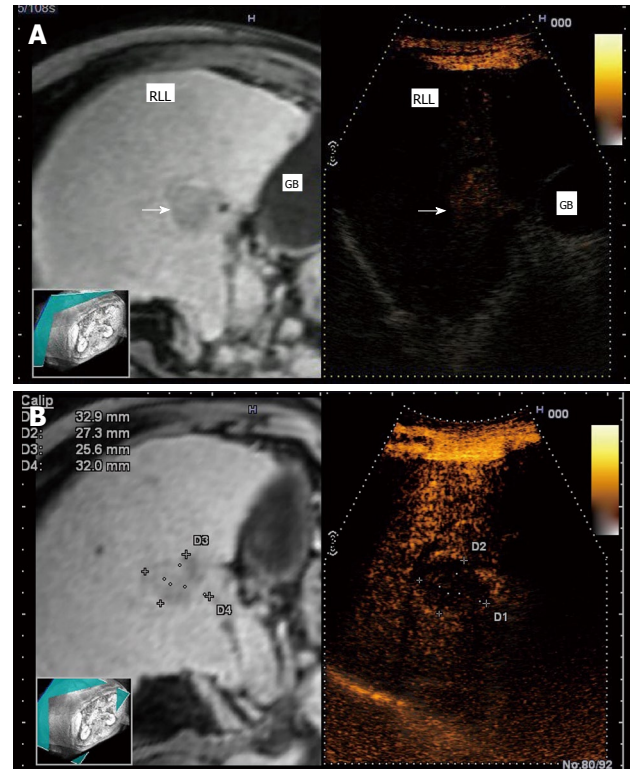


Figure 5 Single hepatocellular carcinoma (3 cm) nodule in a cirrhotic patient. In real-time virtual ultrasonography, after contrast agent injection, a progressive and intense enhancement is observed in the arterial phase (A), followed by wash-out in the tardive phase (B), on contrast-enhanced ultrasound, versus corresponding contrast-enhanced magnetic resonance images.

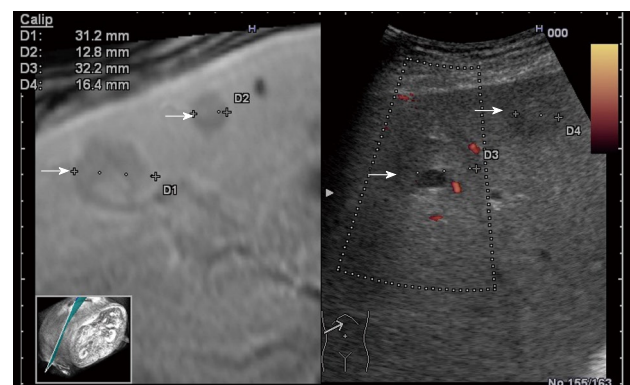


Figure 6 Real-time virtual sonography showing multiple metastases, better depicted on contrast-enhanced computed tomography as compared with ultrasound. A nodule in nodule aspect is visualized with excellent correlation on both contrast-enhanced magnetic resonance and real-time ultrasound.

mechanism of action is clearly different^[8]. Likewise, liver metastases that are poorly visible in 2D ultrasound can also be discovered in real-time virtual ultrasound, especially if contrast-enhancement is used (Figure 6). Although, the combined use of contrast-enhanced images has not yet been described in terms of accuracy for the positive and differential diagnosis of small focal liver lesions, RVS might combine the advantages of both methods (ultrasound and CT/MR).

For oncological patients, the tumor response to che-

motherapy is conventionally assessed by RECIST and WHO size criteria, obtained during CT/MR follow-up^[10]. Anti-angiogenic treatments induce lesion necrosis with no change in volume of the initial tumor; therefore, the use of size criteria appear inappropriate. Several studies have shown that the use of microbubble contrast agents can detect changes in vascularization, by calculation of maximal perfusion parameters, such as peak intensity, time to peak intensity, area under the curve, and slope coefficient of wash-in^[11-14]. Thus, the simultaneous use of real-time virtual sonography with dynamic contrast-enhanced ultrasound can be also employed. The method might allow the quantification of tumor perfusion for early assessment and quantitative monitoring of the efficacy of antiangiogenic agents, based on changes in vascularity, even before morphological changes become apparent^[10].

LIMITS

RVS cannot be used in patients if CT/MR is contraindicated (patients with known contrast medium allergies, renal failure, or metallic implants). Another limitation of RVS is that the technique does not always show the best synchronization between ultrasound and CT/MR images, although the discrepancy can be adjusted with careful visualization of neighboring portal and hepatic veins in most cases^[8]. The use of the RVS module does prolong the examination time; therefore, the clinical impact, in terms of improved decision making, should be further assessed. The technique adds the costs of CT/MR to the costs of the contrast-enhanced ultrasound exam; thus the cost-effectiveness of this approach must be evaluated in future studies^[15].

CONCLUSION

In conclusion, hybrid ultrasound imaging techniques now play a pivotal role in diagnosis, staging, and follow-up during treatment. During local ablation of focal liver lesions hybrid ultrasound imaging allows better control of the procedure and less radiation exposure.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Ma WH

Adrian Săftoiu, MD, PhD, Professor, and Peter Vilmann, MD, PhD, Professor, Series Editors

Optical molecular imaging for detection of Barrett's-associated neoplasia

Nadhi Thekkek, Sharmila Anandasabapathy, Rebecca Richards-Kortum

Nadhi Thekkek, Rebecca Richards-Kortum, Department of Bioengineering, Rice University, Houston, TX 77005, United States
 Sharmila Anandasabapathy, Division of Gastroenterology, Mount Sinai Medical Center, New York, NY 10029, United States
 Author contributions: Thekkek N performed the research; Thekkek N, Anandasabapathy S and Richards-Kortum R reviewed the data; Thekkek N and Richards-Kortum R wrote the paper.

Supported by The National Institute of Health Grants BRP CA103830 and RO1 EB007594

Correspondence to: Rebecca Richards-Kortum, PhD, Professor, Department of Bioengineering, Rice University, 6100 Main St, MS 142, Houston, TX 77005, United States. rkortum@rice.edu

Telephone: +1-713-3483823 Fax: +1-713-3485877

Received: July 2, 2010 Revised: November 17, 2010

Accepted: November 24, 2010

Published online: January 7, 2011

Key words: Barrett's esophagus; Barrett's metaplasia; Dysplasia; Esophageal adenocarcinoma; Endoscopy; Imaging

Peer reviewers: Ralph Graeser, PhD, Group Leader, Molecular and Cellular Biology, ProQinase GmbH, Breisacher Str. 117, Freiburg, 79106, Germany; Alexander S Rosemurgy, MD, FACS, Professor, Department of Surgery, Department of Medicine, University of South Florida, Tampa General Hospital, PO Box 1289, Room F145, Tampa, Florida, FL 33601, United States

Thekkek N, Anandasabapathy S, Richards-Kortum R. Optical molecular imaging for detection of Barrett's-associated neoplasia. *World J Gastroenterol* 2011; 17(1): 53-62 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/53.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.53>

Abstract

Recent advancements in the endoscopic imaging of Barrett's esophagus can be used to probe a wide range of optical properties that are altered with neoplastic progression. This review summarizes relevant changes in optical properties as well as imaging approaches that measures those changes. Wide-field imaging approaches include narrow-band imaging that measures changes in light scattering and absorption, and autofluorescence imaging that measure changes in endogenous fluorophores. High-resolution imaging approaches include optical coherence tomography, endocytoscopy, confocal microendoscopy, and high-resolution microendoscopy. These technologies, some coupled with an appropriate contrast agent, can measure differences in glandular morphology, nuclear morphology, or vascular alterations associated with neoplasia. Advances in targeted contrast agents are further discussed. Studies that have explored these technologies are highlighted; as are the advantages and limitations of each.

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BACKGROUND

The incidence of esophageal adenocarcinoma (EAC) is rapidly increasing; over the last 40 years, the incidence rate of EAC has risen by over 300% in the United States^[1]. This is of particular concern because the overall 5-year survival rate for patients diagnosed with EAC is only 12%^[2]. Although detecting and treating esophageal neoplasia at an early stage has been reported to increase 5-year survival to rates as high as 81%^[3], current methods of early detection have significant limitations. As a result, more than 60% of patients with EAC are diagnosed at a late stage, after local, regional, or distant metastases have occurred^[4].

EAC arises primarily in patients with Barrett's esophagus (BE)^[5,6], which is a highly prevalent condition in which the squamous epithelium of the esophagus is replaced by intestinal metaplasia (IM) near the gastroesophageal (GE) junction^[7-9]. Because of this increased risk, patients with BE undergo regular surveillance endoscopy at designated intervals in an attempt to identify neoplastic lesions at an early stage^[10,11]. Surveillance involves endoscopic examination with random four-quadrant biopsies

taken every 1-2 cm along the BE segment^[10].

Despite surveillance efforts, routine biopsy protocols have been shown to miss up to 57% of neoplastic lesions in patients with BE^[12]. This is largely due to the fact that dysplasia or neoplasia may be focal, flat and endoscopically indistinguishable from non-neoplastic epithelium on routine white-light endoscopy (WLE). The ability to delineate better superficial mucosal changes associated with early neoplasia at a macroscopic level, and subsequently, identify the subcellular changes associated with neoplastic progression would greatly enhance the yield and efficacy of current surveillance practices.

CHANGES IN OPTICAL PROPERTIES

In a standard WLE examination, the endoscopist views white light reflected from the surface of the esophagus; although visual examination of reflected white light can identify some changes in tissue morphology associated with neoplasia, it does not exploit the full range of changes in tissue optical properties that are associated with dysplasia and cancer. Neoplasia alters the light absorption and scattering properties of esophageal tissue^[13,14]; in addition, neoplasia is associated with changes in the autofluorescence properties of esophageal tissue^[13,15-17].

A number of new endoscopic approaches have been developed to more effectively probe neoplasia-related changes in optical properties to improve visualization of early neoplastic lesions. For example, the color of illumination light can be optimized to probe better changes in tissue absorption and/or scattering. Autofluorescence endoscopy can be used to image changes in tissue fluorescence that are associated with neoplasia. Moreover, improving spatial resolution of endoscopic imaging can help reveal changes in cellular architecture and morphology associated with neoplasia. Finally, optically active contrast agents can be used to improve further image contrast and probe specific molecular and morphologic features of neoplastic tissue that may not be associated with changes in native optical properties.

Here, we first review changes in the optical properties of esophageal tissue associated with neoplasia, and then outline new endoscopic imaging approaches to use these changes to improve early detection of esophageal neoplasia. Finally, we discuss the use of targeted contrast agents to expand the range of molecular changes that can be imaged *in vivo*.

Neoplasia-associated changes in tissue light scattering and absorption

Light attenuation in esophageal tissue is governed by a combination of absorption and scattering. In the visible region of the spectrum, the primary source of light absorption in esophageal tissue is hemoglobin. Esophageal neoplasia is associated with increased angiogenesis^[18], and endoscopic imaging approaches to enhance vascular contrast may improve early detection^[19,20]. Oxyhemoglobin has absorption peaks at 420, 542, and 577 nm^[13]; examining the tissue at these illumination wavelengths can enhance

vascular contrast, with vasculature appearing visibly darker than the surrounding tissue due to the increase in light absorption^[21]. Neovascularization is an important quantifiable tool for distinguishing neoplasia from non-neoplastic Barrett's epithelium. Irregular angiogenesis occurs within the lamina propria at various levels of the mucosal layer in high-grade dysplasia (HGD) and cancer. These features have been verified by analysis of microvessels and overexpression of relevant markers such as vascular endothelial growth factor and CD34, which results in a statistically significant difference between the microvessel density in BE versus HGD and cancer^[19,20].

Light scattering in tissue is a result of spatial fluctuations in the refractive index. In general, the scattering of stroma is significantly greater than that of the epithelium and is the dominant source of reflected white light from intact tissue. Neoplasia is associated with a small decrease in stromal scattering that is attributed to degradation in collagen fibers, possibly due to proteases secreted by pre-neoplastic epithelial cells^[18,22,23]. The attenuation of light in tissue is wavelength dependent, with longer red wavelengths able to penetrate more deeply than shorter blue wavelengths. Thus, tuning the illumination wavelength provides some ability to control penetration depth, and highlight vascular contrast.

Neoplasia-associated changes in tissue autofluorescence

Some endogenous constituents of esophageal tissue can reemit absorbed light in the form of fluorescence. Endogenous fluorophores are found in both the epithelium and the stroma of esophageal tissue, and fluorescence imaging provides a way to monitor changes in the concentration and composition of these fluorophores. When esophageal tissue undergoes malignant transformation, endogenous fluorophores undergo alterations^[24-26], which can be probed *via* autofluorescence imaging (AFI), to detect abnormalities that may not be visible during standard WLE. Tuning the excitation wavelength provides a way to selectively probe various fluorophores that can then be quantified by measuring light intensity at specific emission wavelengths^[26].

The primary fluorophores within the epithelium include mitochondrial NADH and FAD found in epithelial cells. Epithelial cells show cytoplasmic autofluorescence attributed to NADH using UV excitation wavelengths (330-370 nm) and FAD using green excitation wavelengths (510-550 nm)^[27,28]. Levels of mitochondrial NADH^[15] and mitochondrial FAD increase due to dysplastic changes in the epithelium^[29,30].

Stromal fluorescence of esophageal tissue is predominantly associated with covalent collagen crosslinks, which are characterized by relatively high autofluorescence intensity across a broad range of UV, blue, and green excitation wavelengths^[16]. Esophageal neoplasia is associated with a loss of stromal autofluorescence, which has been attributed to a decrease in collagen crosslinking^[18,22,23]. Finally, invasive esophageal cancers are often associated with porphyrin fluorescence, with maximal excitation near 400 nm and emission in the red spectral region^[13,31,32].

Table 1 Advantages and disadvantages of optical technologies for identification of neoplasia in Barrett's esophagus

Technology	Advantages	Disadvantages	Stage of clinical translation
Standard WLE	Capable of scanning wide area, widely available outside of tertiary care centers, no exogenous contrast	Limited sensitivity and specificity	Commercially available
High-definition WLE	Capable of scanning wide area, increased image contrast, no exogenous contrast	Performance evaluated in moderate-sized studies	Commercially available
AFI	Capable of scanning wide area, consistently high sensitivity, no exogenous contrast	High rate of false positives, performance evaluated only in small pilot studies	Commercially available
NBI	Capable of scanning wide area, consistently high sensitivity, no exogenous contrast	Performance evaluated in small pilot studies	Commercially available
OCT	Resolves subsurface structure, no exogenous contrast	Technology still under development	Clinical studies
Endocytoscopy	Histology-like imaging, high specificity	Low sensitivity, limited field of view, requires exogenous contrast	Commercially available
CME	Nuclear morphology can be viewed, high sensitivity and specificity	Limited field of view, high cost, uses IV exogenous contrast	Commercially available
High-resolution microendoscopy	Some nuclear morphology can be viewed, lower cost, adaptable to any endoscope	Limited field of view, requires exogenous contrast, technology still in development	Clinical studies

AFI: Autofluorescence imaging; OCT: Optical coherence tomography; CME: Confocal microendoscopy; WLE: White-light endoscopy; NBI: Narrow-band imaging.

High-resolution imaging

The spatial resolution of optical imaging is governed by diffraction, and with visible wavelengths of light, subcellular resolution imaging is possible. Typically, standard endoscopic imaging approaches do not achieve diffraction-limited resolution, however, recent advances in high-resolution imaging techniques such as optical coherence tomography (OCT), endocytoscopy, and endomicroscopy afford the ability to image with subcellular resolution. Such approaches are often termed "optical biopsy", because they allow visualization of glandular and cellular alterations associated with neoplasia. Optical contrast in high-resolution imaging is governed by the same alterations in tissue absorption, scattering and fluorescence described above. In addition, optically active contrast agents are often used to increase contrast for high-resolution imaging.

IN VIVO ASSESSMENT OF IMAGING TECHNOLOGIES

In the past decade, advances in imaging technologies have enabled gastroenterologists to optically image Barrett's-associated neoplasia with better contrast *in vivo*. The development of wide-field imaging technologies affords clinicians a macroscopic view of the tissue, serving as a "red-flag technique" for relevant abnormalities. High-resolution technologies assess microscopic features of the tissue and, if coupled with an ideal source of contrast, may measure biochemical, molecular, and vascular changes. Table 1 summarizes a number of different optical technologies currently under investigation, describes the advantages and disadvantages of each, and describes which stage they have reached in terms of clinical translation. Table 2 summarizes the accuracy of the technologies that have been translated to clinical use and have been used in large clinical trials.

Narrow-band imaging

Narrow-band imaging (NBI) is a wide-field imaging technology that takes advantage of changes in light scat-

Table 2 Summary of performance of emerging optical technologies

Type of detection	Study size	Sensitivity, specificity
AFI	60 patients, 116 images	91%, 43% ^[36]
NBI	63 patients, 175 images	94%, 76% ^[33]
	51 patients, 204 images	100%, 98% ^[34]
	21 patients, 75 images	89%, 95% ^[38]
High-resolution imaging (1-15 μ m resolution)		
OCT	33 patients, 314 images	68%, 82% ^[43]
	55 patients, 177 images	83%, 75% ^[58]
Endocytoscopy	16 patients, 166 images	56%, 68% (425 \times)
		42%, 83% (1125 \times) ^[47]
Confocal imaging	63 patients, 433 images	93%, 98% ^[48]
	38 patients, 296 images	75%, 90% ^[50]

AFI: Autofluorescence imaging; OCT: Optical coherence tomography; NBI: Narrow-band imaging.

tering and absorption in neoplastic tissue. Systems that implement NBI illuminate tissue with one or more narrow-band wavelength ranges corresponding to hemoglobin absorption peaks. Reflected light in these bandwidths is recombined to create a digital image with enhanced vascular contrast. This approach can also enhance visualization of villous mucosal patterns due to lining of vessels in mucosal folds^[21]. An example is shown in Figure 1.

For example, one NBI system combines information from three wavelength ranges: 400-430 nm (blue), 530-550 nm (green), and 600-620 nm (red). Higher relative intensity from the blue region is used to enhance surface level vasculature associated with neoplasia, due to its shallow penetration depth. In a 63 patient study using this approach, researchers in Amsterdam used features such as mucosal morphology and vascular contrast to determine grade of disease. The presence and regularity of these patterns were found to be essential for image evaluation. Out of the 175 areas, 52 were used as training material for endoscopists and the remaining 123 were used as a validation set. In the validation set, 94% of HGD images were



Figure 1 Endoscopic images from an area positive for esophageal adenocarcinoma. Abnormal areas (arrow) can be seen in the high-resolution white light image (A), and the narrow-band image (B) [Copyright (2008), with permission from Elsevier]^[36]; in the narrow-band image, the irregular mucosal morphology is visible (arrow); an abnormal area (arrow) can be seen in the autofluorescence image (C) where areas with loss of fluorescence are indicated as purple regions in the pseudo-colored overlay [Copyright (2005), with permission from Elsevier]^[36].

noted to show irregular or disrupted villous/gyrus mucosal pattern, and 85% were noted to show irregular vascular patterns. Using these features and others, they developed a multi-step hierarchical classification system based on mucosal morphology, including features such as type and regularity of mucosal patterns, regularity of vasculature patterns, and presence and type of abnormal blood vessels. Using this multistep evaluation, they determined the overall sensitivity and specificity to be 94% and 76%, respectively^[33]. Similarly promising performance was also obtained using the same NBI system in a 51 patient study by Sharma and colleagues; sensitivity and specificity for detection of HGD were 100% and 99%, respectively^[34].

Of continued debate, however, is the question of how NBI compares to high-definition white-light endoscopy (HD-WLE) using the current generation of endoscopes. This new generation of endoscopes offers markedly higher pixel densities and high-definition images that result in increased contrast in villous mucosal patterns, and a marked improvement in resolution^[35,36] over standard WLE^[37]. In a study with 65 patients, Wolfsen and colleagues, using a narrow-band system in which only two of the shorter wavelength ranges associated with hemoglobin were used, observed that the combination of HD-WLE and NBI did find higher grades of dysplasia in 18% of the study patients, using fewer biopsies than for standard endoscopy. They also observed that out of five of the cases in which HGD or EAC was detected, three were detected by HD-WLE as well. Although results favored NBI, the study was not designed to determine the efficacy of one modality over the other^[38]. Another study by Curvers and colleagues has observed that, while expert endoscopists preferred the image contrast provided by NBI, this did not improve overall interobserver agreement or accuracy when compared to HD-WLE^[39,40]. Larger studies are needed to determine which is the more accurate technique.

AFI

AFI can also increase contrast between non-neoplastic and neoplastic sites, as a result of the loss of autofluorescence associated with esophageal neoplasia. Typically, tissue auto-

fluorescence is excited in the blue region (395–475 nm) and fluorescence emission is collected at longer wavelengths (> 490 nm) to detect changes in fluorophores associated with malignant transformation. Because the intensity of autofluorescence can be low, this technique requires the use of highly sensitive CCDs to collect the autofluorescence signal. In recent systems, reflected light is also collected through a second CCD. Co-registered images can be used to compensate for changes in fluorescence intensity associated with variations in illumination and distance from the tip of the endoscope to the tissue, thereby further enhancing autofluorescence contrast. The resulting effect is pseudo-colored purple to highlight neoplastic lesions^[36,37]. An example is shown in Figure 1.

In a recent 60 patient study using a standard endoscope with an added AFI component, Kara was able to detect HGD in 22 patients, 14 of which were detected with AFI and WLE, and six of which were detected using AFI alone; thereby increasing the detection rate from 23% to 33% using AFI. Only one of the patients was diagnosed using four-quadrant biopsies alone^[36]. Results suggest that AFI may aid in the detection of additional HGD sites; however, it may not exclude the need for the standard four-quadrant biopsies. Sensitivity and specificity based on the 116 samples used for this study were 91% and 43%, respectively. Although no patient was diagnosed without AFI and four-quadrant biopsies, they cite a high rate of false positives using AFI alone, due in part to the loss of autofluorescence associated with acute inflammation^[36].

Although individually these enhanced endoscopic technologies have shown success, the high rate of false positives is a major drawback. To address this limitation, a combination of modalities is being explored to utilize the benefits of each; potentially increasing the accuracy of detection at the point of surveillance. Kara and colleagues have conducted a 20 patient pilot study in which HD-WLE and AFI were used initially to locate suspicious lesions. Once the lesions were identified, an NBI scope was introduced for detailed inspection of vascular and mucosal patterns. They found that 40% of the HGD lesions were discovered with AFI alone. However, the false-positive rate of

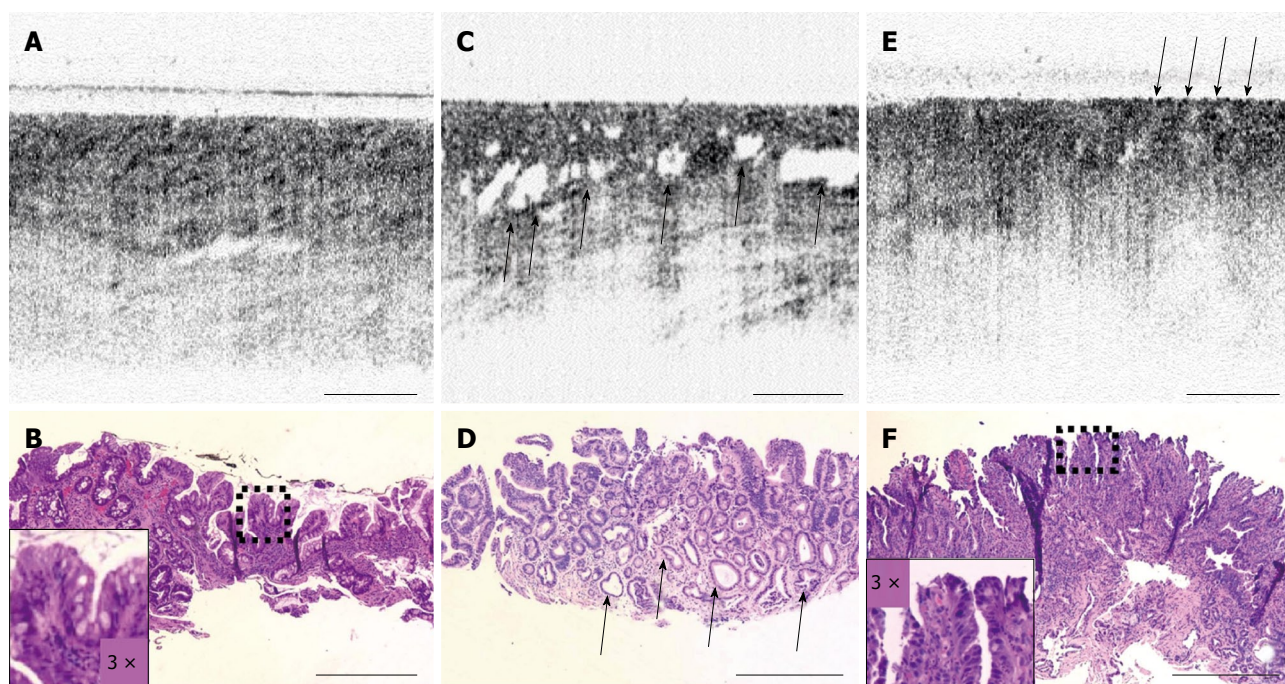


Figure 2 Optical coherence tomography images of intestinal metaplasia (A), and of neoplasia (C, E) are shown with corresponding histological images shown below [Copyright (2006), with permission from Elsevier]^[43], dilated glands (C) and increased surface reflectivity (E) can be seen in the optical coherence tomography images of neoplastic tissue, corresponding histopathology is shown (B, D, F). Scale bars, 500 μm .

the modality was 40% and the positive predictive value was 60%. Following NBI inspection, the false-positive rate was reduced to 10%, which achieved a positive predictive value of 85%^[41]. A more recent study with one scope containing both modalities achieved similar results. In that study, AFI was able to detect more lesions than high-resolution WLE alone, however, the false-positive rate remained a high 81%; following detailed inspection with NBI the rate was reduced to 26%^[42]. In both cases however, random four-quadrant biopsies detected additional lesions that the optical modalities did not identify, which indicates the need for further development of these and other technologies.

High-resolution imaging

Wide-field imaging techniques, such as AFI and NBI, were developed to measure large surface areas of gastrointestinal tissue. More recently, high-resolution systems have been developed to achieve near diffraction-limited imaging from small fields of view. Four primary approaches have been pursued to increase spatial resolution. OCT can image esophageal tissue with 10–15 μm resolution and a penetration depth of 1–2 mm. Endocytoscopy can image surface level esophageal tissue with up to 1–2 μm resolution using the highest magnification setting. Confocal microscopy can image esophageal tissue with 1–2 μm spatial resolution with a penetration depth of 300–400 μm . High-resolution microendoscopy can image surface level esophageal tissue with 4–5 μm spatial resolution. Recent clinical studies with these modalities highlight the benefits and limitations of high-resolution imaging.

OCT uses variations in the time it takes light to be reflected from structures beneath the tissue surface to image

sub-surface tissue structures as seen in Figure 2, in a manner analogous to ultrasound imaging. In a 55 patient study, researchers have determined that OCT could differentiate HGD and EAC from BE with a sensitivity of 83% and a specificity of 75%^[43]. An advantage of OCT is that it relies on endogenous differences in light scattering to generate image contrast. OCT may be a particularly useful tool in the detection and surveillance of sub-squamous BE because of its relatively greater depth of penetration^[44]. However, the technology is still under development^[45] and further clinical studies are needed to assess performance in a wide variety of clinical settings.

Endocytoscopy uses a probe that is passed through the instrumentation channel of an endoscope to image with subcellular resolution. Essentially, high-resolution epi-reflectance microscopy is used with methylene blue contrast to highlight relevant nuclear features (Figure 3A and B). Although models vary, there are generally two types each with different magnifications settings; one at 450 \times where the field of view can be as wide as 300 μm \times 300 μm , and a higher magnification setting of 1125 \times where a field of view as small as 120 μm \times 120 μm is made visible^[46]. A large study evaluating 166 sites in 16 patients with endocytoscopy by Pohl and colleagues reported a sensitivity and specificity of 42% and 83%, respectively^[47]. Although high specificity was encouraging, they did emphasize the need for an initial wide-field surveillance technique to identify suspicious areas. This technology is certainly promising; however, larger studies need to be performed.

Confocal microendoscopy (CME) images subsurface tissue structure with high resolution by using a spatial filter to reduce the background signal produced by scat-

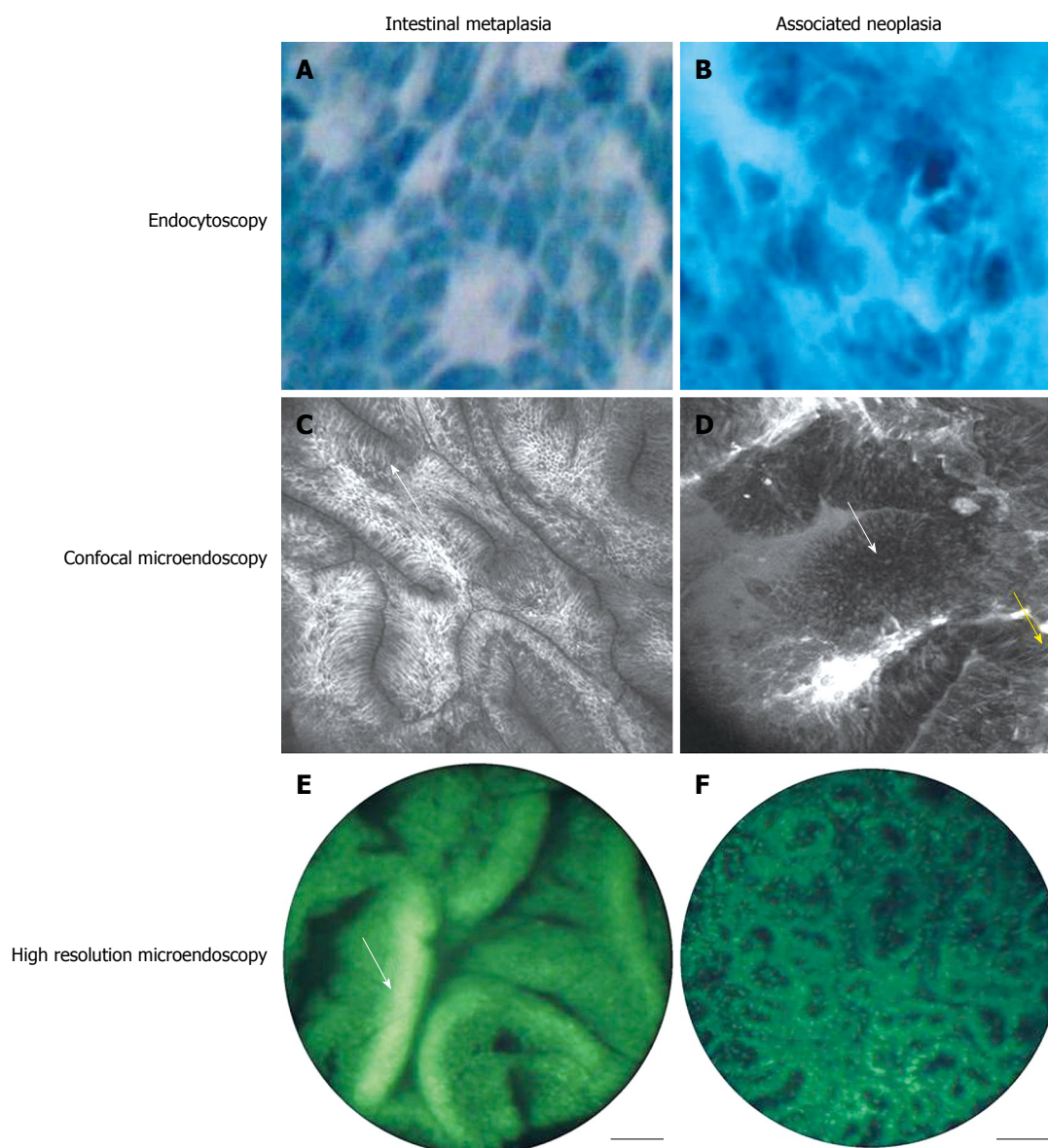


Figure 3 Images representing intestinal metaplasia and neoplasia collected using endocytoscopy (A, B) [Copyright (2007), with permission from Thieme]^[47], confocal microendoscopy (C, D) [Copyright (2006), with permission from Elsevier]^[48], and high-resolution microendoscopy (E, F) [Copyright (2008), with permission from Elsevier]^[51]. Typically applied methylene blue is used in endocytoscopy to highlight nuclear changes (A, B); In metaplasia (A), nuclei appear organized and regular; this is in stark contrast to neoplasia (B) where nuclei appear pleomorphic. Both images were taken using 1125 × magnification. Confocal images were taken using intravenous fluorescein to enhance contrast of subepithelial capillaries (C, D); for intestinal metaplasia (C), confocal microendoscopy allows visualization of mucin-containing goblet cells (white arrow); For Barrett's-associated neoplasia (B), cells are irregularly oriented (white arrow) and malignant invasion of the lamina propria can be seen (yellow arrow). Confocal images are 500 μm × 500 μm . High-resolution microendoscopy uses proflavine for contrast enhancement, highlighting changes in glandular and nuclear patterns (E, F). High-resolution images are 750 μm in diameter.

tered out-of-focus light, which produces images with 1-2 μm spatial resolution. Although CME images can be generated either in reflectance or fluorescence mode, in the context of esophageal imaging, fluorescence CME has been primarily used. Since tissue autofluorescence is weak, typically fluorescent contrast agents are used to generate image contrast in CME. Kiesslich and researchers conducted a 63 patient study in Germany using an endoscope that incorporated standard WLE and confocal microscopy; fluorescein (10% w/v) was administered intravenously to generate vascular contrast. Subepithelial capillaries located in the upper and deeper layers of the lamina propria were identified due to fluorescein con-

trast. Leakage of fluorescein due to irregular capillary formation indicated neoplastic areas (Figure 3C and D). Indeed, due to these irregularities, neoplasia could be detected with a sensitivity and specificity of 94% and 98% respectively^[48]. In a prospective, randomized, double-blind, controlled, crossover study with 39 patients using the same system, CME with targeted biopsy was shown to not only be accurate, but to nearly double the diagnostic yield of collected biopsies. In examining the biopsies identified by standard four-quadrant biopsies and the biopsies identified by CME, there was no statistically significant difference in detection of neoplasia between the two techniques^[49]. However, although accuracy and

diagnostic yield is impressive, the high cost may limit this technology to tertiary care centers.

A fiber-bundle, probe-based confocal system that can be passed through the instrument channel of any standard endoscope was used in a 38 patient study by Pohl and other researchers. A major benefit of this technology is its adaptability to existing endoscopes. This system also requires exogenous contrast; fluorescein was administered intravenously. The sensitivity and specificity of the two study endoscopists were 75% and 89% and 75% and 91%, respectively. They concluded that the confocal fiber probe showed a high negative predictive value for detecting unapparent neoplasia in BE; however, sensitivity was not ideal^[50].

An alternative approach to high-resolution fluorescence imaging uses a coherent fiber bundle placed in direct contact with the surface of tissue labeled with fluorescent dyes to yield high resolution images that reveal subcellular structure (Figure 3E and F)^[51]. This low-cost alternative to confocal imaging may be suited for community-wide surveillance outside of tertiary care centers. In a small pilot study of nine patients, with topical proflavine for contrast enhancement of cell nuclei, researchers achieved a sensitivity and specificity of 87% and 85% using fluorescence microendoscopy^[52].

Contrast enhancement

As optical imaging technology continues to advance, the concurrent development of appropriate contrast agents that target biomarkers of neoplasia is crucial. Two general classes of optical contrast agents have been explored to improve image contrast: vital dyes and targeted contrast agents. Absorbing or fluorescent dyes that have an affinity for specific tissue constituents have often been used to improve the ability to visualize specific features associated with neoplasia. Often referred to as vital dyes, these stains can help delineate features such as angiogenesis, leaky vasculature, and cell morphology. In contrast, targeted contrast agents use a high affinity probe molecule to target a specific molecular biomarker associated with neoplasia^[53]. The probe molecule must be coupled to an optically active component, such as a fluorescent dye or scattering nanoparticle. Here, we briefly review the utility of both types of contrast agents for improved detection of esophageal neoplasia.

Vital dyes can be utilized to delineate better morphological changes associated with epithelial neoplasia. For example, the absorptive dye methylene blue localizes primarily in nuclei and can enhance visualization of nuclei when coupled with appropriate high-resolution instrumentation. Using an endocytoscope, nuclear characteristics associated with neoplasia such as homogeneity, nuclear-to-cytoplasmic ratio, and organization can be resolved. However, since methylene blue dye is known to induce oxidative damage of DNA when exposed to white light illumination^[54], the risks of the contrast agent need to be weighed against the benefits to determine potential use.

Fluorescent vital dyes may be advantageous due to the lack of interference with standard endoscopy. Fluorescein

is a dye that is administered intravenously, thus enhancing the view of vasculature in epithelial tissue. When coupled with confocal imaging, subsurface vasculature can be seen. The illumination and collection wavelengths of commercially available confocal systems correspond to fluorescein excitation (about 490 nm) and emission (about 520 nm)^[48,50]. Acriflavine is another vital fluorescent dye that can be seen using similar excitation (about 450 nm) and emission (about 510 nm) wavelengths. Acriflavine stains cell nuclei, highlighting nuclear characteristics such as size, shape, and spacing, and has been used previously *in vivo* for gastrointestinal imaging^[55].

Targeted contrast agents serve as beacons that signal specific molecular events associated with pre-cancer formation. The benefit of targeted agents is the potential to achieve a high signal to background ratio by virtue of selective binding to a molecular target. Lu and researchers used a phage display library with about 2.8×10^9 unique sequences to select a cancer-specific peptide. The library was biopanned against three cultured human esophageal cell types: adenocarcinoma, metaplasia, and normal, to identify a peptide with specificity for the adenocarcinoma cell line. They used the selected peptide labeled with FITC to image Barrett's-associated neoplasia *in vivo*. The agent was topically applied and imaged with a concurrently developed prototype fluorescence endoscope. Initial results showed a significant increase in binding to Barrett's-associated neoplasia over Barrett's alone when imaged with wide-field fluorescence imaging (Figure 4)^[56]. In a different study, Hsiung and colleagues fluorescently labeled a high-affinity heptapeptide sequence selected with similar phage display techniques for the colon, and were able to differentiate dysplastic from non-dysplastic colonic crypts using confocal imaging^[57]. In both of these cases, the topically applied contrast agent was incubated *in vivo* for a short period of time before the unbound agent was washed off to reduce non-specific signals. Although the excitation and emission wavelengths of these agents correspond well with commercially available confocal endoscopes, another important advantage demonstrated by these studies is the ability to image these agents with both wide-field fluorescence and CME.

DISCUSSION

Recent advances in imaging technologies afford visualization of endogenous optical alterations associated with gastrointestinal neoplasia. NBI shows contrast associated with light absorption due to hemoglobin. High sensitivity and specificity is cited in studies using this technology, however some indicate that there is no significant difference between contrast associated with NBI imaging and HD-WLE, which is becoming increasingly available. AFI measures the signal decrease associated with loss of stromal collagen fluorescence and increased fluorescence associated with porphyrin. Various studies evaluating AFI have cited high sensitivity, but a high rate of false positives. The combination of NBI and AFI may afford better sensitivity and specificity rates; NBI has shown to reduce

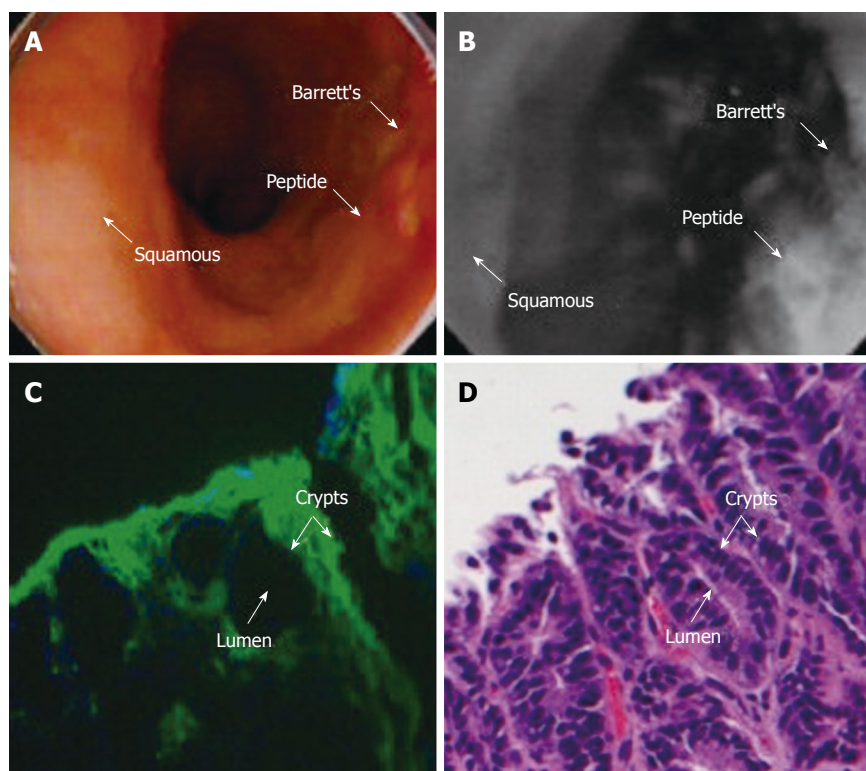


Figure 4 *In vivo* localization of contrast agent localized to a neoplasia region visualized using wide-field fluorescence endoscopy. White light endoscopic image (A) shows no evidence of lesion; topical administration of peptide-targeted fluorescent dye reveals neoplastic area (B) [Copyright (2008), with permission from IOS Press]^[56]; targeted neoplastic crypts seen with fluorescence microscopy (C), and corresponding histology (D) [Copyright (2010), with permission from Elsevier]^[59].

the number of false positives identified by AFI from 81% to 26%^[42].

High-resolution imaging will also play a major role in improving detection, affording clinicians an “optical biopsy” of epithelial tissue. Confocal imaging allows for optical sectioning of up to 250 μm deep, and coupled with vital dyes such as fluorescein, allows evaluation of vascular regularity. High sensitivity and specificity have been cited; however, high cost and the limited field of view remain concerns. Endocytoscopy allows for histology-like reflectance imaging where nuclei appear dark blue due to methylene blue contrast. The technology achieves high specificity; however, the dye has been shown to interfere with white light imaging and image quality has been an issue. When combined with wide-field imaging techniques, high-resolution technologies may reduce false-positive rates if coupled with the appropriate contrast agent.

Unfortunately, despite all the advances in optical imaging methods, there are still lesions that are only detected by standard four-quadrant biopsies. Improvements in contrast agents are also needed to facilitate early detection. A number of contrast agents are commercially available; primarily vital dyes such as fluorescein and methylene blue. However, recent *in vivo* testing of optically labeled high-affinity peptide and heptapeptide sequences has paved the way for molecule-specific contrast agents for gastrointestinal neoplasia^[56,57]. Although advances have translated the use of vital dyes and contrast agents *in vivo*, there are still many unanswered questions regarding their ultimate clinical role. What will be the ideal mechanism of delivery?

How will the development of *in vivo* imaging technologies accommodate the use of new contrast agents? Finally, will the addition of contrast agents create a multifaceted platform that can improve overall accuracy of surveillance?

Although these new imaging technologies may be appropriate for tertiary care centers, additional considerations are necessary as these technologies are disseminated more widely. A potential solution may be a lower cost technology such as the high-resolution microscope, or an adaptable technology such as the confocal miniprobe with topically applied contrast agents; both of which have been cited to achieve reasonably high sensitivity and specificity. Objective, quantitative algorithms will also be important because clinicians outside of tertiary care clinics may not be as familiar with optical characteristics of abnormal lesions detected with new technologies. Various groups have begun work in this area; however, larger trials need to be conducted to determine effectiveness^[52,58].

At this point, larger studies are needed to test the combination of multi-scale, multi-modal technologies against the current surveillance standard, and to test whether the use of contrast agent is advantageous. This multifaceted optical approach has the potential to improve surveillance in BE; once validated, it has the potential to be utilized for surveillance of neoplasia along the gastrointestinal tract and can be further developed for screening.

DISCLOSURE STATEMENT

Thekkek N and Anandasabapathy S have no financial

conflicts to disclose. One of the co-authors (Richards-Kortum R) has a small ownership interest in Remicalm, Inc which has licensed related technology from the University of Texas at Austin and Rice University.

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New endoscopic approaches in IBD

Helmut Neumann, Markus F Neurath, Jonas Mudter

Helmut Neumann, Markus F Neurath, Jonas Mudter, Department of Medicine I, University of Erlangen-Nuremberg, 91054 Erlangen, Germany

Author contributions: Neumann H contributed to the study idea, study design, literature search, manuscript writing and final revision of the article; Neurath MF contributed to the manuscript writing and the final revision of the article; Mudter J contributed to study design, manuscript writing and the final revision of the article.

Correspondence to: Helmut Neumann, MD, Department of Medicine I, University of Erlangen-Nuremberg, Ulmenweg 18, 91054 Erlangen, Germany. helmut.neumann@uk-erlangen.de

Telephone: +49-9131-8535000 Fax: +49-9131-8535209

Received: August 14, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: January 7, 2011

Abstract

Recent advances in endoscopic imaging techniques have revolutionized the diagnostic approach of patients with inflammatory bowel disease (IBD). New, emerging endoscopic imaging techniques visualized a plethora of new mucosal details even at the cellular and subcellular level. This review offers an overview about new endoscopic techniques, including chromoendoscopy, magnification endoscopy, spectroscopy, confocal laser endomicroscopy and endocytoscopy in the face of IBD.

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Key words: Endoscopy; Inflammatory bowel disease; Endomicroscopy; Endocytoscopy; Narrow band imaging; Fujinon intelligent color enhancement; i-Scan; Spectroscopy; Chromoendoscopy; Ulcerative colitis; Crohn's disease; Fluorescence endoscopy

Peer reviewers: Zvi Fireman, Department of Gastroenterology, Hillel- yaffe Med. Ctr., PO Box 169, Hadera 38100, Israel; Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Neumann H, Neurath MF, Mudter J. New endoscopic approaches

in IBD. *World J Gastroenterol* 2011; 17(1): 63-68 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/63.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.63>

INTRODUCTION

Patients with long standing ulcerative colitis and Crohn's disease are at an increased risk for the development of intraepithelial neoplasia (formerly known as dysplasia) and colorectal neoplasia. Therefore, surveillance endoscopy is mandatory in these patients. Nevertheless, standard white light endoscopy with multiple random biopsies may miss a quantum of lesions^[1].

During the last ten years, a variety of new endoscopic techniques were introduced to improve diagnosis and patient outcome in inflammatory bowel disease (IBD). Traditionally, standard white light endoscopy only allows the investigation of the mucosal surface and surrounding blood vessels at low magnification (Figure 1). To overcome these limitations, new endoscopic imaging techniques were developed providing a more detailed view of the mucosa. Emerging endoscopic imaging techniques include chromoendoscopy and magnification endoscopy. Additionally, new endoscopic devices now allow real time *in vivo* histology during ongoing endoscopy.

This review describes the concept of advanced endoscopic imaging techniques in IBD.

CHROMOENDOSCOPY

Chromoendoscopy uses different staining techniques to enhance the mucosal detail and submucosal vascular pattern, thereby improving the detection of pathological lesions and enabling a more precise diagnosis. Currently, chromoendoscopy is distinguished in dye-based and dyeless imaging techniques.

The basic principle of dye-based chromoendoscopy (DBC) is the use of biocompatible dye agents (Figure 2). Dyes include absorptive (methylene blue 0.1%-0.5%, cresyl violet 0.2%) and contrast agents (indigo carmine

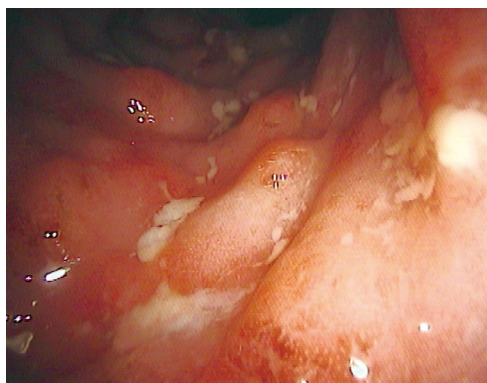


Figure 1 High-resolution standard white light endoscopic image of active Crohn's disease. Endoscopy shows ulcerations, mucosal edema and erythema.

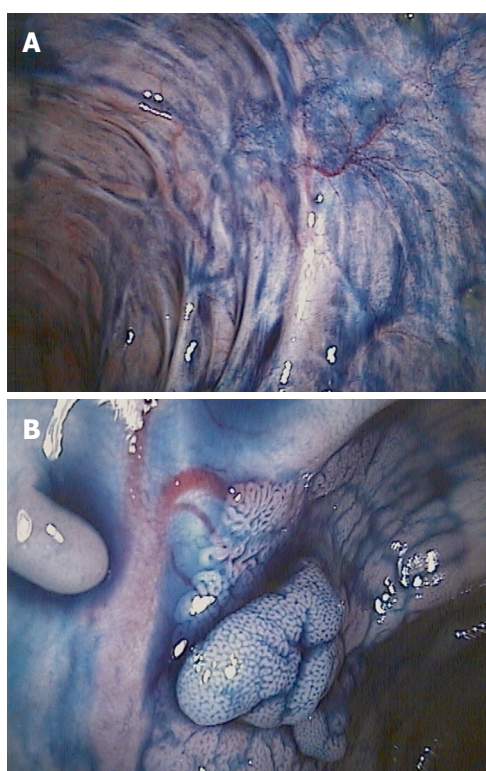


Figure 2 Chromoendoscopy with indigo carmine. A better distinction of mucosal changes in long standing ulcerative colitis (A) and pit pattern analysis of suspicious lesions (B).

0.2%-0.4%). DBC yields an additional diagnostic value with a 3-4 higher detection rate of intraepithelial neoplasia^[2,3]. However, dye-based chromoendoscopy has some potential limitations. There are additional costs for the equipment needed for dye spraying, it is a time consuming procedure, the dye does not always coat the surface evenly and it does not allow for a detailed analysis of the subepithelial capillary network, which is an important feature in the early diagnosis of gastrointestinal neoplasia.

Therefore, dye-less chromoendoscopy (DLC; also called virtual chromoendoscopy) has been developed (Figure 3). DLC includes narrow band imaging (NBI; Olympus, Tokyo, Japan), Fujinon intelligent color enhancement (FICE; Fujinon, Tokyo, Japan) and i-Scan (Pentax, Tokyo, Japan).

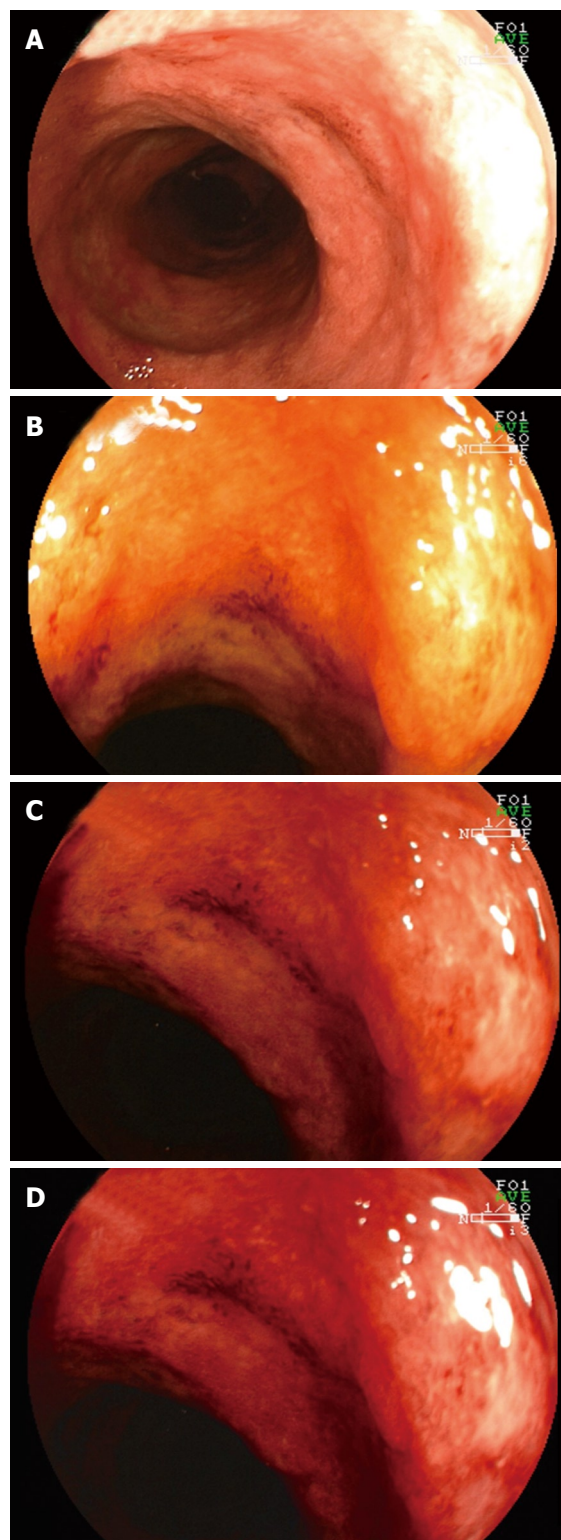


Figure 3 Virtual chromoendoscopy using the Fujinon intelligent color enhancement-system. A: Shows standard white light endoscopic image; B-D: Illustrate different Fujinon intelligent color enhancement settings to improve mucosal detail.

NBI is based on optical filters within the light source of the endoscope which narrow the bandwidth of spectral transmittance such that the blood vessels are enhanced and thus seen more easily.

FICE and i-Scan are based on the same physical prin-

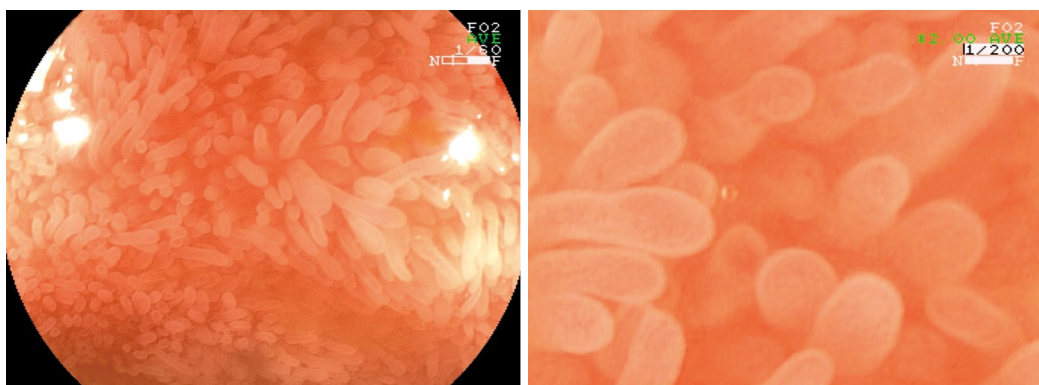


Figure 4 High-magnification endoscopy of ileum mucosa in patient with Crohn's disease without activity. Villi are clearly visualized.

circle as NBI, but due to a computed spectral estimation technology, they are not dependent on the presence of optical filters inside of the video endoscope. In contrast to NBI, FICE and i-Scan use an endoscopic image from the video processor and reconstruct virtual images in real time by increasing the intensity of narrowed blue light to a maximum and by decreasing narrowed red light and green light to a minimum resulting in an improved contrast of the capillary patterns and enhancement of the mucosal surface.

One recent study evaluated magnifying colonoscopy with NBI for the diagnosis of intraepithelial neoplasia in ulcerative colitis. It was found that the tortuous pattern determined by NBI colonoscopy may be a clue for the identification of dysplasia during surveillance for ulcerative colitis^[4]. Another study included 50 patients with longstanding ulcerative colitis and reported on a moderate accuracy (sensitivity 75%, specificity 81%) for the NBI diagnosis of intraepithelial neoplasia^[5]. Additionally, NBI colonoscopy may be of value for determining the grade of inflammation in patients with quiescent ulcerative colitis^[6].

Very recently, it was shown that FICE could not improve the detection or delineation of ulcers and erosions due to Crohn's disease^[7]. Nevertheless, these preliminary data have to be proven in larger prospective trials.

One recent published study tested the efficacy of high definition endoscopy alone compared to i-Scan or chromoendoscopy with methylene blue (0.1%) in screening for colorectal cancer^[8]. It was found that both i-Scan and chromoendoscopy identified more lesions compared to high definition endoscopy alone. Additionally, i-Scan was able to predict neoplasia as precisely as chromoendoscopy.

MAGNIFICATION ENDOSCOPY

Magnification endoscopy (also called zoom endoscopy) utilizes a movable lens to vary the degree of magnification up to 150-fold (Figure 4). By staining the entire colon with methylene blue, it has been shown that chromoendoscopy combined with magnification endoscopy has the potential to improve targeting biopsy examination in patients with long-standing colitis and facilitate early detection of intraepithelial neoplasia and colorectal cancer^[2]. In the chro-

moendoscopy arm a significantly better correlation was found between the endoscopic assessment of degree ($P = 0.0002$) and extent ($P < 0.0001$) of colonic inflammation and the histopathologic findings compared with the conventional colonoscopy group. Additionally, more targeted biopsies were possible, and significantly more intraepithelial neoplasia were detected in the chromoendoscopy group ($P = 0.003$).

These data were confirmed by Hurlstone and colleagues^[3]. In a prospective study, 162 patients with longstanding ulcerative colitis underwent total colonoscopy. After detection of subtle mucosal changes intravital staining with indigo carmine was used. Subsequently, the macroscopic type and the staining pattern were defined. Chromoendoscopy with magnification and targeted biopsies significantly increased diagnostic yield for intraepithelial neoplasia and the number of flat neoplastic changes as opposed to conventional colonoscopy.

The largest prospective study to date comparing conventional endoscopy with magnification endoscopy enrolled 300 patients with ulcerative colitis^[9]. Magnification imaging was significantly better than conventional colonoscopy for predicting disease extent *in vivo* ($P < 0.0001$). The authors concluded that high-magnification imaging provides a sensitive and specific *in vivo* "virtual biopsy" in ulcerative colitis. High-accuracy optical biopsy could limit the number of biopsies required, with significant cost savings for pathology services.

SPECTROSCOPY

Spectroscopy includes several optical techniques, including fluorescence-, reflectance-, light scattering spectroscopy and optical coherence tomography. Spectroscopy depends on the wavelength of the light source and on tissue characteristics. Based on differences between the spectra of light that is backscattered between cells, different spectra can be identified that are specific for various diseases such as ischemia, inflammation, and malignancy^[10].

One study assessed fluorescence endoscopy for the detection of intraepithelial neoplasia in ulcerative colitis by taking optical guided biopsies. By using 5-aminolevulinic acid as an exogenous fluorophore agent, sensitivity

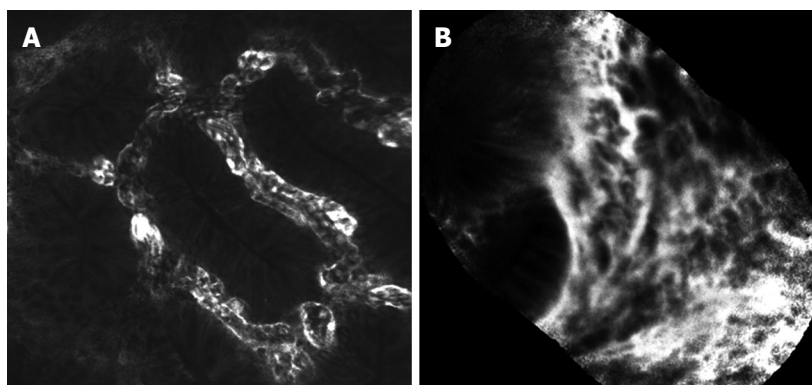


Figure 5 Confocal laser endomicroscopy using either the integrated system (iCLE, A) or the probe-based system (pCLE, B) visualizes dilated microvessels, leakage and disturbed crypt architecture in active ulcerative colitis.

and specificity for dysplastic lesions was 100% and 62%, respectively^[11]. Very recently, the detection of invisible flat intraepithelial neoplasia with protoporphyrin IX fluorescence was compared to standard 4-quadrant biopsies^[12]. Flat intraepithelial neoplasia was detected in 7% of patients by standard white light 4-quadrant biopsies and in 24% of patients using fluorescence-guided endoscopy ($P = 0.02$). Sensitivity and specificity for differentiating patients with and without dysplasia were 100% and 81%, respectively. Additionally, dysplastic and non-dysplastic mucosa could be discriminated with a sensitivity and specificity of 73% and 81%, respectively.

CONFOCAL LASER ENDOMICROSCOPY

In 2004, confocal laser endomicroscopy was introduced, allowing real time *in vivo* histology of 1000-fold magnification during ongoing endoscopy^[13]. Currently, two FDA approved devices are available (Figure 5). One is integrated into the distal tip of a high resolution endoscope (iCLE; Pentax, Tokyo, Japan), one represents a stand-alone probe which is capable of passage through the working channel of most standard endoscopes (pCLE; Cellvizio, Mauna Kea Technologies, Paris, France). A blue laser light source delivers an excitation wavelength of 488 nm and returning light is detected at > 505 nm. Endomicroscopy requires the application of fluorescence agents, either systemically (fluorescein) or topically (e.g. acriflavine, cresyl violet)^[14].

While endomicroscopy only covers a limited field of view within the mucosa, pan-endomicroscopy of the whole gastrointestinal tract is not feasible. Therefore, macroscopic visualization of suspected areas is necessary before performing targeted endomicroscopy.

To date, different studies have addressed the utility of endomicroscopy for the *in vivo* diagnosis of IBD associated mucosal changes.

To compare endomicroscopic imaging of inflamed and non-inflamed rectal mucosa in patients with ulcerative colitis, Watanabe *et al.*^[15] enrolled 17 patients with ulcerative colitis and 14 controls. Confocal images were compared to standard histopathology. Endomicroscopy was able to visualize crypt architecture, capillaries and inflammatory cells, providing equivalent information to histopathology.

Recently, a new classification of inflammation activity in ulcerative colitis using endomicroscopy was proposed^[16], including crypt architecture and microvascular alterations. In this study, endomicroscopy was reliable for real-time assessment of inflammation activity in ulcerative colitis showing good correlations with histological results (Spearman's rho, both $P < 0.001$).

Our group addressed the utility of endomicroscopy for the *in vivo* evaluation of Crohn's disease associated changes. Using the pCLE system, endomicroscopy was able to diagnose Crohn's disease associated changes with high accuracy. Furthermore, pCLE could detect residual macroscopic non-visible mucosal inflammation as precisely as histology (κ values 0.8, unpublished data).

Current guidelines recommend a large number of biopsy specimens during surveillance colonoscopy in ulcerative colitis. Nevertheless, flat lesions still may be missed. In a trial of longstanding ulcerative colitis, chromoendoscopy was used to unmask lesions for endomicroscopy and compared with standard white light endoscopy with random biopsies^[17]. Chromoendoscopy in combination with endomicroscopy detected 4.75-fold more neoplasia compared to conventional colonoscopy ($P = 0.005$). Additionally, 50% fewer biopsy specimens were required ($P = 0.008$). The presence of neoplastic changes could be predicted with high sensitivity, specificity and accuracy (94.7%, 98.3%, 97.8%, respectively).

One recent study prospectively evaluated the clinical applicability and predictive power of endomicroscopy for the *in vivo* differentiation of dysplasia-associated lesion mass (DALM) or adenoma-like mass (ALM)^[18]. Accuracy of endomicroscopy was 97% and an excellent agreement between endomicroscopy and histopathological diagnosis was found ($\kappa = 0.91$).

ENDOCYTOSCOPY

Endocytoscopy (Olympus, Tokyo, Japan) is a new imaging technique, enabling microscopic imaging of the mucosal layer of the gut at a magnification up to 1400-fold (Figure 6). Endocytoscopy is based on a contact light microscope which enables real-time visualization of cellular structures of the superficial epithelial layer in a plane parallel to the

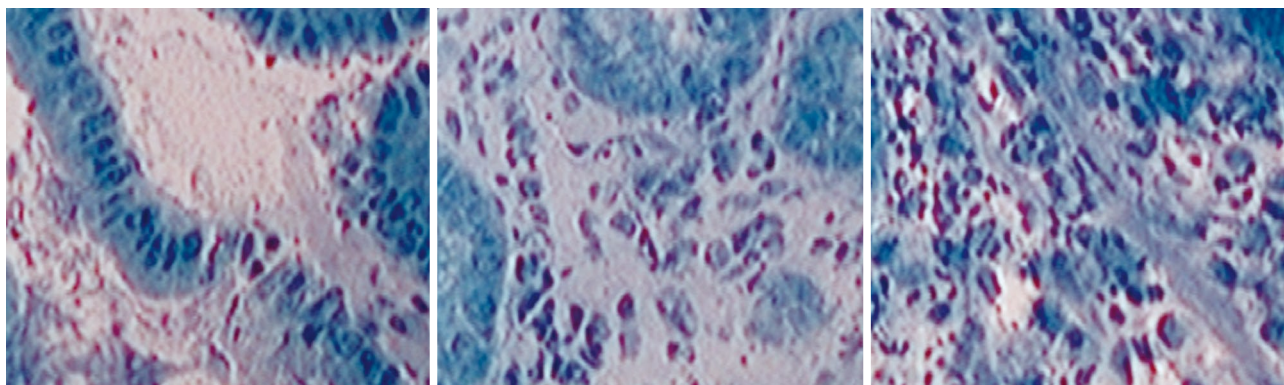


Figure 6 Endocytoscopy enables visualization of different cytological and architectural features, including size, arrangement, and density of cells.

mucosal surface. Currently, systems integrated into the distal tip of an endoscope (iEC) and probe-based (pEC) systems are available. Probe-based systems consist of handheld miniproboscopes, which are capable of being inserted through the accessory channel of a standard endoscope. The device provides ultra high magnification imaging at $\times 570$ (pEC), $\times 580$ (iEC) or $\times 1400$ (pEC) on a 19-inch monitor from an optical sampling site of about 0.5 mm in diameter. Endocytoscopy requires preparation of the mucosal layer with absorptive contrast agents like methylene blue or toluidine blue^[19].

Recently, endocytoscopy has been established as a useful tool to examine mucosal surfaces^[20-23]. Different studies suggest the potential of endocytoscopy for the *in vivo* evaluation of duodenal mucosa in celiac disease^[21]. Furthermore, endocytoscopy was able to detect tissue abnormalities in normal mucosa surrounding colorectal cancer and to identify neoplasia in aberrant crypt foci^[22]. Additionally, endocytoscopy was shown to distinguish neoplastic from non-neoplastic lesions, and also to differentiate invasive colon cancer from adenoma^[23,24].

Currently, data on endocytoscopy for *in vivo* diagnosis of IBD are still lacking. Nevertheless, this new imaging technique is a promising development allowing surface magnification at cellular and subcellular resolution.

CAPSULE ENDOSCOPY

In order to evaluate the small intestine capsule endoscopy (CE) was introduced. Currently two CE systems are available. One is distributed by Given Imaging (Norcross, Ga), and one by Olympus (Tokyo, Japan). The capsule is passively propelled through the intestine by peristalsis while transmitting color images of the intestine^[25].

CE is useful for the evaluation of the small intestine in patients in whom the diagnosis of Crohn's disease is elusive^[26]. Due to the danger of capsule retention in patients with established Crohn's disease, a patency capsule is available which is self-dissolved approximately 30 h after ingestion. In addition, balloon-guided endoscopy could be used to remove impacted capsules^[27]. Dubcenco and coworkers studied CE findings in patients with established and suspected small-intestine Crohn's disease and correlated the findings with radiologic, endoscopic and histologic

findings. Final diagnosis of active small-intestine Crohn's disease was made in 74% of patients. In addition, CE yielded a sensitivity and specificity of 89.6% and 100%, respectively, and a positive predictive value and a negative predictive value of 100% and 76.9%, respectively^[28]. Furthermore, CE was shown to be superior compared to push enteroscopy and enteroclysis^[29].

CONCLUSION

Modern endoscopy has revolutionized the diagnosis and management of patients with IBD. The newly developed endoscopic devices offer features that allow more and more mucosal and submucosal details to be seen. According to high magnification and respective reduced field of view, prior assessment of suspicious lesions is mandatory. Chromoendoscopy, using either vital or virtual staining techniques unmasks circumscribed lesions and confocal laser endomicroscopy or endocytoscopy can then be used to predict intraepithelial neoplasia with high accuracy. Nevertheless, the assessment of these new endoscopic imaging modalities in clinical practice still warrants further investigation. In addition, currently there is no reimbursement for advanced endoscopic imaging methods including endomicroscopy, endocytoscopy and spectroscopy. Therefore, endoscopy with multiple random biopsies remains the gold standard for surveillance in patients with IBD.

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S- Editor Sun H L- Editor O'Neill M E- Editor Ma WH

S100A4 over-expression underlies lymph node metastasis and poor prognosis in colorectal cancer

Li-Yong Huang, Ye Xu, Guo-Xiang Cai, Zu-Qing Guan, Wei-Qi Sheng, Hong-Fen Lu, Li-Qi Xie, Hao-Jie Lu, San-Jun Cai

Li-Yong Huang, Ye Xu, Guo-Xiang Cai, Zu-Qing Guan, San-Jun Cai, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China

Li-Yong Huang, Ye Xu, Guo-Xiang Cai, Zu-Qing Guan, Wei-Qi Sheng, Hong-Fen Lu, San-Jun Cai, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Wei-Qi Sheng, Hong-Fen Lu, Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai 200032, China

Li-Qi Xie, Hao-Jie Lu, Department of Chemistry, Fudan University, Shanghai 200032, China

Li-Qi Xie, Hao-Jie Lu, Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China

Author contributions: Huang LY, Xu Y, Cai GX, Guan ZQ, Lu HJ and Cai SJ designed the research; Huang LY and Xie LQ performed the research; Huang LY, Cai GX, Sheng WQ and Lu HF analyzed the data; Huang LY and Cai SJ wrote the paper.

Supported by Shanghai Momentous Program, Grant No. 07dz19505; and Shanghai Rising-star Program from the Science and Technology Commission of Shanghai Municipality, No. 10QA1401400, China

Correspondence to: San-Jun Cai, MD, Professor of Medicine, Chief, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, 270 Dong'an Road, Shanghai 200032, China. caisanjun@csco.org.cn

Telephone: +86-21-64175590 Fax: +86-21-64035387

Received: April 20, 2010 Revised: August 10, 2010

Accepted: August 17, 2010

Published online: January 7, 2011

Abstract

AIM: To develop lymph node metastasis (LNM)-associated biomarkers for colorectal cancer (CRC) using quantitative proteome analysis.

METHODS: Differences in protein expression between primary CRC with LNM (LNM CRC) and without LNM (non-LNM CRC) were assessed using methyl esterification stable isotope labeling coupled with 2D liquid chromatography followed by tandem mass spectrometry (2D-LC-MS/MS). The relationship to clinicopatholog-

ical parameters and prognosis of candidate biomarkers was examined using an independent sample set.

RESULTS: Forty-three proteins were found to be differentially expressed by at least 2.5-fold in two types of CRC. S100A4 was significantly upregulated in LNM CRC compared with non-LNM CRC, which was confirmed by Western blotting, immunohistochemistry and real-time quantitative polymerase chain reaction. Further immunohistochemistry on another 112 CRC cases showed that overexpression of S100A4 frequently existed in LNM CRC compared with non-LNM CRC ($P < 0.001$). Overexpression of S100A4 was significantly associated with LNM ($P < 0.001$), advanced TNM stage ($P < 0.001$), increased 5-year recurrence rate ($P < 0.001$) and decreased 5-year overall survival rate ($P < 0.001$). Univariate and multivariate analyses indicated that S100A4 expression was an independent prognostic factor for recurrence and survival of CRC patients ($P < 0.05$).

CONCLUSION: S100A4 might serve as a powerful biomarker for LNM and a prognostic factor in CRC.

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Key words: Colorectal cancer; Lymph node metastasis; Prognosis; Proteome analysis; S100A4

Peer reviewers: Mark De Ridder, MD, PhD, Professor, Dienst Radiotherapie, UZ Brussel, Vrije Universiteit Brussel, Laarbeeklaan 101, B-1090 Brussel, Belgium; Shashi Bala, PhD, Post Doctoral Associate, Department of Medicine, LRB 270L, 364 Plantation street, UMass Medical School, Worcester, MA 01605, United States

Huang LY, Xu Y, Cai GX, Guan ZQ, Sheng WQ, Lu HF, Xie LQ, Lu HJ, Cai SJ. S100A4 over-expression underlies lymph node metastasis and poor prognosis in colorectal cancer. *World J Gastroenterol* 2011; 17(1): 69-78 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/69.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.69>

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent human cancer worldwide, with 1 million estimated new cases annually, of which, about 50% die^[1]. CRC frequently migrates through the lymphatic route, depositing tumor cells into local lymph nodes, namely lymph node metastasis (LNM). The status of the local lymph nodes delivers crucial information concerning cancer staging, prognosis, and clinical decision making, on the understanding that the existence of LNM notably reduces the chance of CRC survival^[2]. Unfortunately, the mechanisms related to LNM remain poorly understood at present because LNM is a complicated process that involves cancer cell detachment from the primary tumor, migration, invasion, adhesion and implantation in the new environment. A variety of dysregulated molecules play a significant role in this highly sophisticated process^[3,4]. Therefore, LNM-related investigations have attracted much attention.

Clinicopathological features such as poorly differentiated cancer, depth of wall penetration, lymphovascular invasion, and tumor size are considered to be associated with CRC with LNM (LNM CRC)^[5,6]; however, these characteristics are still insufficient to predict the existence of LNM. In order to improve the diagnosis and prognosis of CRC, there is an urgent need to identify specific tumor molecular markers to recognize patients with LNM, which can define a subset of CRC patients who could benefit from rational management.

It is presently in progress to develop new strategies for the identification of cancer-related molecular markers. Proteomics, the emerging technology that examines the overall characteristics of the expressed proteins, has identified many differential proteins associated with tumor development and progression in various diseases^[7-10]. The recent development of proteomic technology, which presents better sensitivity than conventional gel-based strategies - coupling stable isotope labeling with liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) - introduces a powerful approach to accurate qualitative and quantitative proteomic analysis of clinical samples, as successfully applied to research on difference cancers^[11-14]. This procedure can provide new opportunities to develop biomarkers associated with LNM for CRC.

In the present study, we employed the combination of methyl esterification stable isotope labeling and 2D-LC-MS/MS to perform an accurate quantitative analysis. A total of 43 proteins were identified that were differently expressed by at least 2.5-fold, including S100A4, which was significantly upregulated in LNM CRC compared with non-LNM CRC. S100A4 was recently reported in association with LNM in several studies that attracted our interest^[15-17]. Meanwhile, there have been a limited number of similar studies on the association of S100A4 with CRC. As a result, we focused our attention on S100A4. After confirmation by Western blotting, immunohistochemistry and real-time quantitative polymerase chain reaction (PCR), we further investigated the relationship between S100A4 expression and the lymph node metastatic phe-

notype of CRC, and determined its prognostic value on another independent set of 112 CRC cases.

MATERIALS AND METHODS

Patients and samples

A total of 144 colorectal carcinoma samples were collected after obtaining informed consent in our hospital (Fudan University Shanghai Cancer Center, Shanghai, China). None of the patients received chemotherapy or radiotherapy before surgery. Resected specimens were reviewed by two senior pathologists according to the criteria described in the American Joint Committee on Cancer Cancer Staging Manual (6th edition, 2002)^[18]. For the screening and confirmation study, 32 primary CRC tissue samples that were obtained from patients who underwent curative resection in 2009 were collected and divided into two groups of LNM CRC and non-LNM CRC, with 16 cases in each group. The number of lymph nodes retrieved was not less than 12 in the non-LNM CRC. None of them had distant metastasis. The fresh colorectal tumor tissues were obtained immediately after surgery, washed twice with chilled phosphate buffered saline (PBS), immediately stored in liquid nitrogen and at -80°C in our tissue bank until further use. The detailed clinical data of these patients is provided in Table 1. For the S100A4 expression study, paraffin-embedded tissues in another independent set of 112 primary CRC samples between January and August 2004 were used for immunohistochemistry assessment. Ethical approval was obtained from the Cancer Center Research Ethics Committee.

Sample preparation, methyl esterification stable isotope labeling and 2D-LC-MS/MS

Frozen samples were crushed to powder in liquid nitrogen, and dissolved in lysis buffer [7 mol/L urea, 2 mol/L thiourea, 100 mmol/L DTT and 1 × protease inhibitor cocktail (Roche, Penzberg, Germany)] by continuous vortex at 4°C for 1 h. After centrifugation at 15000 r/min for 45 min at 4°C, equal amounts of each sample from LNM CRC and non-LNM CRC groups were pooled together. One hundred micrograms of proteins from each sample pool were reduced with 10 mmol/L dithiothreitol (60 min, 56°C) and alkylated with 12 mmol/L iodoacetamide in darkness (45 min, 37°C), followed by digestion with 1:20 (w/w) ratio of trypsin (Promega, Madison, WI, USA) overnight at 37°C. The lyophilized peptides from LNM CRC sample were tagged with d0-methanolic HCl, whereas those from non-LNM CRC samples were labeled with d3-methanolic HCl (Sigma-Aldrich, St. Louis, MO, USA) as previously described^[13,19]. Briefly, 100 µL of d0-methanolic HCl or d3-methanolic HCl (created by dropwise addition of 160 µL acetyl chloride with stirring to 1 mL d0-methanol or d3-methanol) was added to the corresponding sample. The reaction was allowed to proceed for 2 h at room temperature. After lyophilization and re-dissolving in 100 µL 5% acetonitrile in 0.1% formic acid, the two peptides were mixed, followed by desalination using Sep-Pak Vac C18 (Waters, Milford, MA, USA).

Table 1 Clinicopathological characteristics of colorectal cancer patients included in screening and confirmation study

Patient No.	TNM	Sex	Age (yr)	Location
Non-LNM CRC				
1	T2N0M0	Male	73	Colon
2	T4N0M0	Male	44	Rectum
3	T2N0M0	Male	49	Colon
4	T3N0M0	Female	53	Colon
5	T3N0M0	Male	54	Colon
6	T4N0M0	Female	52	Colon
7	T3N0M0	Male	69	Rectum
8	T3N0M0	Female	59	Colon
9	T4N0M0	Male	66	Colon
10	T2N0M0	Male	73	Colon
11	T2N0M0	Female	72	Rectum
12	T3N0M0	Male	68	Rectum
13	T2N0M0	Male	71	Colon
14	T4N0M0	Male	68	Colon
15	T3N0M0	Male	59	Rectum
16	T2N0M0	Female	43	Rectum
LNM CRC				
1	T4N2M0	Male	41	Colon
2	T3N1M0	Male	60	Rectum
3	T2N1M0	Male	63	Rectum
4	T2N2M0	Female	37	Rectum
5	T4N1M0	Female	40	Colon
6	T4N2M0	Male	80	Colon
7	T4N1M0	Male	32	Colon
8	T4N2M0	Female	65	Colon
9	T3N1M0	Female	49	Colon
10	T2N1M0	Female	47	Rectum
11	T2N2M0	Female	55	Rectum
12	T4N1M0	Male	86	Colon
13	T3N1M0	Female	71	Colon
14	T3N2M0	Male	79	Colon
15	T3N1M0	Male	70	Colon
16	T4N1M0	Male	56	Colon

LNM: Lymph node metastasis; CRC: Colorectal cancer.

They were lyophilized again and re-dissolved in 40 μ L of the above-mentioned solution. Subsequently, the resultant sample was separated by a 2D microcapillary HPLC system, followed by MS/MS analysis using an LTQ Orbitrap (Thermo Fisher, San Jose, CA, USA). LC solvent gradients were controlled by the chameleon 6.5 (Dionex, Amsterdam, The Netherlands). The MS scan was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Fragment ion selection was based on ion intensity (above 10 counts) and charge state (+2, +3).

Data analysis

Bioworks 3.3.1 was used to generate the peaklists of all acquired MS/MS spectra, which were then automatically searched against the International Protein Index human protein sequence database, version 3.43 using SEQUEST (University of Washington, licensed to Thermo Fisher), with a 95% confidence level. Except for 57 Da reductive alkylation modification on Cys, static modifications were set on Asp, Glu, C-terminal (+14 Da and +17 Da for light and heavy isotope labeling, respectively). The mass tolerance of the peptides and fragment ions was 10 ppm and 1.0 Da, respectively. The identified peptides were further ana-

lyzed with two computer software programs, PeptideProphet and ProteinProphet. PeptideProphet with a probability score of 0.9 and ProteinProphet with a probability score of 0.95 were used to ensure an overall false-positive rate below 0.005. Quantification of the ratio of each protein was achieved using the Xpress program. Proteins with expression fold change > 2.5, $P < 0.05$ were defined as differentially expressed proteins.

Western blotting

The same protein samples for screening were used for Western blotting. Briefly, 30- μ g protein samples from each case were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes. The membranes were incubated with rabbit polyclonal antibody against S100A4 (1:1000 dilution; Abcam, Cambridge, UK) and then incubated with a horseradish-peroxidase-conjugated secondary antibody (1:100 dilution; Proteintech, Chicago, IL, USA). β -actin was detected simultaneously as a loading control (anti- β -actin, 1:1000 dilution; Kangchen, Beijing, China). All blots were visualized using an ECL detection system (Amersham, Arlington Heights, IL, USA) and quantitated by densitometry using an LAS-3000 imager.

Immunohistochemistry

S100A4 expression was examined immunohistochemically using paraffin-embedded tissues. In brief, 4- μ m-thick tissue sections were heated in 6.5 mmol/L citrate buffer (pH 6.0) at 100°C for 28 min, and incubated with antibody against S100A4 (1:200 dilution). Immunostaining was performed employing the DAKO En-Vision System (Dako Diagnostics, Zug, Switzerland). In the negative control group, PBS was used instead of primary antibody. S100A4 expression was scored by two independent experienced pathologists. Each sample was graded according to the intensity and extent of staining as described previously^[11]. The intensity of staining was scored as 0 (no staining), 1 (weak staining), and 2 (strong staining). The extent of staining was based on the percentage of positive tumor cells: 0 (no staining), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The final score was assessed by summarizing the results of intensity and extent of staining. The case was considered negative if the final score was 0 or 1 (-) or 2 or 3 (\pm), and positive if the score was 4 or 5 (+) or 6 or 7 (++). In most cases, the two reviewers provided consistent results. Any inconsistencies were resolved by discussion to achieve a consensus score.

Real-time quantitative PCR

Total tissue RNA was extracted using the Rneasy Mini Kit (Qiagen, Valencia, CA, USA). Real-time quantitative PCR analysis was performed according to the manufacturer's instructions (the Quant SYBR Green PCR Kit, TIAN-GEN BIOTECH, Beijing, China). β -actin was applied as an internal control. The primers for β -actin (205 bp) were 5'-TGACGTGGACATCCGCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAGCGAGG-3' (antisense). The primers for S100A4 (185 bp) were 5'-GCCCTGGATGT-

Table 2 Protein expression upregulated and downregulated at least 2.5-fold in lymph node metastasis colorectal cancer compared with non-lymph node metastasis colorectal cancer

Accession No.	Protein name	Protein ratio ¹	SD	Peptide	Coverage rate (%)
Upregulated					
IPI00171494	Isoform 2 of cytoplasmic dynein 2 heavy chain 1	21.8	0	1	2.4
IPI00103253	Isoform 5 of pyrin and hin domain-containing protein 1	8.68	2.85	19	4.9
IPI00298520	Putative uncharacterized protein dkfzp686m09245	7.39	0	1	2.5
IPI00027194	Syntaxin-18	6.30	0	1	3.9
IPI00410639	Isoform 2 of fch and double sh3 domains protein 2	5.25	1.03	5	2.1
IPI00300631	Scaffold attachment factor B1	4.69	0	1	1.51
IPI00009236	Isoform α of caveolin-1	4.42	3.75	2	3.0
IPI00216654	Isoform β of nucleolar phosphoprotein P130	4.35	0	1	1.6
IPI00004273	RNA binding motif protein 25	3.87	0	1	1.5
IPI00239077	Histidine triad nucleotide-binding protein 1	3.73	0.38	5	7.91
IPI00010320	Chromobox protein homolog 1	3.56	1.90	4	1.8
IPI00010274	Isoform 1 of tryptase α -1 precursor	3.52	0.09	2	9.0
IPI00010414	Pdz and lim domain protein 1	3.41	0	1	4.0
IPI00014852	Isoform 1 of phosphoglucomutase-like protein 5	3.24	1.07	4	11.1
IPI00000156	Ligase III, DNA, ATP-dependent isoform β precursor	3.10	0.60	2	2.1
IPI00102821	Isoform 1 of proapoptotic caspase adapter protein precursor	3.09	0.40	2	36.1
IPI00032313	Protein S100-A4	3.04	0.69	2	7.9
IPI00008750	Metallothionein-1H	2.95	0.11	2	27.9
IPI00216153	40S Ribosomal protein S15	2.94	0.57	4	9.0
IPI00654777	Eukaryotic translation initiation factor 3 subunit 5	2.90	0.24	2	5.1
IPI00024933	60S Ribosomal protein L12	2.86	1.02	11	14.4
IPI00025366	Citrate synthase, mitochondrial precursor	2.85	0.47	4	5.9
IPI00007928	Pre-mRNA-processing-splicing factor 8	2.81	0	1	1.3
IPI00024976	Mitochondrial import receptor subunit tom22 homolog	2.78	0	1	8.5
IPI00577039	Annexin A2	2.77	0.32	2	3.6
IPI00062151	Similar to 60s ribosomal protein L15	2.70	0.37	6	6.5
IPI00219757	Glutathione S-transferase P1	2.55	0.37	2	9.1
IPI00396437	Isoform 2 of drebrin-like protein	2.51	0.45	5	2.8
Downregulated					
IPI00023673	Galectin-3-binding protein precursor	0.09	0	1	2.2
IPI00011062	Isoform 1 of carbamoyl-phosphatesynthase , mitochondrial precursor	0.10	0	1	1.0
IPI00030279	Isoform 1 of zinc finger ran-binding domain-containing protein 3	0.11	0	1	7.6
IPI00219682	Erythrocyte band 7 integral membrane protein	0.20	0	1	9.8
IPI00433499	Rhomboid, veinlet-like 6 isoform 1	0.20	0	1	2.1
IPI00000230	Tropomyosin 1 α chain isoform 2	0.26	0.06	32	51.8
IPI00029631	Enhancer of rudimentary homolog	0.27	0	1	7.7
IPI00009950	Vesicular integral-membrane protein vip36 precursor	0.30	0	1	3.4
IPI00384603	Isoform 2 of ribonuclease p protein subunit P21	0.30	0.11	13	10.7
IPI00177543	Peptidylglycine α -amidating monooxygenase isoform A	0.31	0.08	2	8.1
IPI00000330	Isoform 1 of uncharacterized protein c9orf80	0.35	0	1	17.6
IPI00298949	Cyclin G-associated kinase	0.35	0	1	1.3
IPI00008964	RAS-related protein rab-1B	0.37	0	1	7.5
IPI00013860	3-Hydroxyisobutyrate dehydrogenase, mitochondrial precursor	0.37	0	1	5.4
IPI00296053	Isoform mitochondrial of fumarate hydratase, mitochondrial precursor	0.39	0.18	1	2.4

¹Protein ratio represents the abundance ratio between lymph node metastasis group and non-lymph node metastasis group. LNM: Lymph node metastasis; CRC: Colorectal cancer.

GATGGTGT-3' (sense) and 5'-TCGTTGTCCCTGTT-GCTGTC-3' (antisense). Each assay was done in triplicate, and the average was calculated. For relative quantification, $2^{-\Delta\Delta C_t}$ was calculated and used as an indication of the relative expression levels.

Statistical analysis

The Student *t* test was used to evaluate the differences in S100A4 expression between LNM CRC and non-LNM CRC. The χ^2 test was used to assess the relationships between S100A4 expression and clinicopathological factors. The cumulative recurrence and survival probability were

estimated using the Kaplan-Meier method, and differences were calculated by log-rank test. Prognostic factors were determined using Cox regression analysis. The recurrence-free and overall survival times were calculated from the first resection of the primary tumor to first evidence of recurrence or to death from any cause, respectively. The diagnosis of recurrence was based on the typical features presented on computed tomography/magnetic resonance imaging and elevated serum carcinoembryonic antigen. All *P* values were two-sided, and *P* < 0.05 was considered to be significant. Statistical analyses were performed using SPSS 13.0 software.

RESULTS

Quantitative proteome analysis with methyl esterification stable isotope labeling combined with 2D-LC-MS/MS

To perform accurate quantitative analysis, we compared the protein expression profiles between LNM CRC and non-LNM CRC using methyl esterification stable isotope labeling combined with 2D-LC-MS/MS. The quantitative differential expression of 644 proteins was identified, after calibration with β -casein. Significantly, 43 of these (6.7%) proteins were displayed differentially (at least 2.5-fold) in LNM CRC compared with non-LNM CRC. Among these 43 proteins, 28 were found to be upregulated in LNM CRC (Table 2), and 15 were downregulated (Table 2). The identification of S100A4 significantly upregulated in LNM CRC is shown in Figure 1 as an example. These differentially expressed proteins formed the possible protein profiles associated with LNM in CRC.

Confirmation of S100A4 expression by Western blotting, immunohistochemistry and real-time quantitative PCR

We extended the experiments to confirm the differential expression of S100A4 in the same samples described above.

Thirty micrograms of total proteins from LNM CRC and non-LNM CRC were analyzed *via* Western blotting. The expression of S100A4 was dramatically higher in LNM CRC compared with non-LNM CRC ($P < 0.001$). A representative Western blotting result is presented in Figure 2A.

To confirm upregulation of S100A4 at the protein level, we used immunohistochemistry to evaluate S100A4 expression *in situ*. In normal mucosa, there was no immunoreactivity in cells. In non-LNM CRC, there was weak staining in cancer cells. In LNM CRC, there was notable brown staining in both primary and matched metastatic lymph node cancer cells. Positive staining was present mainly in the cytoplasm and/or nucleus of cancer cells (Figure 2B).

Real-time quantitative PCR revealed that S100A4 mRNA level was higher in LNM CRC than in non-LNM CRC ($P < 0.001$, Figure 2C), which is consistent with the trend at the protein level.

Association of S100A4 expression with clinicopathological features and postoperative prognosis of CRC patients

To detect the relationship between S100A4 expression and clinicopathological features and whether S100A4 could be a prognostic factor in predicting clinical outcomes of CRC patients, we evaluated S100A4 expression in an additional archived 112 CRC samples. In the group of 53 LNM CRC samples, 83% were positive for S100A4 expression, whereas 16.9% of the 59 non-LNM CRC samples had positive expression.

After division of these patients into S100A4-positive and S100A4-negative groups, statistical analysis revealed that positive expression of S100A4 was significantly associated with LNM, and advanced TNM stage ($P < 0.001$).

Table 3 Relationships of S100A4 expression with clinicopathological factors in colorectal cancer

Clinicopathological factors	n	S100A4 expression		P value ¹
		Negative	Positive	
Sex				
Male	59	32	27	0.252
Female	53	23	30	
Age (yr)				
≤ 60	76	39	37	0.497
> 60	36	16	20	
Tumor size (cm)				
≤ 5	74	32	42	0.083
> 5	38	23	15	
Tumor location				
Colon	47	23	24	0.975
Rectum	65	32	33	
Tumor differentiation ²				
I - II	82	43	39	0.244
III-IV	30	12	18	
Tumor status ²				
T1-2	34	18	16	0.592
T3-4	78	37	41	
Lymph node metastasis ²				
N0	59	49	10	< 0.001
N1-2	53	9	44	
TNM stage ²				
I - II	57	46	11	< 0.001
III-IV	55	9	46	

¹Statistical analysis was estimated with χ^2 test, and $P < 0.05$ was considered statistically significant; ²Grading of differentiation status and TNM classification for colorectal cancer were based on the American Joint Committee on Cancer Cancer Staging Manual (6th edition, 2002). The tumors were classified into two groups: well differentiated (grades I and II) and poorly differentiated (grades III and IV).

However, no significant correlations were observed between S100A4 expression and other clinicopathological parameters of sex, age, tumor size, tumor differentiation and tumor location (Table 3).

Furthermore, we found that patients with S100A4-positive CRC had significantly poorer prognosis than those with S100A4-negative CRC. The 5-year cumulative recurrence rate was significantly higher in patients with S100A4-positive CRC than in the S100A4-negative group ($P < 0.001$, Figure 3A). The 5-year cumulative survival rate in patients with S100A4-positive CRC was much lower than in those with S100A4-negative CRC ($P < 0.001$, Figure 3B). Univariate analyses revealed that LNM, TNM stage and S100A4 expression were associated with recurrence and overall survival. In multivariate analysis, LNM, TNM stage and S100A4 expression were also independent prognostic factors of recurrence and overall survival ($P < 0.05$, Table 4).

DISCUSSION

Metastasis remains one of the major challenges in management of CRC patients. LNM is the most common form of metastasis in CRC. To develop LNM-associated biomarkers for CRC, we employed the quantitative proteomic strategy of methyl esterification stable isotope

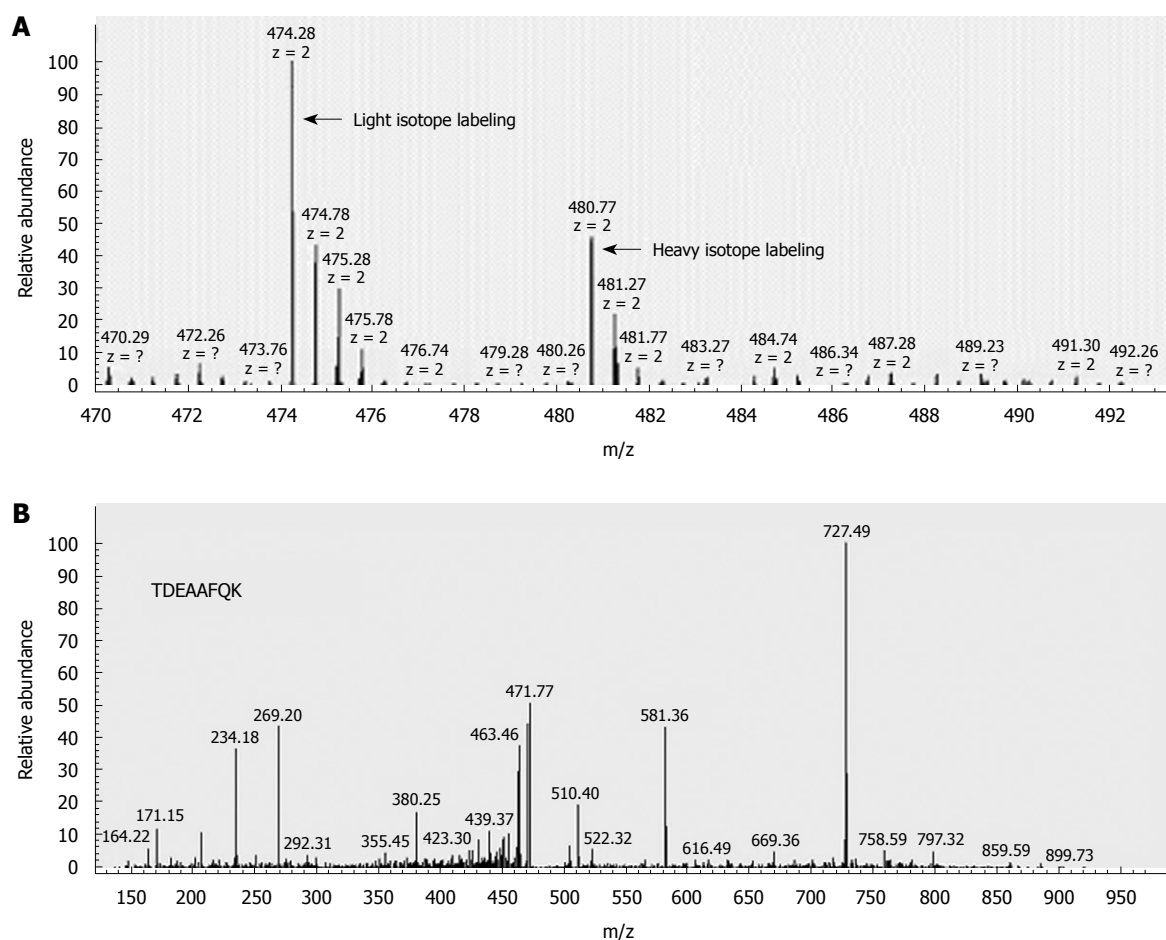


Figure 1 Identification of quantitatively dysregulated expression of S100A4. A: Quantification of S100A4 through the isotopically labeled fragment ion signals of the peptide "TDEAAFQK". The areas under the monoisotopic peaks represent the relative abundance of peptides, light [lymph node metastasis (LNM)]/heavy (non-LNM) = 3.04:1; B: Identification of the peptide "TDEAAFQK" from S100A4 by MS/MS.

labeling coupled with 2D-LC-MS/MS. A total of 644 proteins were identified, including 43 that were differentially expressed by at least 2.5-fold between LNM CRC and non-LNM CRC. We found many of the 43 proteins that possibly participate in the biological processes associated with tumor metastasis, such as cell motility and adhesion, migration, and signal transduction.

Among the upregulated proteins, annexin A2, one of the calcium- and phospholipid-binding proteins, has been widely reported in various cancers with the regulation of cell growth, motility, invasion and signaling pathways^[20]. The increase in caveolin-1 performs the functions of signal transduction, cell transformation and anti-apoptotic activity^[21]. Moreover, caveolin-1 has been found to be overexpressed in several multidrug-resistant cancer cell lines^[22,23]. In addition, some downregulated proteins identified in our study have also been observed to possess similar biological effects, including galectin-3-binding protein^[24] and cyclin-G-associated kinase^[25].

Previously, Pei *et al.*^[26] have carried out a proteomic study on 10 CRC samples using conventional 2D electrophoresis coupled with MALDI-TOF-MS, and have reported a pattern of four differentially expressed proteins potentially associated with LNM. In contrast, our results revealed up to 43 proteins that were differentially

expressed by at least 2.5-fold. Annexin A2 and glutathione S-transferase P1 both correlated with LNM in our study and that of Pei *et al.* Heat shock protein-27 and liver fatty acid binding protein (L-FABP) were found to correlate with LNM by Pei *et al.*, but not in our study, whereas 41 proteins identified in our study were not listed by Pei *et al.* This discordance is probably due to the different clinical background of the samples included in the studies, and the different proteomic strategies used. It is required to analyze systemically and integrate all the complementary data from various institutions into a common databank to elucidate exactly the molecular background of CRC. In addition, Pang *et al.*^[27] have identified and confirmed six differentially expressed proteins (e- fatty acid binding protein 5, methylcrotonoyl Coenzyme A carboxylase 2, pyrophosphatase 2, synaptotagmin-like protein 2, Ezrin, and smooth muscle protein) that are associated with LNM in prostate cancer by DIGE-based proteome analysis. However, there was no concordance between the results in that study and our study, which is probably mainly due to the different cancers and proteomic approaches included.

Recently, several studies have shown that S100A4 is an important factor relevant to progression and prognosis in various human cancers, such as thyroid^[28], breast^[29], pancreatic^[30], bladder^[31], gastric^[17] and colorectal^[32] cancer.

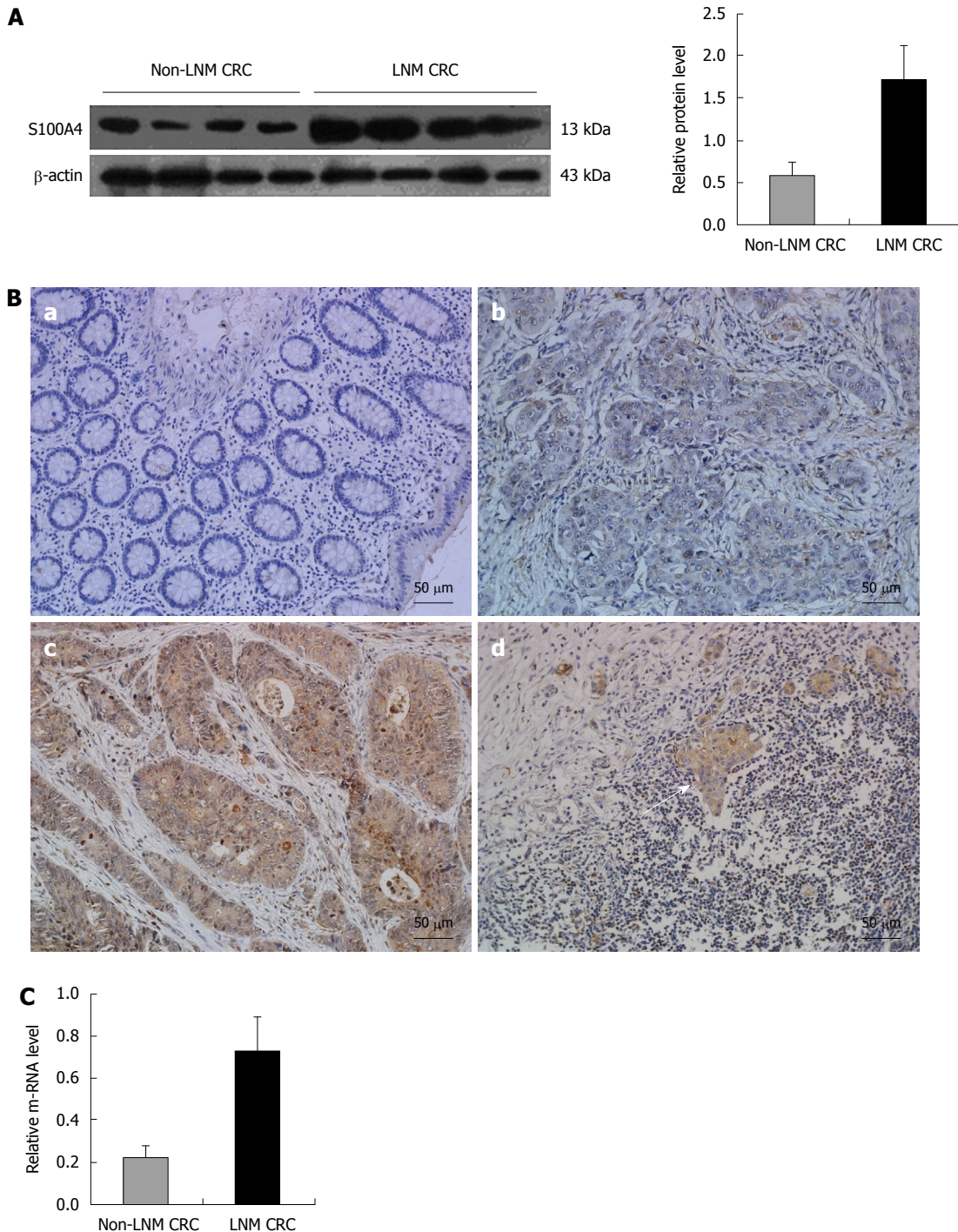


Figure 2 Confirmation of the overexpression of S100A4 in colorectal cancer. A: Western blotting analysis for S100A4 expression in colorectal cancer (CRC) specimens. β -actin was used as the internal loading control. The histogram shows the relative expression levels of S100A4 in non-LNM (16 cases) and lymph node metastasis (LNM) (16 cases) groups. Data represent the mean \pm SE ($P < 0.001$, Student *t* test); B: Immunohistochemical study of S100A4 distribution and expression in CRC specimens at 20×10 magnification. a: There was no immunoreactivity in the normal mucosa; b: Weak staining in cancer cells in the non-LNM group; c: Strong staining in cancer cells in the LNM group; d: Marked metastatic lymph nodes (arrow); C: mRNA level of S100A4 via real-time quantitative polymerase chain reaction. S100A4 was consistently increased in the LNM group (16 cases) compared with non-LNM group (16 cases). The mRNA level was normalized to that of β -actin. Data represent the mean \pm SE ($P < 0.001$, Student *t* test).

In particular, several studies have revealed that overexpression of S100A4 strongly indicates the presence of LNM^[15-17], which agrees with our original study aim. However, similar investigations have been limited between S100A4 expression and LNM in CRC. In view of the above reasons, S100A4, one of the significantly upregulated proteins identified in LNM CRC compared with non-

LNM CRC, which has been confirmed at the protein and mRNA levels, attracted our attention and interest.

S100A4, also known as 18A2/mts1, CAPL, PEL-98, 42A, p9Ka, and metastasin, belongs to the S100 superfamily of calcium-binding proteins^[33]. S100A4-mediated calcium signaling plays a major role in crucial biological functions that influence various aspects of cell physiology,

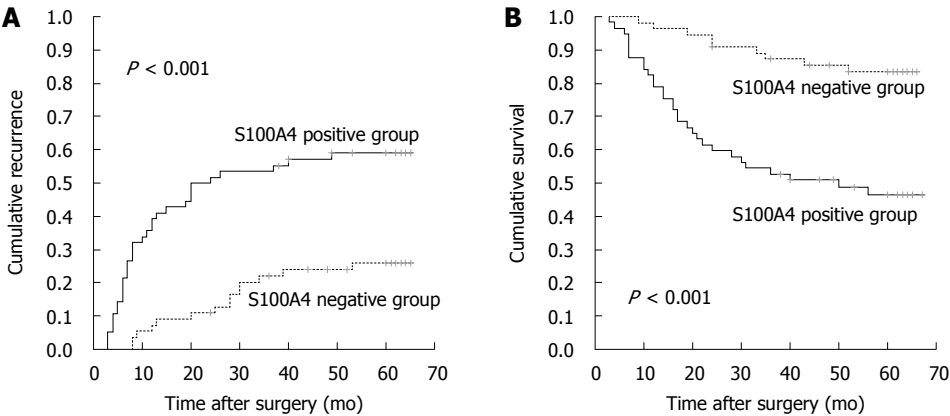


Figure 3 Overexpression of S100A4 correlated with poor prognosis in 112 colorectal cancer patients. A: Cumulative recurrence between the S100A4-positive and S100A4-negative groups ($P < 0.001$, log-rank test); B: Cumulative survival between the S100A4-positive and S100A4-negative groups ($P < 0.001$, log-rank test).

Table 4 Univariate and multivariate analyses of recurrence and survival (Cox regression)

Variables	Recurrence		Survival	
	HR (95% CI)	P value	HR (95% CI)	P value
Univariate analysis				
Sex				
Male/female	0.872 (0.490-1.549)	0.640	0.857 (0.452-1.624)	0.636
Age (yr)				
≤ 60/> 60	1.517 (0.842-2.732)	0.165	1.842 (0.967-3.508)	0.063
Tumor size (cm)				
≤ 5/> 5	0.881 (0.165-0.685)	0.687	0.880 (0.444-1.745)	0.715
Tumor location				
Colon/rectum	0.823 (0.463-1.464)	0.508	0.920 (0.483-1.752)	0.799
Tumor differentiation				
I - II / III-IV	1.234 (0.660-2.305)	0.511	1.171 (0.581-2.361)	0.659
Tumor status				
T1-2/T3-4	0.886 (0.484-1.620)	0.693	1.026 (0.517-2.033)	0.942
Lymph node metastasis				
N0/N1-2	2.727 (1.510-4.923)	0.001	2.852 (1.463-5.559)	0.002
TNM stage				
I - II / III-IV	3.560 (1.940-6.534)	< 0.001	3.393 (1.680-6.850)	< 0.001
S100A4 expression				
Negative/positive	3.666 (1.929-6.964)	< 0.001	4.154 (1.963-8.792)	< 0.001
Multivariate analysis				
LNM				
N0/N1-2	0.205 (0.055-0.769)	0.019	0.193 (0.044-0.857)	0.031
TNM stage				
I - II / III-IV	8.915 (2.081-38.189)	0.003	9.057 (1.705-48.108)	0.010
S100A4 expression				
Negative/positive	2.454 (1.056-5.705)	0.037	2.888 (1.131-7.379)	0.027

HR: Hazard ratio; CI: Confidence interval.

including proliferation and apoptosis, and differentiation and morphogenesis. It is also significantly involved in cell adhesion and motility, and cancer invasion and metastasis^[34,35]. A large body of evidence suggests that S100A4 is involved in cell metastatic phenotype by modulating the cytoskeletal dynamics, cadherin/catenin complex cytoskeletal linkage, CD44/cytoskeletal linkage, and extracellular-matrix-associated proteolytic enzyme. Furthermore, S100A4 can participate in the activation of the matrix metalloproteinase/tissue inhibitor of metalloproteinase system and angiogenic factor vascular endothelial growth factor, which in turn can lead to tumor neovascularization^[36,37].

To study further the relationship between S100A4 expression and the LNM phenotype of CRC, and determine whether S100A4 could be a prognostic factor in predicting clinical outcomes of CRC patients, we examined an additional 112 archived CRC samples for S100A4 expression. We found that the elevation in S100A4 expression level was significantly correlated with LNM and advanced TNM stage, which suggests that S100A4 plays an important part in the progression of CRC from a localized to lymph node metastatic disease. In addition, patients with S100A4-positive CRC had an increasing risk of recurrence and significantly reduced overall survival. Univariate and

multivariate analyses indicated that S100A4 expression is a powerful independent prognostic factor for recurrence and overall survival in CRC, which indicates the considerable prognostic value of S100A4 expression.

In conclusion, our current study employed a quantitative proteome analysis to profile the differently expressed proteins associated with LNM in CRC. S100A4 was identified and confirmed to be significantly overexpressed in LNM CRC. Further evaluation in an independent sample set has suggested that S100A4 acts as a powerful biomarker for LNM and prognosis in CRC. However, many questions remain to be answered with respect to the cellular function of S100A4 and how it exerts its influence on metastatic progression, with further investigations on our part in progress. We also identified a number of proteins besides S100A4 that might provide a more profound insight into the mechanism of LNM in CRC and merit further research.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, and it is estimated that half of the patients die from cancer annually. Lymph node metastasis (LNM) is the most common form of metastasis although the mechanism is largely unknown. Studies on metastasis of CRC were performed in order to improve the diagnosis and prognosis.

Research frontiers

LNM is a complicated process that involves a variety of dysregulated molecules playing a significant role. Increasingly, it has become a hot research topic to employ proteome analysis to identify proteins associated with tumor development and progression in various diseases.

Innovations and breakthroughs

To date, there has been a limited number of studies regarding specific tumor molecular markers associated with LNM in CRC. In this study, the authors employed more sensitive proteome analysis than conventional strategies to identify a set of differently expressed proteins associated with LNM. Furthermore, the authors confirmed the significant correlation between overexpression of S100A4 and LNM, advanced TNM stage, increased recurrence rate and decreased overall survival rate.

Applications

By identifying the protein S100A4 as being associated with LNM, the authors evaluated the biological features and prognosis in CRC, which could improve our understanding of CRC, and provide a scientific basis for the application of S100A4 inhibitors in the treatment of CRC.

Terminology

Proteome: all proteins that derive from the genome of cells, a tissue or an organism. It is a dynamic collection that reflects both the intrinsic genetic information of the cell and the impact of its immediate environment. Compared with gene analysis, proteome analysis can provide a more accurate view of the biological status and be expected to be more useful for evaluating, for example, disease development, progression and response to treatment.

Peer review

The authors performed also an extensive proteomics analysis, and identified 43 proteins that were differentially regulated in metastatic cancer, including 16 proteins that were upregulated even more than S100A4. The results indicate that overexpression of S100A4 could be used as biomarker for LNM in CRC. However, none of the latter candidate biomarkers were further examined in terms of prognostic value.

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S- Editor Shi ZF L- Editor Kerr C E- Editor Lin YP

MiRNA profile in esophageal squamous cell carcinoma: Downregulation of miR-143 and miR-145

Bing-Li Wu, Li-Yan Xu, Ze-Peng Du, Lian-Di Liao, Hai-Feng Zhang, Qiao Huang, Guo-Qiang Fang, En-Min Li

Bing-Li Wu, Hai-Feng Zhang, Qiao Huang, Guo-Qiang Fang, En-Min Li, Department of Biochemistry and Molecular Biology, Medical College of Shantou University, Shantou 515041, Guangdong Province, China

Li-Yan Xu, Ze-Peng Du, Lian-Di Liao, Institute of Oncologic Pathology, Medical College of Shantou University, Shantou 515041, Guangdong Province, China

Author contributions: Li EM and Xu LY designed the research; Wu BL, Du ZP, Liao LD, Zhang HF, Huang Q and Fang GQ performed the research and analyzed the data; Wu BL wrote the paper.

Supported by Grants from the NSFC-Guangdong Joint Fund, No. U0932001 and the National Natural Science Foundation of China, No. 30900560

Correspondence to: En-Min Li, Professor, Department of Biochemistry and Molecular Biology, Medical College of Shantou University, Shantou 515041, Guangdong Province, China. nmli@stu.edu.cn

Telephone: +86-754-88900413 Fax: +86-754-88900847

Received: June 10, 2010 Revised: July 29, 2010

Accepted: August 5, 2010

Published online: January 7, 2011

Abstract

AIM: To investigate the expression profile of miRNA in esophageal squamous cell carcinoma (ESCC).

METHODS: The expression profile of miRNA in ESCC tissues was analyzed by miRNA microarray. The expression levels of miR-143 and miR-145 in 86 ESCC patients were determined by real-time polymerase chain reaction (PCR) using TaqMan assay. The mobility effect was estimated by wound-healing using esophageal carcinoma cells transfected with miRNA expression plasmids.

RESULTS: A set of miRNAs was found to be deregulated in the ESCC tissues, and the expression levels of miR-143 and -145 were significantly decreased in most of the ESCC tissues examined. Both miR-143 and miR-145 expression correlated with tumor inva-

sion depth. The transfection of human esophageal carcinoma cells with miR-143 and miR-145 expression plasmids resulted in a greater inhibition of cell mobility, however, the protein level of the previously reported target of miR-145, *FSCN1*, did not show any significant downregulation.

CONCLUSION: These findings suggest that the deregulation of miRNAs plays an important role in the progression of ESCC. Both miR-143 and miR-145 might act as anti-oncomirs common to ESCC.

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Key words: Esophageal squamous cell carcinoma; MicroRNA; miR-143; miR-145; Tumor invasion depth

Peer reviewer: Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

Wu BL, Xu LY, Du ZP, Liao LD, Zhang HF, Huang Q, Fang GQ, Li EM. MiRNA profile in esophageal squamous cell carcinoma: Downregulation of miR-143 and miR-145. *World J Gastroenterol* 2011; 17(1): 79-88 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/79.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.79>

INTRODUCTION

MicroRNAs (miRNAs) are an endogenous conserved class of non-coding 20-22 nt small RNAs that regulate gene expression at post-transcriptional level by mainly binding to 3'-UTR of target mRNAs, leading to mRNA degradation or translation inhibition^[1]. Many miRNAs show sequence and function conservation between distantly related organisms, suggesting that this class of small RNAs is an integral part of essential cellular processes^[2]. It was predicted that about 30% of human genes are

regulated by miRNAs^[3]. miRNAs regulate a variety of biological processes, including developmental timing, signal transduction, cell growth, and cell death^[4]. The importance of microRNA in cancer is highlighted by the observation that about 50% of miRNAs are located in cancer-associated genomic regions or fragile sites, which are frequently amplified or deleted in tumorigenesis^[5]. Moreover, accumulated evidence shows that miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate^[6]. Recent reports demonstrate a role for miRNA expression in disease progression and outcome^[7].

Esophageal carcinoma is one of the most lethal malignancies in China and other Asian areas, with a significant low 5-year survival rate after curative surgery^[8,9]. There are two major histologic types of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. To date, many studies focusing on miRNA expression profiles in Barrett's esophagus and esophageal adenocarcinoma have been reported^[10]. Yang *et al.*^[11] identified 11 miRNAs showing statistically significant differences between the different progression stages of esophageal adenocarcinoma. Nevertheless, there is still little information available on specific miRNA expression patterns and their roles in ESCC. Feber *et al.*^[12] identified a set of differentially expressed miRNAs that could distinguish different esophageal tissue types and also discriminate malignant from normal esophageal tissue, including adenocarcinoma, squamous cell carcinoma, Barrett's esophagus and high-grade dysplasia. These data suggest that miRNA expression profiling is now a promising method to identify key miRNAs which play important roles in esophageal carcinogenesis.

Chaoshan Area in China is the main coastal area and has a high ESCC morbidity rate^[13]. The specific geographical environment and dietary habits of the population may characterize some of the specific features of ESCC in this area, which might also be reflected in the miRNA expression profile in ESCC tissues. To develop novel diagnostic and therapeutic targets for esophageal squamous cell cancer, we first investigated the expression profile of miRNA in three pairs of clinical ESCC samples and confirmed the differences in expression of relevant miRNAs using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 86 pairs of ESCC samples. The associations between miRNA expression and clinicopathological features were analyzed. Furthermore, we tried to identify the targets of these differentially expressed miRNAs.

MATERIALS AND METHODS

Cell lines

Human ESCC cell lines EC8712, KYSE150, EC109, EC18, SHEEC, KYSE180, KYSE70, and KYSE140 were cultured in 199 or DMEM medium (Invitrogen, Carlsbad, CA, USA) plus 10% newly born calf serum.

Table 1 Clinical characteristics of esophageal squamous cell carcinoma used for quantitative reverse transcription-polymerase chain reaction analysis

Clinical parameter	<i>n</i>
Gender	
Male	62
Female	24
Age (yr)	
< 55	35
≥ 55	51
Diameter	
< 5 cm	57
≥ 5 cm	29
LNM	
N0	53
N1	33
Invasion	
T1	4
T2	18
T3	64
Histological type	
Ulcerative	49
Medullary	24
Fungating	10
Others	3
Differentiation	
I	23
II	51
III	12
TNM stage	
I	3
II a	49
II b	4
III	30

Specimen collection

ESCC tissues and matched normal tissues were obtained from surgical specimens immediately after resection from patients undergoing primary surgical treatment of esophageal carcinoma from Oct 2007 to Dec 2008 in the Department of Tumor Surgery of Shantou Central Hospital, China. No patient had received preoperative irradiation or chemotherapy. The samples were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Among these samples, three were used for microRNA microarray analysis and 86 were using for qRT-PCR analysis. Tumor specimens underwent histological examination by a pathologist to confirm the diagnosis, verify the presence of tumor, select those samples with at least 75% tumor tissue, and establish the pathological stage. Clinical and pathological information was extracted from the patients' medical charts and pathology reports. The clinical data used for qRT-PCR analysis are shown in Table 1. Written consent for tissue donation (for research purposes) was obtained from the patients before tissue collection and the protocol was approved by the Institutional Review Board of Shantou Central Hospital.

miRNA microarray

RNA labeling and hybridization were completed by KangChen Bio-tech Inc. (Shanghai, China) according to

the manufacturer's instructions. Briefly, total RNA from three pairs of esophageal carcinoma and matched normal tissues were isolated using Trizol (Invitrogen, USA) and purified using the RNeasy mini kit (QIAGEN, Germany). The concentration and quality of total RNA were measured by NanoDrop ND-1000 at 260 and 280 nm (A260/280) and confirmed by gel electrophoresis. Each RNA sample from three pairs of ESCC was separately labeled using the miRCURY Hy3/Hy5 labeling kit and hybridized on the six miRCURYTM locked nucleic acid (LNA) array version 11.0 (Exiqon, Denmark), which contains probes for 1700 mature miRNAs. Scans were quantified using GenePix software (Molecular Devices). The data were exported to Microsoft Excel worksheets, log₂ transformed, normalized using global Lowess (Locally Weighted Scatter plot Smoothing) regression algorithm (MIDAS, TIGR Microarray Data Analysis System), which we previously found to produce the best within-slide normalization to minimize the intensity-dependent differences between the dyes. Replicated spots on the same slide were averaged by obtaining a median ratio of replicated spots. Between slides normalization was performed by scale normalization to reduce between-slide variability. Only those with a greater than 2-fold increase or 2-fold decrease in expression in two samples were considered significantly changed. Samples were clustered according to their miRNA profile using Cluster 3.0 and shown using Treeview.

miRNA real-time RT-PCR quantification

qRT-PCR analysis of miRNA expression was carried out using TaqMan MicroRNA Assay kits according to the manufacturer's protocol (Applied Biosystems, USA). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, USA) from clinical samples and ESCC cell lines. cDNAs were synthesized from total RNA using gene-specific primers. Reverse transcriptase reactions contained 10 ng RNA samples, 50 nmol/L stem-loop RT primer, 1 × RT buffer, 0.25 mmol/L each of the dNTPs, 3.33 U/μL MultiScribe reverse transcriptase and 0.25 U/μL RNase inhibitor. The 15 μL reactions were incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The 20 μL PCR reaction included 1.33 μL RT product, 1 × TaqMan Universal PCR master mix and 1 μL primers and probe mix of the TaqMan MicroRNA Assay kit. Reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR reactions were run on a 7500 Real Time PCR machine (Applied Biosystems) and analyzed using 7500 System SDS software.

U6 small nuclear RNA was used as an internal control to normalize RNA input. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The fold change was calculated using the 2^{-ΔΔCt} method, presented as the fold-expression change in tumors relative to their corresponding normal tissues after normalization to the endogenous control. All experiments were carried out in triplicate.

Plasmid construction

Human genomic fragments of miR-145 and miR-143 pre-

cursors with flanking about 200 bp were amplified by PCR using human genomic DNA as a template. PCR primers were designed as follows: miR143 forward: 5'-AAGCT-TAAGGTCAAGGTTTGGTCCT-3'; miR143 reverse: 5'-CTCGAGTGCTAAGATGGACACACTGG-3'; miR145 forward: 5'-AAGCTTCAGAGGGTTTCCGGTACTT-3'; miR-145 reverse: 5'-CTCGAGAGCCTCACAGGGAT-GTTATG-3'. The PCR products were cloned into the pDNA3.0 vector and named pcDNA-miR145 and pcDNA-miR143, respectively.

Transfection

Approximately 2 × 10⁵ of KYSE150 and KYSE180 cells were seeded and cultured in 6-well plates, respectively. For each well, 2.0 μg of plasmids were added to 100 μL Opti-MEM medium and 10 μL of Superfect (QIAGEN, Germany). The mixture was added to the cells and incubated for 2 h before replacing the medium. Stable clones were generated by selection in complete culture medium containing 400 mg/L of G418.

In vitro wound-healing assay

KYSE150 and KYSE180 cells were seeded in a 6-well dish, and incubated overnight yielding a confluent monolayer for wounding. Wound healing was performed using a tip with a flat point. An image was taken at different time points in each visual field at 50 ×.

miRNA target prediction

TargetScan (release 5.1, <http://www.targetscan.org/>) was used to analyze potential target genes for the deregulated microRNAs.

Western blotting

Total cell lysates were prepared in RIPA buffer [50 mmol/L TrisHCl, pH 8.0, 150 mmol/L NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium desoxycholate, 0.1% (wt/vol) SDS] containing the complete protease inhibitor cocktail. Western blotting analysis was performed as described with the following primary antibodies: monoclonal mouse anti-fascin (DAKO, Denmark) and mouse anti-β-actin (Sigma, MO, USA). Experiments were repeated in triplicate.

Statistical analysis

Statistical differences between tumor and normal tissue were evaluated using the paired *t*-test. Statistical differences between clinicopathologic parameters and miRNA fold change were evaluated using ANOVA. The correlation coefficients of miR-143 and miR-145 were calculated using the Spearman correlation. *P* values less than 0.05 were considered statistically significant. All calculations were performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

The group specimen used for qRT-PCR was obtained

from 62 males and 24 females and their details are shown in Table 1. The average age of these patients was 54 years and ranged from 40 to 75 years. Fifty-seven patients (66.3%) had tumors smaller than 5 cm, and 29 (33.7%) had tumors greater than 5 cm. Lymph node metastases were observed in 38.4% of the patients ($n = 33$), while the remaining patients had no lymph node metastases. Of these patients, 4 (4.7%) of 86 were diagnosed at invasion T1, 18 (20.9%) at invasion T2 and 64 (74.4%) at invasion T3. Histological type was ulcerative in 49 cases, medullary in 24 cases, fungating in 10 cases and 3 cases were unidentified. Seventy-four cases were well differentiated (I + II) and 12 cases were poorly differentiated. Taken together, the numbers diagnosed in the four clinical stages were 3, 49, 4 and 30, respectively. After two years of follow-up, the overall survival rate of the 86 ESCC patients was as high as 84.9% with only 13 deaths reported. The impact of the expression of miRNAs on patient survival will be analyzed in a future study.

Differential expression of miRNAs found in ESCC

Using miRNA microarray, we then identified the miRNAs that were differentially expressed in tumor and non-tumor samples. Only miRNAs that were altered by at least 2-fold in at least two of the samples were considered significant candidates. Using these strict criteria, we identified 33 upregulated miRNAs and 40 downregulated miRNAs between normal and cancer tissues. Heat maps depict the relative expression level of mature miRNAs indicated by microarray analyses of the samples from three patients (Figure 1). This showed that the changes from two chips in each clinical case were consistent.

In accordance with previous reports^[12,14], miR-21 was observed to be the most upregulated miRNA with an average 24.4-fold change. miR-203 was the most downregulated with an average 4.3-fold change. miR-203, miR-99a and miR-100 were also found to be downregulated in this study. With the exception of these, several miRNAs such as miR-143 and miR-145 were found to be changed in esophageal squamous cell carcinoma (Figure 1). On average miR-143 was downregulated 4.3-fold, while miR-145 was downregulated 3.2-fold. miR-25 was only detected in one chip and was upregulated 2.3-fold. However, a previous report of significant differential expression of miRNAs in ESCC^[12,14-16], including miR-106b, -103, -107, -34b, -139 and -129, did not show significant changes in our study. All raw and normalized miRNA expression data are available from GEO publicly accessible server (<http://www.ncbi.nlm.nih.gov/geo/>) with the accession number: GSE23142.

Downregulation of miR-143 and miR-145 in ESCC

To confirm our microarray data and to determine the clinical significance of deregulated expression of miRNAs in esophageal carcinoma, we evaluated the expression of two cancer-associated miRNAs, miR-143 and miR-145, in 86 clinical samples of esophageal carcinoma and their matched normal tissues using qRT-PCR. These microR-

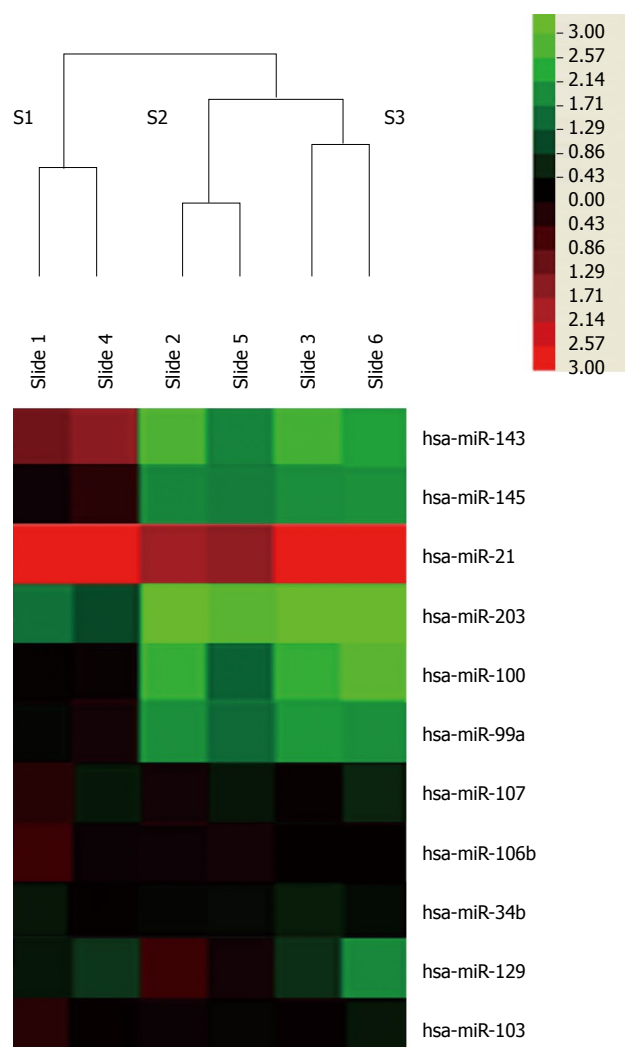


Figure 1 miRNAs are deregulated in esophageal squamous cell carcinoma as detected by microRNA microarray. Three pairs of esophageal carcinoma and normal tissue matches were analyzed by miRCURY LNA™ microRNA Arrays v.11.0. Each RNA sample was dye-swap labeled. Unsupervised hierarchical cluster analysis of miRNA expression in three esophageal carcinoma patients. Rows: miRNAs; columns: cases. The analysis showed that two chips in each case were consistent. For each miRNA, red represents higher expression and blue represents lower expression than the average expression. S1, sample 1; S2, sample 2; S3, sample 3.

NAs were chosen as they had previously been shown to be differentially expressed in various tumors. U6 RNA expression did not differ significantly between tumor and non-tumor tissue in our study population (data not shown). As shown in Figure 2, the expression level of miR-145 was significantly downregulated in ESCC compared to matched normal tissues ($P = 0.001$), and no statistically significant difference in miR-143 expression between the two groups was observed ($P = 0.436$).

Using the $2^{-\Delta\Delta Ct}$ method and a 2-fold change criterion, the qRT-PCR results showed that the expression of miR-143 was downregulated in 47.7% (41/86) and miR-145 was downregulated in 61.6% (53/86) of the clinical samples of esophageal carcinoma (Figure 3). The qRT-PCR results showed good consistency with the microRNA microarray results. In order to investigate the

Table 2 Associations between expression levels of miR-143 and miR-145 and clinicopathological features

	miR-143		miR-145	
	median \pm SD	P value	median \pm SD	P value
Gender				
Male	1.43 \pm 2.99	0.805	0.82 \pm 1.61	0.718
Female	1.27 \pm 1.72		0.95 \pm 1.32	
Age (yr)				
< 55	1.67 \pm 3.54	0.418	1.15 \pm 1.99	0.138
\geq 55	1.19 \pm 1.92		0.65 \pm 1.08	
Diameter (cm)				
< 5	1.47 \pm 2.02	0.701	0.93 \pm 1.31	0.497
\geq 5	1.23 \pm 3.71		0.69 \pm 1.89	
LNM				
N0	1.47 \pm 3.17	0.717	0.88 \pm 1.71	0.815
N1	1.25 \pm 1.70		0.80 \pm 1.16	
Tumor stage				
T1	6.31 \pm 9.33	0.000 ^b	3.33 \pm 4.70	0.001 ^b
T2	0.80 \pm 1.32		0.34 \pm 0.30	
T3	1.25 \pm 1.87		0.84 \pm 1.26	
Histological type				
Ulcerative	1.45 \pm 3.28	0.996	0.79 \pm 1.76	0.955
Medullary	1.29 \pm 1.72		0.90 \pm 1.23	
Fungating	1.32 \pm 1.70		0.90 \pm 1.13	
Others	1.48 \pm 1.18		1.27 \pm 0.98	
Differentiation				
I	1.60 \pm 2.11	0.907	0.87 \pm 1.31	0.925
II	1.30 \pm 3.12		0.81 \pm 1.70	
III	1.38 \pm 1.60		1.00 \pm 1.15	
TNM stage				
I	0.76 \pm 1.00	0.971	0.94 \pm 1.39	0.998
II a	1.41 \pm 2.18		0.83 \pm 1.31	
II b	1.06 \pm 1.47		0.95 \pm 1.48	
III	1.46 \pm 3.60		0.87 \pm 1.90	

^b $P < 0.01$.

clinical values of miR-143 and miR-145, we analyzed the relationships between the expression levels of these two miRNAs in cancer tissues and clinicopathological factors of patients with esophageal carcinoma. The mean fold changes of miRNAs in esophageal carcinoma samples and their possible connections with cancer are presented in Table 2. Significant correlations between miR-143 and miR-145 levels in the primary tumors and tumor invasion were observed ($P = 0.000$ and $P = 0.001$, respectively, Figure 4). Furthermore, the co-expression of miR-143 and miR-145 was analyzed with the Spearman correction and showed a value of 0.967 ($P = 0.000$).

miR-143 and miR-145 inhibit cell mobility

The expression levels of miR-143 and miR-145 were significantly downregulated in ESCC cell lines (Figure 5A). Following transfection with pcDNA-miR143 and pcDNA-miR145, the expression levels of these two microRNAs were significantly increased (Figure 5B). In the wound-healing assays, the distance KYSE150 and KYSE180 stably transfected cells moved in a wounded cell monolayer on plastic was determined, together with pcDNA transfected cells which were used as controls. The results showed that the cells transfected with

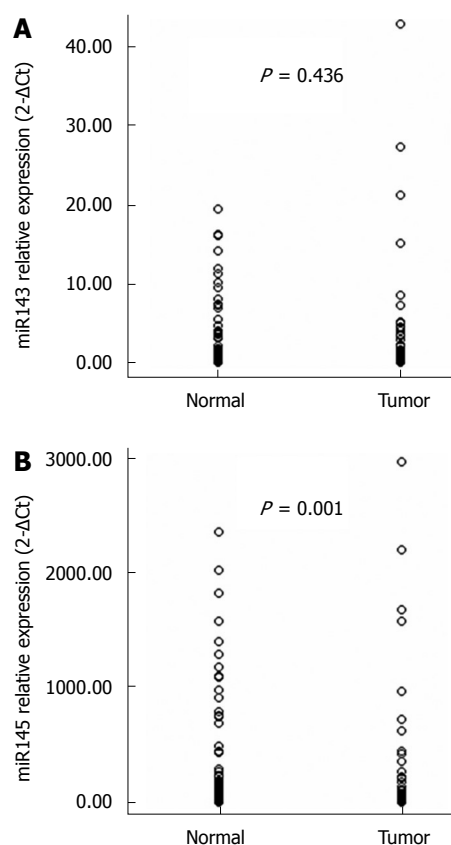


Figure 2 Differential expression of miR-143 (A) and miR-145 (B) in esophageal squamous cell carcinoma compared to normal tissue. Each value represents the relative expression using the 2- Δ Ct method with U6 RNA as an endogenous control. miR-145 is significantly deregulated in esophageal carcinoma patients ($P = 0.001$).

miR-145 and miR-143 migrated very small distances and were unable to achieve wound closure (Figure 5C and D, respectively). For stably transfected KYSE150 cells, 89% of the wounds containing the control cells (16/18 visual fields) were closed or were healing, while only 58% of the miR-143 transfectant (7/12 visual fields) and 18% of the miR-145 transfectant (3/17 visual fields) were healing. For stably transfected KYSE180 cells, the percentage of healing in the control, miR-143 transfectant and miR-145 transfectant was 50% (6/12), 42% (5/12) and 0% (0/6), respectively.

miRNA target prediction and identification

Since miR-143 and miR-145 can inhibit the mobility of esophageal carcinoma cells, we tried to identify their targets that related to cell mobility. Recently, *FSCN1* has been proved to be a target of miR-145 both in the esophageal squamous cell lines TE2 and TE13 and in bladder cancer^[17,18]. However, our results showed that the protein level of *FSCN1* did not significantly change in the miR-143 and miR-145 stable expressing KYSE150 and KYSE180 cells, respectively (Figure 6A and B). Using TargetScan, we found that the 3'-UTR of *FSCN1* contained hundreds of miRNA binding sites, especially four conserved binding sites for miR-145 and two non-conserved

Table 3 miR-143 and miR-145 predicted targets related to cell mobility

Predicted targets	Functions
miR-143	
LASP1 (LIM and SH3 protein 1)	Functions as an actin-binding protein and possibly in cytoskeletal organization
RICTOR (RPTOR independent companion of MTOR, complex 2)	Functions upstream of Rho GTPases to regulate the actin cytoskeleton
ARHGAP26 (Rho GTPase activating protein 26)	Regulates the organization of the actin-cytoskeleton
EPB41 [erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)]	Together with spectrin and actin, constitute the red cell membrane cytoskeletal network.
MYO3A (myosin III A)	Belongs to the myosin superfamily, an actin-dependent motor protein
MARCKS (myristoylated alanine-rich protein kinase C substrate)	An actin filament crosslinking protein involved in cell motility
SVIL (supervillin)	Tightly associated with both actin filaments and plasma membranes
miR-145	
ARF6 (ADP-ribosylation factor 6)	Regulates vesicular trafficking, remodeling of membrane lipids, and signaling pathways that lead to actin remodeling.
ABLIM2 (actin binding LIM protein family, member 2)	Bound strongly to F-actin, localized to actin stress fibers
ADD3 [adducin 3 (γ)]	Involved in assembly of the spectrin-actin network in erythrocytes and at sites of cell-cell contact in epithelial tissues
CAPZB (c apping protein (actin filament) muscle Z-line, β)	Regulates growth of the actin filament by capping the barbed end of growing actin filaments.
ELMO1 (engulfment and cell motility 1)	Mediates cytoskeletal rearrangements during phagocytosis of apoptotic cells and cell motility
PHACTR2 (phosphatase and actin regulator 2)	Coprecipitated with both PP1 and actin
TMOD1 (tropomodulin 1)	Inhibits depolymerization and elongation of the pointed end of actin filaments

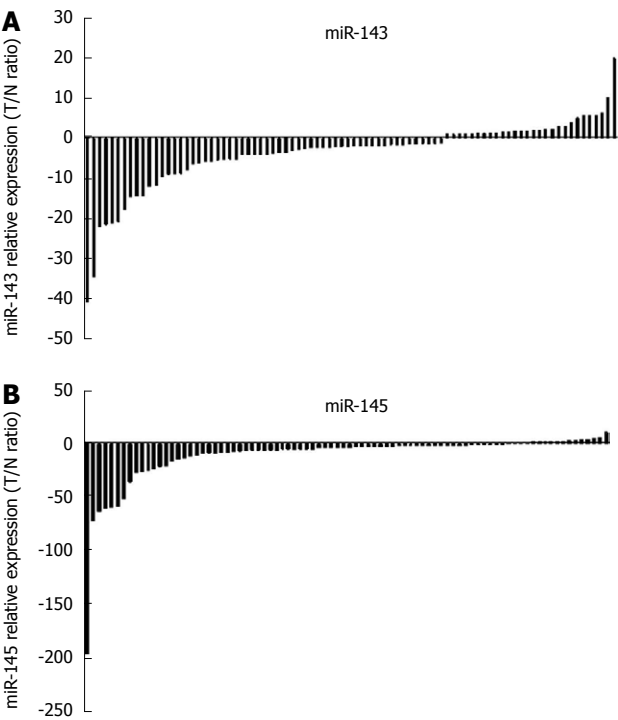


Figure 3 Downregulation of miR-143 and miR-145 in esophageal squamous cell carcinoma. Using the $2^{-\Delta\Delta Ct}$ method, those with a greater than 2-fold change were considered significant. The expression of miR-143 was downregulated in 48.9% (46/94) and miR-145 was downregulated in 60.6% (57/94) clinical samples of esophageal carcinoma.

binding sites for miR-143 (Figure 7). Moreover, we also found other predicted targets of miR-143 and miR-145 that related to cell mobility using TargetScan (Table 3).

DISCUSSION

Accumulated studies have indicated that microRNAs,

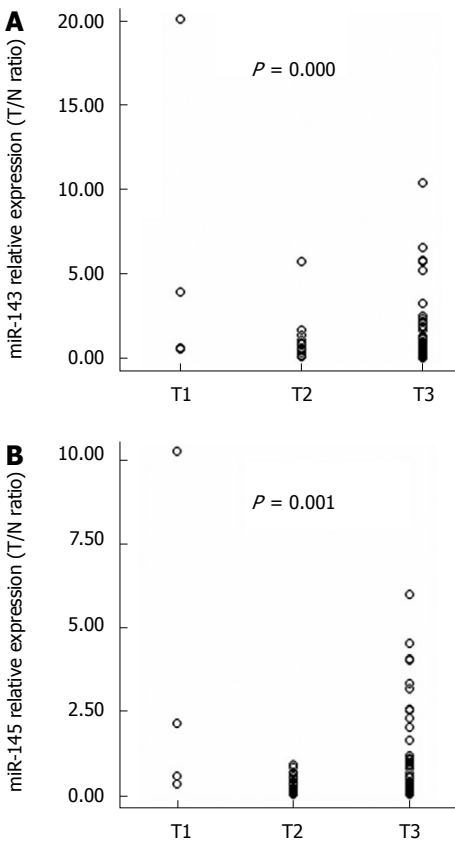


Figure 4 Associations between the expression of miR-143 and miR-145 and clinicopathologic features. The expression of both miR-143 and miR-145 were significantly associated with esophageal squamous cell carcinoma tumor invasion ($P = 0.000$ and $P = 0.001$, respectively).

posttranscriptional modulators of gene expression, are involved in the initiation and progression of various malignancies. Many studies have demonstrated that some human miRNAs are consistently deregulated in human

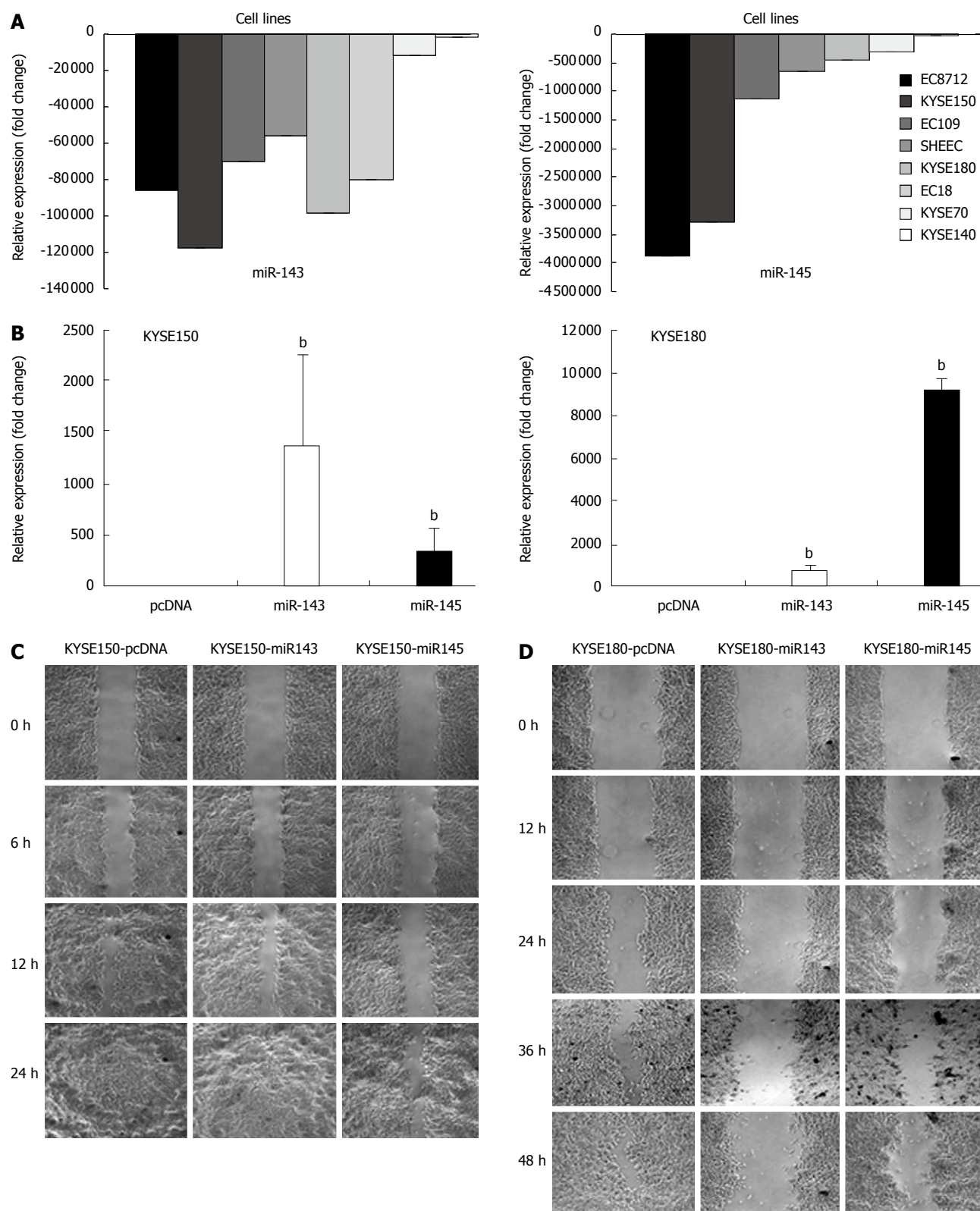
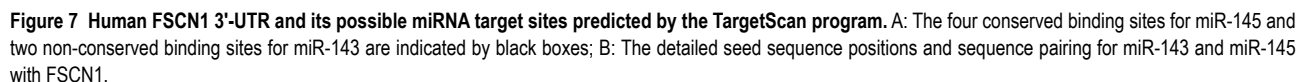
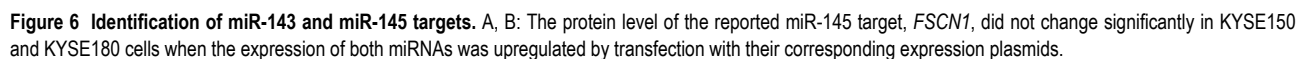


Figure 5 miR-143 and miR-145 inhibited esophageal squamous cell carcinoma cell mobility. A: The expression levels of miR-143 and miR-145 in esophageal squamous cell carcinoma (ESCC) cell lines. Using normal tissue as the control, both miRNAs were significantly downregulated in ESCC cell lines; B: The increased expression of miR-143 and miR-145 following stable transfection. Quantitative reverse transcription-polymerase chain reaction demonstrated a significant increase in the expression levels of miR-143 and miR-145 in KYSE150 and KYSE180 cells transfected with the respective expression plasmids compared with the levels in control cells ($P < 0.01$); C, D: The increased expression of miR-143 and miR-145 inhibited the mobility of both KYSE150 and KYSE180 cells. The miRNA stably transfected cells were seeded in a 6-well plate, streaks were made using a tip when the cells were grown to almost confluence 24 h later. Streaks were photographed at different intervals at 50 \times . This showed that upregulation of miR-143 and miR-145 can prevent ESCC cell wound healing. These findings are shown in (C) and (D).

cancer, suggesting a role for these genes in tumorigene-

sis^[19]. A comparison between human cancers and adjacent



In this study, our miRNA microarray results partially agree with Feber's and Guo's findings, including miR-21 and miR-203, miR-99a and miR-100, respectively, which further support the robustness of our results. With the exception of deregulated miRNAs identified in Feber's and Guo's reports, we also found common differentially expressed miRNAs as reported recently by Ogawa *et al.*^[16], including the upregulation of miR-21 and the downregulation of miR-133b and miR-145. Nevertheless, this is the

A number of reports have shown that miR-143 and miR-145 are downregulated in various cancer cells, including colorectal cancer, nasopharyngeal carcinoma, lung cancer and B-cell malignancies^[20-23]. To our knowledge, we have performed the largest study to date which assesses the potential diagnostic and prognostic utility of

miRNAs in esophageal squamous cell cancer. Consistent with the aforementioned previous reports, our miRNA microarray and qRT-PCR results revealed that miR-143 and miR-145 were under-expressed in most esophageal carcinoma specimens, compared with matched normal tissue samples. These results imply that these two miRNAs might serve as novel diagnostic and therapeutic targets for esophageal squamous cell cancer.

However, the pathobiological significance of aberrant miRNA expression in human esophageal squamous cell carcinoma has not been well documented. In this study, we found that both miR-143 and miR-145 correlated with tumor invasion. This suggested that both of these miRNAs might be associated with esophageal carcinoma progression. To date, little information is available on the correlations between miR-143 and miR-145 and the clinicopathologic features of other tumors. It was reported that there was no relationship between miR-143 and miR-145 expression and other clinicopathological features, except that miR-145 expression was related to cancer site following the analysis of 98 primary colorectal cancer specimens^[20]. Slaby *et al.*^[24] also found that colorectal cancer tumors larger than 50 mm in maximum diameter were characterized by low expression of miR-143 and miR-145. Clapé *et al.*^[25] showed that miR-143 expression levels were inversely correlated with advanced stages of prostate cancer. Therefore, the roles of miR-143 and miR-145 in esophageal squamous cell carcinoma progression remain to be revealed.

Xin *et al.*^[20] showed that miR-143 and miR-145 selectively target genes that regulate the actin cytoskeleton in smooth muscle cells, which is intimately coupled to cell migration. Migration of esophageal squamous carcinoma cells was consistently reduced by over-expression of miR-143 and miR-145 in our wound-healing experiments. It has been reported that *FSCN1* is a target of miR-145 both in bladder cancer and esophageal carcinoma^[17,18]. However, no significant downregulation of *FSCN1* protein was found in ESCC KYSE150 and KYSE180 cells, while the expression of miR-143 and miR-145 was rescued. This controversial result may be due to the specificity of these cell lines. Using TargetScan, we also found other predicted targets of miR-143 and miR-145 related to cell mobility, such as *LASP1* and *ARF6*. These results implied that *FSCN1* could be regulated by other miRNAs in ESCC cell lines except the TE series^[16]. The targets of miR-143 and miR-145 in KYSE150 and KYSE180 cells remain to be identified.

Interestingly, the chromosome loci of both miR-143 and miR-145 are very close to each other within approximately 2 kb on 5q32, which led us to speculate that both precursors originate from the same primary transcript. In our study, we found that these two miRNAs were highly co-expressed in 86 esophageal tumors, with Spearman correlation coefficients of 0.967 ($P = 0.000$). This indicated that miR-143 and miR-145 might be regulated by the same factor(s) and generated from the same primary transcript (pri-miRNAs). However, various results have suggested that there may be different mechanisms for the transcriptional regulation of this locus. miR-143 and

miR-145 could be transcribed individually and co-transcribed. Zhang *et al.*^[27] demonstrated that miR-143 is transcribed by nuclear factor κ B, and the expression levels of miR-143 were dramatically increased in metastatic HBV-HCC and HCC patients. Xu *et al.*^[28] found that the 1.5 kb sequence upstream of miR-145 has an intrinsic promoter activity, and such activity is repressed by OCT4, which is one of the targets of miR-145. Cordes *et al.*^[29] also found that miR-143 and miR-145 were direct transcriptional targets of the serum response factors, myocardin and Nkx2-5, in multipotent murine cardiac progenitors. The transcription regulation of miR-143/-145 seems to be tissue-specific.

In summary, we report that miRNAs were deregulated and miR-143 and miR-145 were downregulated in ESCC. Furthermore, rescued expression of miR-143 and miR-145 can inhibit cell mobility. This study provides the first evidence for the anti-oncogenic activity of miR-143 and miR-145 in the development of esophageal squamous cell cancer. Targets of these two miRNAs remain to be defined. These results indicate that miRNAs may eventually constitute useful biomarkers as well as therapeutic targets.

COMMENTS

Background

MicroRNAs (miRNAs) regulate gene expression by mainly binding to the 3'-UTR of target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

Research frontiers

Esophageal squamous cell carcinoma is one of the most lethal malignancies in China. Many studies have reported the miRNA expression profiles in Barrett's esophagus and esophageal adenocarcinoma. In this study, the authors report the expression profile of miRNA in esophageal squamous cell carcinoma (ESCC) and investigate the expression and functions of miR-143 and miR-145 in ESCC.

Innovations and breakthroughs

Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. A set of miRNAs was found to be deregulated in ESCC and the expression levels of miR-143 and miR-145 were significantly decreased in most of the ESCC tissues examined. The authors performed the largest study to date which assessed the potential diagnostic and prognostic utility of miRNAs in ESCC. It is the first study to show that both miR-143 and miR-145 were correlated with tumor invasion depth. The anti-oncogenic role of miR-143 and miR-145 was demonstrated as significant cell mobility inhibition.

Applications

This study provides the first evidence of the anti-oncogenic activity of miR-143 and miR-145 in the development of ESCC. These results indicated that miRNAs may eventually constitute useful biomarkers as well as therapeutic targets.

Peer review

Many miRNAs are found deregulated in ESCC tissue and miR-143 and -145 are significantly decreased in ESCC. Both miR-143 and miR-145 correlated with tumor invasion depth. This study provides the evidence for an anti-oncogenic activity of miR-143 and miR-145 in the development of ESCC and may develop to be useful biomarkers or therapeutic targets in ESCC. It's an excellent study.

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S- Editor Wang JL L- Editor Webster JR E- Editor Ma WH

Impact of diet on long-term decline in gastric cancer incidence in Poland

Mirosław Jarosz, Włodzimierz Sekuła, Ewa Rychlik, Katarzyna Figurska

Mirosław Jarosz, Ewa Rychlik, Department of Dietetics and Nutrition in Hospitals with Clinic of Metabolic Diseases and Gastroenterology, National Food and Nutrition Institute, 02-903 Warsaw, Poland

Włodzimierz Sekuła, Katarzyna Figurska, Independent Laboratory of the Economics of Food and Nutrition, National Food and Nutrition Institute, 02-903 Warsaw, Poland

Author contributions: Jarosz M designed the research; Sekuła W and Figurska K performed the research; Sekuła W, Rychlik E and Figurska K analyzed the data; Jarosz M, Sekuła W and Rychlik E wrote the paper.

Supported by Statutory action of the National Food and Nutrition Institute

Correspondence to: Mirosław Jarosz, Professor, Department of Dietetics and Nutrition in Hospitals with Clinic of Metabolic Diseases and Gastroenterology, National Food and Nutrition Institute, Powsińska St. 61/63, 02-903 Warsaw, Poland. jarosz@izz.waw.pl

Telephone: +48-22-5509677 Fax: +48-22-8421103

Received: July 6, 2010 Revised: September 7, 2010

Accepted: September 14, 2010

Published online: January 7, 2011

of refrigerators in the household (-0.77 and -0.80; $P < 0.0001$). A decline in these rates could also be linked to reduction in salt intake.

CONCLUSION: The decline of gastric cancer incidence probably resulted from increased consumption of vegetables, fruit and vitamin C and a decrease in salt consumption.

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Key words: Vegetables; Fruit; Vitamin C; Salt; Gastric cancer

Peer reviewer: Maria Gabriella Caruso, MD, Piazza Giuseppe Garibaldi 49, 70122 Bari, Italy

Jarosz M, Sekuła W, Rychlik E, Figurska K. Impact of diet on long-term decline in gastric cancer incidence in Poland. *World J Gastroenterol* 2011; 17(1): 89-97 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/89.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.89>

Abstract

AIM: To examine the relationship between the trends in food consumption and gastric cancer morbidity in Poland.

METHODS: The study was based on gastric cancer incidence rates and consumption of vegetables, fruit, vitamin C and salt in Poland between 1960 and 2006. Food consumption data were derived from the national food balance sheets or household budget surveys. Spearman correlation coefficients were used to estimate the relationship between the variables.

RESULTS: A negative correlation was found between vegetables (-0.70 both for men and women; $P < 0.0001$), fruit (-0.65 and -0.66; $P < 0.0001$) and vitamin C (-0.75 and -0.74; $P < 0.0001$) consumption and stomach cancer incidence rates. The same applied to the availability

INTRODUCTION

Gastric cancer ranks fourth in morbidity and second in mortality for malignant cancers worldwide^[1]. However, in numerous countries, in particular in economically developed ones, gastric cancer morbidity within the past few decades has reduced significantly. Several epidemiological studies have shown that this phenomenon is most probably related to favorable changes in dietary pattern in the developed countries, that is, mostly to increased consumption of fresh fruit and vegetables, and decreased consumption of salt-preserved food^[2] and greater availability of refrigerators^[3]. It is assumed that lower gastric cancer morbidity rates are also related to lower prevalence of *Helicobacter pylori* (*H. pylori*) infection, which is considered as an essential factor for the development of a majority of gastric cancers^[4,5]. In these countries, prevalence of

H. pylori infections affects 20%-40% of the population.

Against this background, Poland seems to be a particular case. Despite the fact that, according to national epidemiological studies, the percentage of people infected with *H. pylori* is unusually high and amounts to 73% of the total population and 85%-95% of those aged > 25 years^[6], in 2006, gastric cancer morbidity rate was twofold lower compared to that in 1960^[7,8].

What was the reason for such a significant decrease in morbidity rate, taking such widespread dissemination of *H. pylori* as a major carcinogen into consideration? This intriguing phenomenon might be most probably assigned to specific changes in dietary pattern, as in the case of western countries.

Data that reflect average food consumption per capita in Poland in 1950-2006 show that this period was highly diversified with regard to dietary trends^[9-11]. In relation to the above, the two following periods can be distinguished: 1950-1989 and 1990-2006. Within the first period, consumption of products of animal origin (meat and meat products, animal fat, milk and dairy products, butter, fish and eggs) as well as sugar and sugar products showed a growing trend. At the same time, consumption of cereals and potatoes had decreased. Growth of vegetable consumption had come to an end in the 1950s and 1960s. Fruit consumption was low, particularly compared to other countries, due to frequent fluctuations in crops and limited fruit import, which was insufficient to mitigate the effect of such fluctuations.

Within the second period, a reversal in these trends was noticeable, particularly in relation to consumption of butter and other animal fats, red meat as well as milk and dairy products. This period was also characterized by a significant increase in fruit consumption and, despite no increasing trend, also in relatively high vegetable consumption^[9-11].

The above-mentioned favorable phenomena are considered to have brought about a significant improvement in the health situation in Poland. It can be assumed that positive changes in diet have influenced the decline in overall mortality seen since 1992, and stabilization of total malignant cancer mortality as well as a significant decrease in morbidity.

MATERIALS AND METHODS

Data on gastric cancer incidence rates were derived from the National Cancer Registry administered by the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw^[7,8]. They showed standardized gastric cancer incidence rates for men and women covering individual years between 1960 and 2006, excepting 1984, 1986, 1997 and 1998, for which no such data were available; in the case of the missing data for 1997-1998, the physicians' strike was the main contributing reason.

The source of information on the dietary pattern in the same time period was the database that was established and maintained for several decades by the National Food and Nutrition Institute^[9-11]. This database covers both

published and unpublished data derived from the national food balance sheets and shows major food quantities available for consumption per capita/year. They are converted into energy and nutrients with the use of a set of nutrient conversion factors developed at the institute and based on the national food composition tables^[12]. The resultant estimates show energy and nutrient amounts derived from food and available for consumption per capita/d.

In Poland, no data on average consumption of table salt per capita over the long term are available. Such data are available only for the period before World War II^[13]. After the war, this practice has not been continued. Data on salt consumption re-appeared, however, in 1998. They show average monthly consumption of salt per capita in households, which participated in budget surveys, carried out on annual basis using a sampling method that allowed for generalization of the results to all households in the country^[14]; these data were used for the analyses in the present study.

The features of the data on food quantities available for consumption and the derived estimates of the amounts of energy and nutrients made them particularly useful in the analysis of the trends over time, and to compare them with the trends in the health situation. In such a way, they were utilized in the present study. The study was focused on identification and measurement of the relationship between gastric cancer incidence rates and variables related to dietary pattern represented by the consumption of fruit, vegetables, vitamin C and kitchen salt. A trend in the equipment of Polish households with refrigerators was taken into consideration also as an important factor that affected perishable food quality and protected against nutrient loss.

Spearman's rank correlation coefficients (r_s) were estimated as a measure of the relationship between stomach cancer incidence rates and selected parameters.

RESULTS

Gastric cancer has a much higher incidence among men than women. In a base year of our analysis, i.e. 1960, the standardized rate for men amounted to 25.1/100 000, which exceeded twice the incidence rate for women (10.4/100 000)^[7]. For men, this rate reached a maximum level in 1970 and was 38% higher compared to 1960. In the same year, the maximum morbidity level was observed among women also; the rate was 39% higher than in 1960. The following years brought a decline in morbidity, and in 2006, the rates for men and women were approximately two times lower compared to the base year^[8].

Due to the fact that gastric cancer is one of two major types of cancer for which the risk is commonly agreed to be modified mainly by food and nutrition^[15], correlations between morbidity rates and consumption of certain food products and nutrients, which had both a positive and negative impact on this type of cancer, were analyzed. Due to reliable evidence that diets rich in fruit and vegetables protect against gastric cancer, these groups of products were analyzed first.

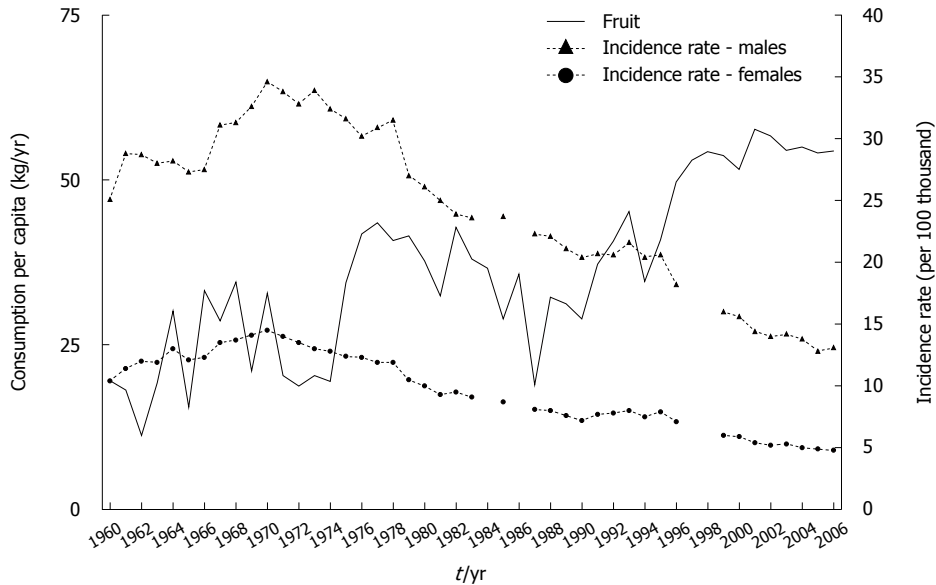


Figure 1 Fruit consumption and gastric cancer morbidity in 1960-2006.

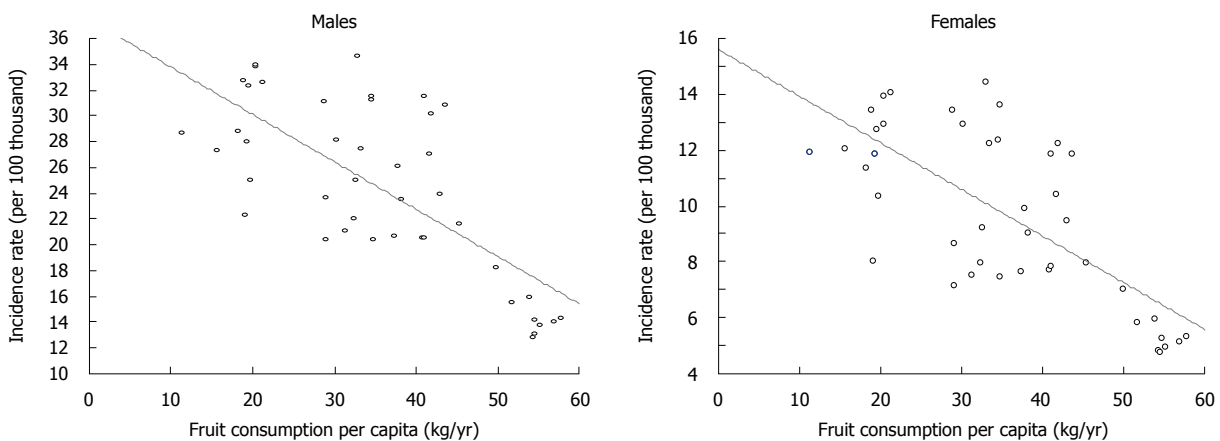


Figure 2 Correlation between fruit consumption and gastric cancer morbidity. Spearman's rank correlation coefficient (r_s) for men: $r_s = -0.65$ ($P < 0.001$), and for women: $r_s = -0.66$ ($P < 0.001$).

At the beginning of the 1960s, fruit was of minor importance in the average dietary pattern in our country, which resulted from low domestic production levels and no imports (Figure 1)^[10]. In the following years, the role of fruit has been gradually increased due to its greater availability. A particular improvement has been observed within the past 12 years, i.e. after initiation of political, economic and social transformation, accompanied by an increase in domestic fruit production and import^[9,11]. Also, changes in retail price relations between fruit and other food products have stimulated consumer demand for fruit and this has had a great impact.

Spearman's rank correlation coefficient, which covered fruit consumption in 1960-2006 and gastric cancer morbidity in the same period, showed a high correlation between the increase in fruit consumption and decrease in morbidity rate (Figure 2).

Vegetables traditionally have been more prominent than fruit in the dietary pattern in Poland, which has resulted from their greater availability and, in consequence,

lower prices. Consumption of this group of products, similarly to fruit, has been subject to a growing trend, and in 2006 was almost 30% higher compared to 1960 (Figure 3)^[9-11].

Estimate of the Spearman's rank correlation coefficient showed a very high correlation between the trend in vegetable consumption and gastric cancer morbidity (Figure 4).

Increased consumption of both food groups resulted in the growth of vitamin C content in the diet, despite a reduction in potato consumption: total vitamin C content grew from approximate 100 mg/d in 1960 to 124 mg in 2006, and a rise in the proportion contributed by fruit and vegetables was noted (Figure 5)^[9-11]. Calculations made for the purposes of this study showed a very high correlation between increased vitamin C intake and decreased gastric cancer morbidity (Figure 6).

Reliable evidence that confirmed that storing food in refrigerators protects against gastric cancer^[15-17] has focused attention on the trend in their availability in Polish

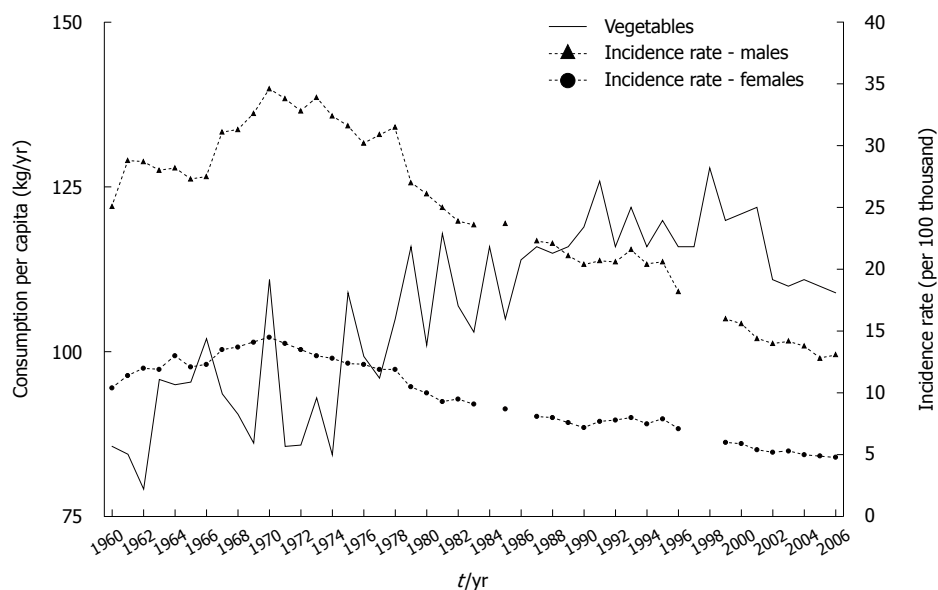


Figure 3 Vegetables consumption and gastric cancer morbidity in 1960-2006.

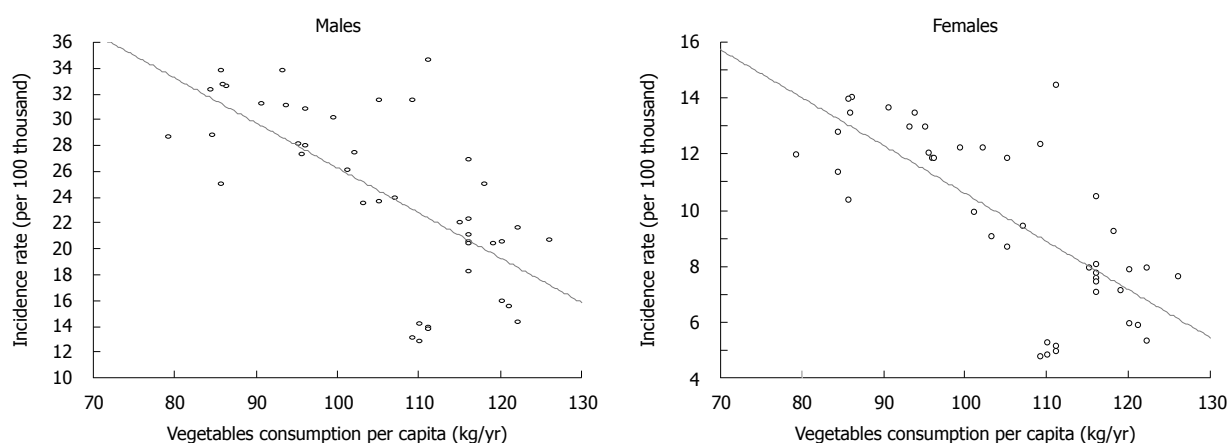


Figure 4 Correlation between vegetable consumption and gastric cancer morbidity. Spearman's rank correlation coefficient (r_s) for men: $r_s = -0.70$ ($P < 0.001$), and for women: $r_s = -0.70$ ($P < 0.001$).

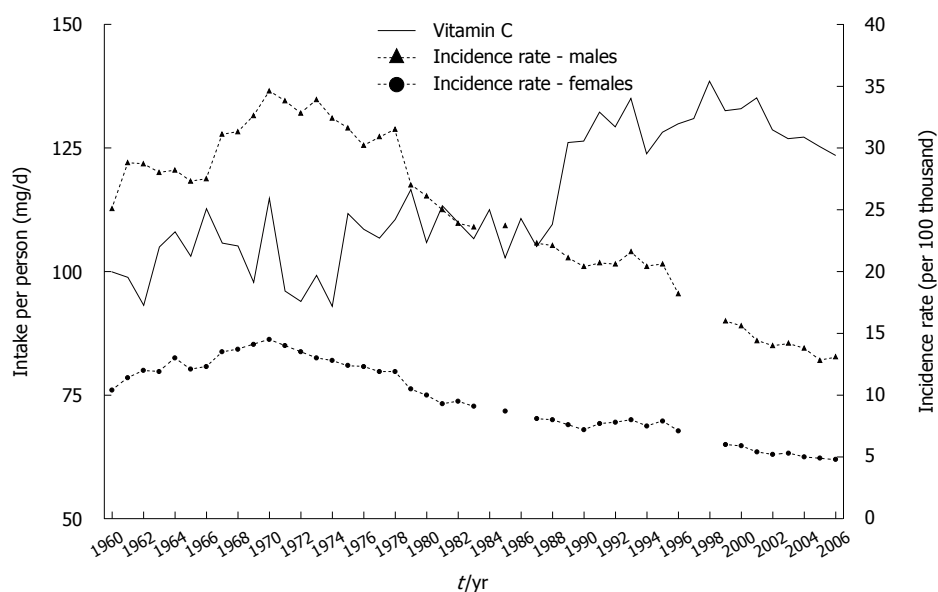


Figure 5 Vitamin C intake and gastric cancer morbidity in 1960-2006.

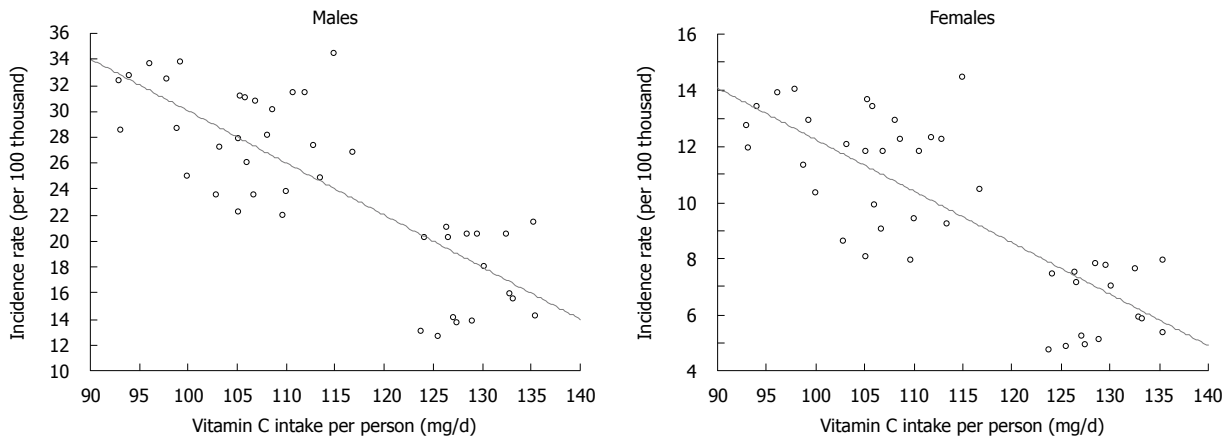


Figure 6 Correlation between vitamin C intake and gastric cancer morbidity. Spearman's rank correlation coefficient (r_s) for men: $r_s = -0.75$ ($P < 0.001$), and for women $r_s = -0.74$ ($P < 0.001$).

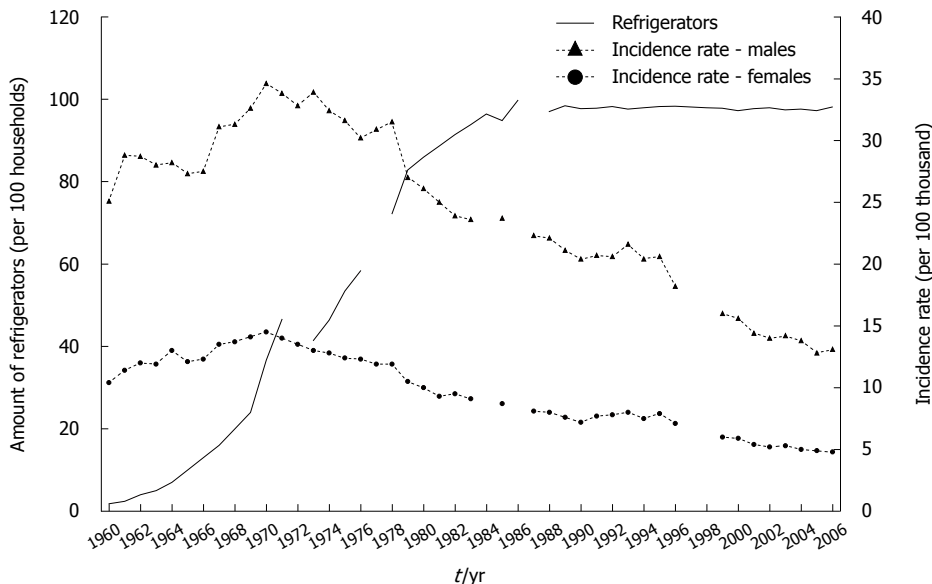


Figure 7 Equipment of households with refrigerators and gastric cancer morbidity in 1960-2006.

households. This equipment has become popular relatively late in our country. In 1960, there were only an average of 1.8 refrigerators per 100 households, which contrasts with western countries (Figure 7). The above situation has also related to the availability of refrigerators in the retail and catering trades.

Up to 1970, the number of refrigerators increased to approximate 37 per 100 households, although still only about one-third of the total number of households. Only at the beginning of the 1980s were > 90% of households equipped with refrigerators.

Estimates made for the purposes of this study demonstrated a very high correlation between the equipment of households with refrigerators and gastric cancer morbidity (Figure 8).

It has been proven that diets with high salt content have an unfavorable effect on gastric cancer. Consumption of table salt in 1929 amounted to 9.9 kg per capita (27 g/d), and decreased to 8.4 kg in 1938 (23 g/d). Despite the decrease,

this amount was still very high, which resulted mainly from its common usage as a preserving agent, which in turn, was determined by insufficient development of commercial food processing. Slow progress in this area in the early years after World War II allows us to assume that salt consumption was maintained at the same very high level observed in the pre-war period, and then decreased. According to the results of household budget surveys, table salt consumption in 2006 amounted to 0.25 kg/mo per person, that is, 3 kg/year (8.2 g/d)^[14]. This is almost threefold lower compared to consumption in 1938, but the fact that data from food balance sheets and budget surveys are not fully comparable should be mentioned. Despite this reservation, the decrease in salt consumption is unquestionable.

Data on total sodium chloride consumption, including both table salt and salt contained in food products (calculated on the basis of sodium content) showed that between 1999 and 2006, this consumption decreased significantly (Figure 9). At the same time, similar to previous

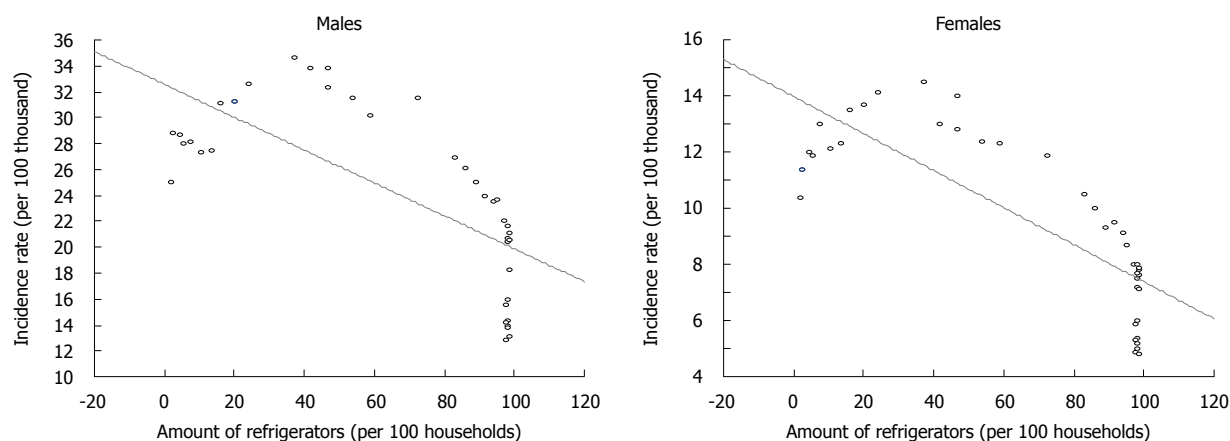


Figure 8 Correlation between equipment of households with refrigerators and gastric cancer morbidity. Spearman's rank correlation coefficient (r_s) for men: $r_s = -0.77$ ($P < 0.001$), and for women: $r_s = -0.80$ ($P < 0.001$).

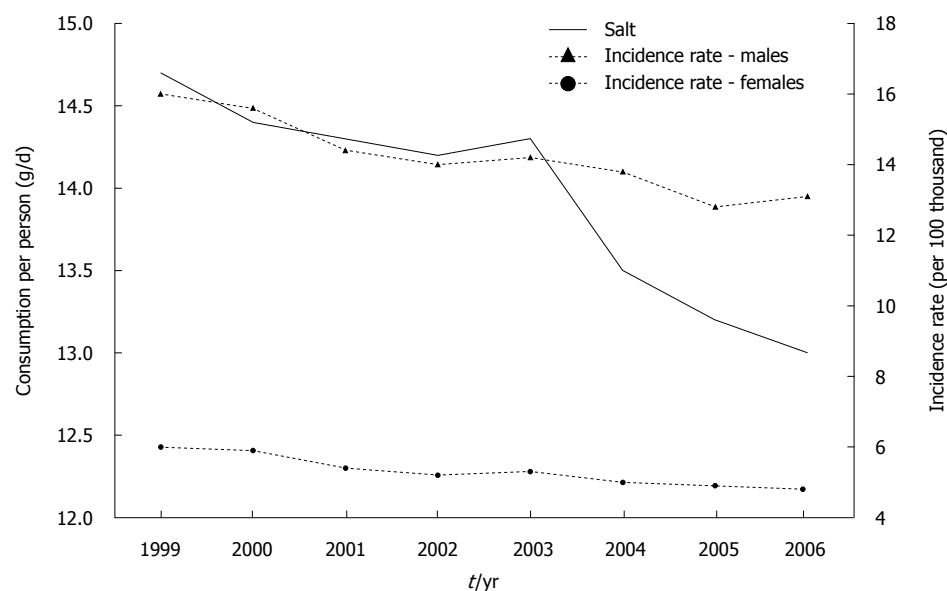


Figure 9 Total salt consumption and gastric cancer morbidity in 1999-2006. Kitchen salt and salt calculated from sodium content in food products.

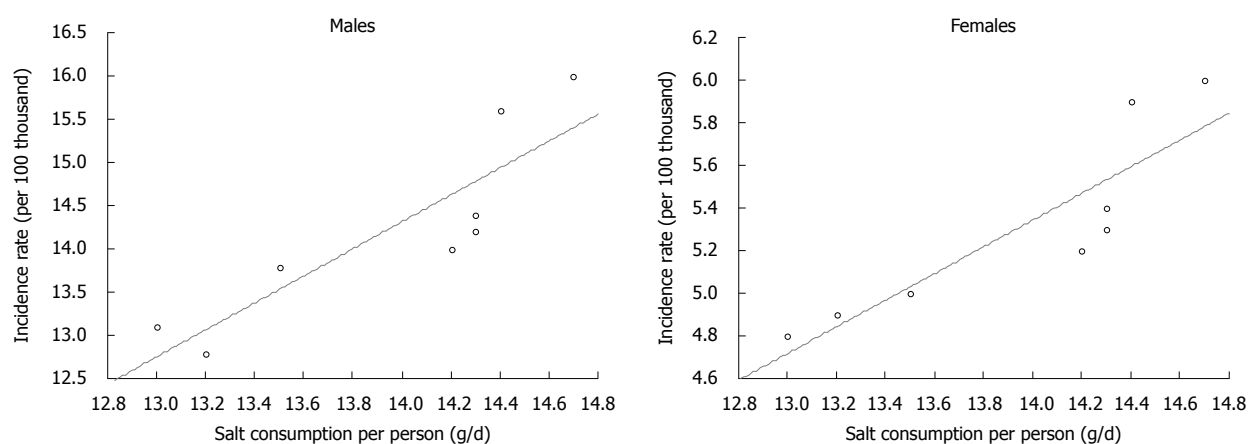


Figure 10 Correlation between total salt consumption and gastric cancer morbidity. Spearman's rank correlation coefficient (r_s) for men: $r_s = 0.97$ ($P < 0.001$), and for women: $r_s = 0.99$ ($P < 0.001$). Kitchen salt and salt calculated from sodium content in food products.

years, a regular decrease in gastric cancer morbidity has been recorded, both for men and women. Calculations

made for the purposes of this study demonstrated almost full correlation between the analyzed factors (Figure 10).

DISCUSSION

Our results enabled us to conclude that dietary factors have had a significant impact on gastric cancer risk in Poland. Their importance was underlined by the very high decrease in gastric cancer morbidity rate within the past few decades, despite a significant prevalence of *H. pylori* infection (73% of total population and 85-95% of adults aged > 25 years)^[6] and smoking, which are important, commonly accepted factors that increase gastric cancer risk.

Infection with *H. pylori* (first-class carcinogen) is considered to be an essential factor for initiation of a series of inflammatory lesions in gastric mucous membrane, from chronic superficial inflammation to pre-cancer lesions (atrophic inflammation, metaplasia, dysplasia), from which gastric cancer may develop^[18,19]. Gastric cancer grows as a result of complex interactions between genetic factors, virulence of *H. pylori* and environmental factors (dietary pattern, smoking). Our study shows that nutritional factors probably constitute one of the most important elements in these complex interactions.

For many years vitamin C has been considered as a significant factor for decreasing the risk of gastric cancer morbidity. As some studies have demonstrated, high concentrations of this vitamin in gastric juice and gastric mucous membrane can establish unfavorable conditions for development of *H. pylori* in the stomach, and its administration in high doses can even lead to eradication of this bacterium^[20]. It has been proven that vitamin C reduces growth of bacteria in culture, and the activity of urease produced by these bacteria^[21]. This enzyme enables *H. pylori* to survive in the acid environment of gastric juice, because it alkalizes the microenvironment by decomposition of urea to ammonium and carbon dioxide. However, it should be emphasized that the most important mechanism by which vitamin C can reduce the carcinogenic effect of *H. pylori* infection is destruction of free oxygen radicals that are produced in great amounts during infection^[22]. They damage the genetic material of gastric epithelial cells, which leads to mutations.

It can be assumed that increased vitamin C intake reduces the deficiency in vitamin C in smokers. Smoking, similar to *H. pylori* infection, results in lower concentration of vitamin C in gastric juice. This decrease is greater in smokers infected with *H. pylori* compared with non-smokers^[21]. An increase in consumption of fruit, vegetables and fruit juices could reduce the risk of gastric cancer development in smokers.

The impact of vitamin C on the decreased concentration of N-nitro compounds in the stomach in patients with chronic atrophic gastritis might also be significant^[23,24]. A previous study has proven that vitamin C has the ability to slow down the nitrosation process, which results in the presence of nitrosamines in the stomach; compounds that have a mutagenic effect and are considered as carcinogens^[25]. *In vitro* and *in vivo* tests have shown that ascorbic acid is the strongest inhibitor of this process^[26]. It reacts with nitrites to produce nitrogen oxides. It oxidizes itself to dehydroascorbic acid, which reduces the nitrosation reaction; details of which remain unknown. In addition,

ascorbic acid reduces transformation of nitrates to nitrites, which are direct substrates for nitrosamine production. The above is of significant importance in *H. pylori* infection, because, in the course of such infection, there is a significant increase in the concentration of nitrous compounds that are the source for nitrosamine production.

We found a very high correlation between decrease in gastric cancer morbidity and increase in dietary vitamin C content, which was related to growing consumption of fruit and vegetables. The purpose of many studies has been to examine the association between fruit and vegetable consumption and the incidence of gastric cancer. In a prospective study of Swedish women and men, consumption of vegetables was inversely associated with risk of gastric cancer after controlling for potential confounders^[27]. However, no significant association was observed between fruit consumption and gastric cancer risk. In a Canadian study that evaluated associations between dietary patterns and incident gastric cancer risk, dietary patterns characterized by increased consumption of fruit and vegetables were associated with lower risk^[28]. The association between vegetables and fruit consumption and gastric cancer risk was investigated in the Japan Public Health Center-based Prospective Study^[29]. That study suggested that vegetable and fruit intake, even in low amounts, was associated with a lower risk of stomach cancer. In contrast, a different Japanese study (Japan Collaborative Cohort Study) found no association between stomach cancer mortality and consumption of fruit and vegetables^[30]. The Netherlands Cohort Study found evidence for an inverse association between stomach cancer and the consumption of vegetables and fruit, however, it became weaker and non-significant in multivariate analysis^[31].

The nested case-control EPIC study is one of the largest prospective analyses of the association of nutrition with cancer. That study found no association between total vegetable intake or specific groups of vegetables and gastric cancer risk, except for the intestinal type, for which a negative association was found for total vegetable intake. There was no evidence of any association between fresh fruit intake and gastric cancer risk^[32]. Within the EPIC cohort, dietary vitamin C also showed no significant association with gastric cancer risk at any level of intake^[33].

In Poland, decreased salt consumption within the past few decades has probably also contributed to the decrease in gastric cancer morbidity. High concentrations of sodium chloride in the stomach facilitate damage and inflammation of mucous membranes, which leads to development of atrophic lesions, which are pre-cancerous lesions^[34]. A high-salt diet is considered to alter the viscosity of the protective mucous barrier and facilitate exposure to carcinogenic factors such as nitrates. Some information suggests that high salt intake in some way facilitates colonization with *H. pylori*^[35,36]. However, other studies have suggested that high salt intake *per se* does not promote *H. pylori* infection^[37].

According to the WCRF/AICR report, any effect of salt on stomach cancer is principally the result of regular consumption of salted or salt-preserved food, rather than salt as such^[15]. Some studies have investigated only salt added in cooking or at the table, but this is usually a small

proportion of total salt consumption. The results from such studies are liable to produce different conclusions. For example in the Netherlands Cohort Study, an inverse association was found between stomach cancer and salt added to hot meals^[38].

However many studies have confirmed the association between salt and/or salty food intake and the risk of stomach cancer. In a case-control study in Lithuania, a higher risk of gastric cancer was found in subjects who added salt to prepared meals or those who liked salty food^[39]. In a cohort study conducted in a rural area of Japan, the frequent intake of highly salted food remained as a significant risk factor for gastric cancer mortality^[40].

It is not entirely clear whether high salt intake and *H. pylori* infection are independent or interdependent risk factors. In a prospective study of a Japanese population, the effect of high salt intake on gastric carcinogenesis was strong in subjects who had atrophic gastritis and *H. pylori* infection^[37]. A study of 67 Chinese rural counties has suggested an interaction between high salt consumption and *H. pylori* infection^[41]. The significant correlation between *H. pylori* prevalence and stomach cancer mortality was only observed in counties with high levels of urinary sodium, and the significant correlation between urinary sodium and stomach cancer mortality only existed in counties with high *H. pylori* prevalence. In a case-control study in Korea, subjects with positive *H. pylori* infection and a high salt preference had a 10-fold higher risk of early gastric cancer than those without *H. pylori* infection and a low salt preference^[42].

In light of the above-mentioned results, the observed decrease in gastric cancer morbidity in Poland could be explained by the fact that, in the years when salt consumption was very high, the impact of *H. pylori* infection on carcinogenesis was significantly higher compared to the time in which dietary salt content was lower. Another factor that could have influenced the decrease in gastric cancer morbidity in Poland could be improvement in food storage, which has resulted from better equipment of households with refrigerators. This method of food storage protects fruit and vegetables against loss of vitamin C. It also prevents growth of microorganisms that can reduce nitrates contained in food products to nitrites.

The WCRF/AICR report has concluded that there is convincing evidence that use of refrigeration indirectly protects against gastric cancer^[15]. Some surveys have shown a significant association between the use of refrigeration and reduced risk of gastric cancer. According to a case-control study conducted in Northern Italy, 5% of all gastric cancer cases were attributable to less than 30 years use of an electric refrigerator^[16]. In a case-control study in Germany, the use of a refrigerator at home for ≥ 30 years compared to ≤ 24 years showed an inverse relationship with stomach cancer risk^[17]. However, in the Netherlands Cohort Study, no association was observed for duration of refrigerator use^[38]. Similarly, in a Polish case-control study, no association was found between stomach cancer risk and long-term refrigerator use^[43].

To summarize, our study shows that the effect of certain nutritional factors (increase in fruit and vegetable

consumption and related increase in dietary vitamin C and decrease in salt consumption) within the past few decades could have had a positive preventive impact on gastric cancer. The preventive effect probably depends on recognized and well-documented mechanisms reducing the risk of carcinogenesis, and perhaps on still unknown mechanisms. The prevention might also occur in populations that are exposed to other, strong, unfavorable environmental factors (carcinogens), such as *H. pylori* or smoking. Our results constitute a crucial argument for aiming to improve diet in the population through nutritional education, food production and mass catering.

COMMENTS

Background

In many countries, gastric cancer morbidity within the past few decades has reduced significantly. This phenomenon is most probably related to favorable changes in the dietary pattern in developed countries; mostly to increased consumption of fresh fruit and vegetables and decreased consumption of salt-preserved food. Lower gastric cancer morbidity is also related to lower prevalence of *Helicobacter pylori* (*H. pylori*) infection, which is considered to be essential for development of the majority of gastric cancer cases. However, Poland seems to be a particular case. Despite the fact that the percentage of people infected with *H. pylori* is unusually high, in 2006, gastric cancer morbidity rate was twofold lower compared to that in 1960.

Research frontiers

Gastric cancer ranks fourth in morbidity and second in mortality among malignant cancers worldwide. Despite the decline in incidence of this cancer, it is important to investigate the factors that could decrease or increase the risk of the disease. In this study, the relationship between diet and gastric cancer morbidity during the past few decades in Poland was evaluated.

Innovations and breakthroughs

The relationship between diet and cancer morbidity is mainly based on the results of case-control studies or prospective cohort studies. The present study was conducted using national data on diet that were derived from food balance sheets or household budget surveys.

Applications

The results suggest a positive influence of increased fruit and vegetable consumption, and related increase in dietary vitamin C content and reduced salt consumption, on the decline in gastric cancer morbidity. These results could be useful in preparation of dietary guidelines for cancer prevention.

Terminology

Metaplasia is the reversible replacement of one differentiated cell type with another mature differentiated cell type. Dysplasia is an expansion of immature cells, with a decrease in the number and location of mature cells. Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia. Nitrosation is a process of converting organic compounds into nitroso derivatives.

Peer review

The manuscript is well written and underlines in an exhaustive manner the cause of the decline in gastric cancer incidence in Poland.

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Endoscopic management of occluded biliary uncovered metal stents: A multicenter experience

Panagiotis Katsinelos, Athanasios Beltsis, Grigoris Chatzimavroudis, Dimitris Paikos, George Paroutoglou, Dimitris Kapetanios, Sotiris Terzoudis, Georgia Lazaraki, Ioannis Pilpilidis, Kostas Fasoulas, Stefanos Atmatzidis, Christos Zavos, Jannis Kountouras

Panagiotis Katsinelos, Athanasios Beltsis, Grigoris Chatzimavroudis, Sotiris Terzoudis, Kostas Fasoulas, Stefanos Atmatzidis, Department of Endoscopy and Motility Unit, "G. Gennimatas" General Hospital, 55132, Thessaloniki, Greece
Dimitris Paikos, Georgia Lazaraki, Ioannis Pilpilidis, Department of Gastroenterology, Theagenion Anticancer Hospital, 54642, Thessaloniki, Greece

George Paroutoglou, Department of Gastroenterology, University Hospital of Thessaly, 41222, Larissa, Greece

Dimitris Kapetanios, Department of Gastroenterology, "G. Papanikolaou" General Hospital, 55236, Thessaloniki, Greece

Christos Zavos, Jannis Kountouras, 2nd Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, Ippokraton Hospital, 54642, Thessaloniki, Greece

Author contributions: Katsinelos P, Paikos D, Paroutoglou G, Kapetanios D, Lazaraki G and Pilpilidis I performed the endoscopies; Beltsis A, Chatzimavroudis G, Terzoudis S, Fasoulas K and Atmatzidis S analyzed and interpreted the patient data and reviewed the relative literature; Chatzimavroudis G performed the statistical analysis of data; Katsinelos P designed the study and wrote the manuscript; Zavos C and Kountouras J were major contributors in revising the manuscript critically for important intellectual content.

Correspondence to: Dr. Panagiotis Katsinelos, Assistant Professor, Head, Department of Endoscopy and Motility Unit, "G. Gennimatas" General Hospital, 55132, Thessaloniki, Greece. gchatzim@med.auth.gr

Telephone: +30-2310-211221 Fax: +30-2310-210401

Received: May 6, 2010 Revised: July 13, 2010

Accepted: July 20, 2010

Published online: January 7, 2011

Abstract

AIM: To compare diverse endoscopic interventions in the management of occluded uncovered self-expanding metal stents (SEMSs) that had been placed for palliative treatment of unresectable malignant biliary obstruction.

METHODS: A retrospective review was undertaken

in 4 tertiary endoscopic centers to determine optimal management of different types of occluded SEMSs. The technical success of performed treatment in occluded SEMSs, the patency of the stent, the need for re-intervention and the financial costs of each treatment were analyzed.

RESULTS: Fifty four patients were included in the analysis; 21 received Hanaro, 19 Wallstent and 14 Flexus. For the relief of obstruction, a plastic stent was inserted in 24 patients, a second SEMS in 25 and mechanical cleaning was performed in 5 patients. The overall median second patency rates between second SEMSs and plastic stents did not differ (133 d for SEMSs vs 106 d for plastic stents; $P = 0.856$). Similarly, no difference was found between the overall survival of SEMS and plastic stent groups, and no procedure-related complications occurred. Incremental cost analysis showed that successive plastic stenting was a cost-saving strategy at least in Greece.

CONCLUSION: Insertion of uncovered SEMSs or plastic stents is a safe and effective treatment for occluded uncovered SEMSs; insertion of plastic stents appears to be the most cost-effective strategy.

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Key words: Biliary obstruction; Gastrointestinal neoplasms; Stents; Cost effectiveness

Peer reviewers: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia; Yuk-Tong Lee, MD, Department of Medicine and Therapeutics, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China

Katsinelos P, Beltsis A, Chatzimavroudis G, Paikos D, Paroutoglou G, Kapetanios D, Terzoudis S, Lazaraki G, Pilpilidis I, Fasoulas K, Atmatzidis S, Zavos C, Kountouras J. Endoscopic

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INTRODUCTION

The treatment of choice for patients with unresectable malignant biliary obstructions with survival time beyond 6 mo, is the insertion of self-expanding metal stents (SEMSs), either endoscopically or percutaneously^[1-4]. The main advantages of SEMSs over plastic stents in the palliation of malignant biliary strictures are their longer patency, greater complication-free survival, and cost-effectiveness, despite the initial cost; stent patency is critical because it significantly affects patient survival^[5-7]. However, despite their large lumen, SEMSs are prone to occlusion by tissue ingrowth or overgrowth and biliary sludge/debris^[8,9], resulting in recurrent jaundice or cholangitis; unlike plastic stents, the major disadvantage of SEMSs is the difficulty with repositioning or extraction once deployed^[10]. In contrast, plastic stents are less expensive and easier to remove or to change, although they have a shorter duration of patency and a higher risk of clogging and dislocation^[11]. There are currently limited data comparing the efficacy of different treatment options and reporting on the follow-up of SEMS occlusion; only 3 studies^[12-14] described the management of occluded Wallstents and one with different types of SEMSs^[15]. As SEMSs with different characteristics are more frequently used worldwide, we anticipate that occluded SEMSs of different types will be more commonly encountered in current clinical practice. Since experience and consensus regarding the optimal management of occlusion of different types of SEMSs is lacking, such data would be useful in clinical decisions. In this respect, in countries (e.g. Greece) where endoscopic retrograde cholangiopancreatography (ERCP) costs are very low compared to those with SEMSs, initial endoscopic retrograde biliary drainage by a plastic stent appears to be less expensive.

We conducted this study to compare diverse endoscopic interventions, but with significant cost difference, in the management of different types of occluded SEMSs by 4 tertiary level centers over a 10-year period.

MATERIALS AND METHODS

A retrospective study was performed between September 1999 and December 2008 in 4 tertiary endoscopic centers in Northern ($n = 3$) and Central ($n = 1$) Greece, in patients undergoing therapeutic ERCP for unresectable malignant distal biliary obstruction. Medical records were reviewed to determine the management and clinical course until death of patients with occluded SEMSs or the end of the study period (cut-off date, January 31, 2009). Additional information was also obtained by phone contact with the patients' referring physicians or relatives. The study was approved by the institutional re-

view boards of all participating hospitals.

We identified patients through the endoscopy database. Age, sex, indication for first SEMS placement, information from outpatient visits, diagnostic tests, interventions and treatments that patients underwent at the 4 centers were reviewed. Three types of uncovered SEMS had been used. The Wallstent (Boston Scientific, USA) original SEMS is considered the industry standard. It consists of a braided stainless steel mesh with soft barbed ends, is available in 40, 60 and 80 mm lengths, and 8 or 10 mm diameter, and costs 2200 Euros. The Flexus (formerly Mometherm and Luminex) (ConMed, USA) is a highly flexible nitinol stent with flared ends. The stent is made from a laser-cut single piece of nitinol, a nickel-titanium alloy that provides a high degree of flexibility, and the interstices of the lattice work are large enough to permit cannulation and, after dilatation, placement of another stent in "Y" configuration for palliation of hilar strictures and costs 1950 Euros. The Hanaro (MI Tech, Korea) is also made of nitinol and costs 1650 Euros. The interstices of the lattice are larger compared to those of a Wallstent and similar to those of the Flexus stent.

Stent occlusion was diagnosed when a patient developed symptoms and/or signs of cholangitis (fever, right upper quadrant tenderness, and/or a ≥ 2 -fold increase in bilirubin concentration above baseline at the post endoscopic retrograde biliary drainage period) or when bilirubin concentration increased ≥ 2 -fold above baseline after endoscopic retrograde biliary drainage, even without cholangitis symptoms and/or signs or an imaging study confirming biliary obstruction recurrence. The reason for stent occlusion was classified as predominantly tumor ingrowth, tumor proximal or distal overgrowth or obstruction from sludge/debris. Tumor ingrowth and proximal overgrowth were identified when cholangiography showed a stricture within the stent (Figure 1A) or a new stricture proximal to the stent, respectively. Distal overgrowth was diagnosed by direct endoscopic visualization (Figure 1B). Occlusion from debris or sludge was diagnosed when cholangiography showed filling defects within the lumen of the stent and cleaning of the stent produced passage of debris/sludge confirmed endoscopically. Management of occluded SEMSs included insertion of either an additional uncovered SEMS (Figure 1C and D) or a 10 Fr plastic stent (length 7, 9 or 10 cm) (Figure 1E) within the first or mechanical cleaning of the occluded SEMS. Mechanical cleaning was accomplished by flushing the obstructed SEMS with normal saline solution and repetitive passage of an inflated stone extraction balloon through the SEMS. Successful endoscopic management of stent occlusion was defined as a significant decrease in bilirubin level after the procedure and/or resolution of cholangitis or imaging improvement. First stent patency was defined as the time (in days) elapsed from the initial stent placement to the first occlusion that required one of the aforementioned interventions (placement of a second SEMS, plastic stent or mechanical cleaning). Second stent patency was defined as the time (in days) elapsed from the intervention to resolve the first occlusion to the first subsequent intervention or patient death with a

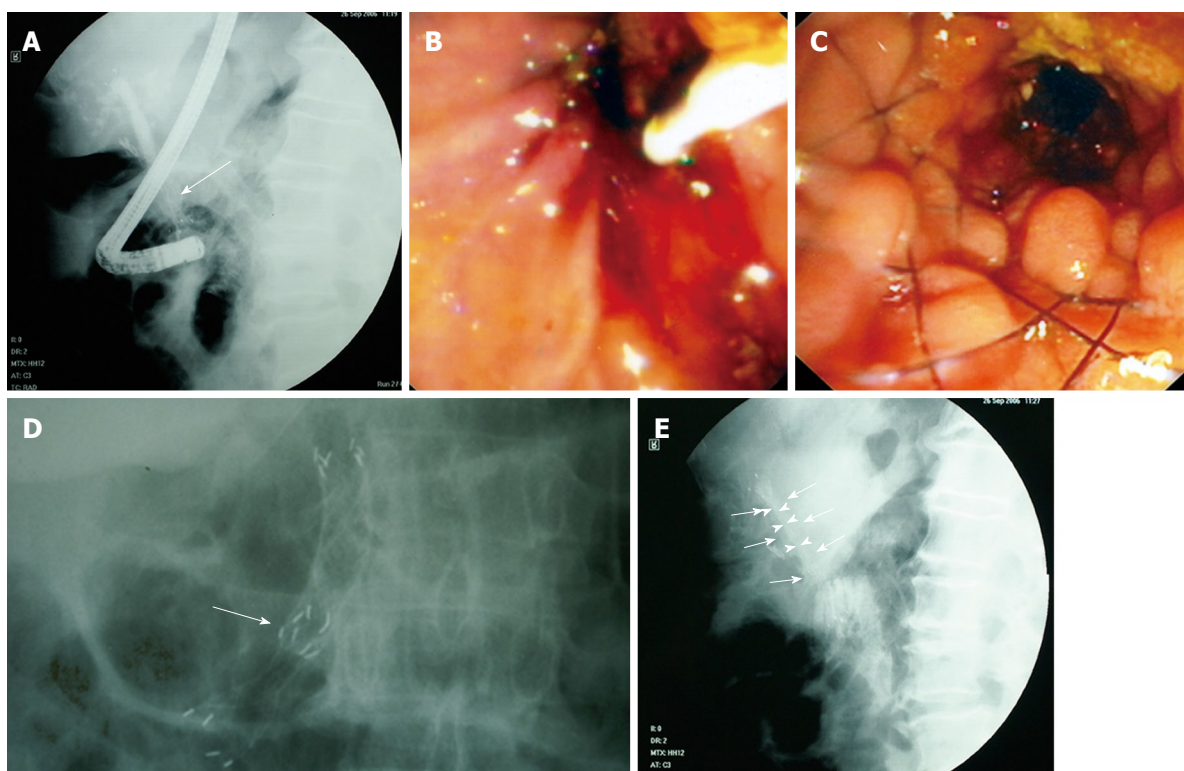


Figure 1 The reasons for stent occlusion (A, B) and the management of occluded self-expanding metal stents (C-E). A: Cholangiogram showing self-expanding metal stent (SEMS) occlusion by tumor ingrowth (arrow); B: Endoscopic view showing the distal end of a SEMS completely occluded by tumor overgrowth; C: Insertion of a new SEMS within the distally occluded stent; D: The second SEMS (arrows) was placed to resolve the occlusion by tumor overgrowth; E: Plastic biliary stent (arrowheads) passed through the occluded SEMS (arrows) that had been placed for palliation of pancreatic carcinoma.

patent stent. Overall second stent patency was defined as the time until a second SEMS became obstructed or the patient died with a patent SEMS, or, in the case of plastic stents, the time until no further stent exchanges could be performed or the patient died with a patent stent. For each treatment strategy to resolve the first SEMS obstruction (new SEMS, plastic stent or mechanical cleaning) we retrospectively counted the total amount of stents of either type used until the patient's death and calculated the cost per patient.

The incremental cost per patient (excluding the cost of the initial SEMS) was calculated by multiplying the number of stents of each type used by their price adding the cost of a balloon catheter (160 Euros) for each mechanical cleaning procedure, 70 Euros for daily hospital charges and cost of hospitalization because of cholangitis until the endpoint, and 150 Euros for each ERCP, according to the financial policy of the Greek National Health System.

Statistical analysis

The analysis was performed using the statistical program Statistical Package for Social Sciences (SPSS, version 13.0, Chicago, IL, USA). The estimation of patient survival and stent patency in the various groups of the study was performed using the Kaplan-Meier method, supplemented by the log-rank test used for comparisons of groups in relation to their survival and the duration of patency of the stents.

For the purpose of statistical data analysis the χ^2 test,

Fisher's exact test, the Mann-Whitney *U* test and the Kruskal-Wallis test were used. For comparisons between groups the Bonferroni adjusted *P*-value was used. Significance was set at $P < 0.025$.

RESULTS

Between September 1999 and December 2008, 219 patients with distal unresectable biliary obstruction received uncovered SEMSs of 3 different types, at 4 tertiary endoscopy centers. Hanaro stents were used in 74, Wallstents in 81 and Flexus stents in 64 patients. Sixty-three (28.8%) patients who underwent an ERCP because of SEMS (Hanaro 26, Wallstent 20 and Flexus 17) occlusion as determined by an increasing serum bilirubin level and/or an imaging study confirming recurrence of biliary obstruction were identified. Nine patients were excluded from the study because data were incomplete for analysis. There were 54 patients, 31 male, 23 female; median age 71 (range, 54-86 years) who were followed up until death and were included in the analysis. Of the 54 uncovered SEMSs that were occluded, 21 were Hanaro, 19 Wallstent and 14 Flexus stents (Table 1). Indications for SEMSs insertion were: pancreatic carcinoma in 28, cholangiocarcinoma in 10, papillary cancer in 8, metastatic lymphadenopathy in 7 and hepatocellular carcinoma in 1 case (Table 1). Hepatic metastases were present in 6 patients with metastatic lymphadenopathy (Table 1). The cause of first SEMS occlusion was ingrowth in 35 patients (64.8%), overgrowth

Table 1 Patient characteristics

	Flexus	Hanaro	Wallstent	All stents
No. of patients	14	21	19	54
Sex (M/F)	5/9	9/12	9/10	23/31
Median age (range)	75 (59-86)	69 (54-83)	73 (60-83)	71 (54-86)
Indication				
Pancreatic cancer	7	12	9	28
Biliary cancer	3	3	4	10
Hilar metastatic lymphadenopathy	1	4	2	7
Papillary tumor	3	2	3	8
Hepatocellular carcinoma			1	1
Hepatic metastases	1	3	2	6
Prior plastic stent	5	2	2	9
Presentation of first occlusion				
Painless jaundice	3	12	8	23
Cholangitis	11	9	11	31
Cause of obstruction				
Ingrowth	7	16	12	35
Overgrowth	4	5	5	14
Sludge and/or debris	3		2	5
Median time to first occlusion (range) (d)	228.5 (178-398)	248 (74-582)	176 (98-754)	242.5 (7-754)
Type of intervention for first occlusion				
Self-expanding metal stent	5	15	5	25
Plastic stent	6	6	12	24
Mechanical cleaning	3		2	5

Table 2 Outcomes of interventions and financial cost in the course of treatment of first self-expanding metal stent occlusion, median (range) (d)

	Plastic stent	Second SEMS	Mechanical cleaning	All types	P
Intervention for first occlusion					
No. of patients	24	25	5	54	
No further interventions required					
No. of patients	13 (54.2%)	17 (68%)	1 (20%)	31 (57.4%)	
Survival after first occlusion without further interventions	84 (40-179)	116 (62-227)	39	97 (39-227)	
Further intervention(s) required					
No. of patients	11 (45.8%)	8 (32%)	4 (80%)	23 (42.6%)	
Types of intervention	11 plastic stents insertion	5 plastic stents insertion 3 mechanical cleaning	3 plastic stents insertion 1 insertion of SEMS	19 plastic stents insertion 1 insertion of SEMS 3 mechanical cleaning	
Time to second occlusion (d)	91 (60-74)	144 (26-331)	112 (21-180)	96 (21-331)	
Overall second stent patency	106 (39-645)	133 (26-331)			0.856
Second stent patency	88 (40-179)	133 (26-331)			< 0.001
Survival after first occlusion	106 (40-645)	177 (60-870)	210 (39-390)	142 (39-870)	0.180
Number of interventions per patient	1 (1-5)	1 (1-4)	2 (1-3)	1 (1-5)	0.199
Cost per patient in euros	590 (380-2550)	2170 (1870-3620)	1020 (380-2550)	1685 (380-3620)	< 0.001

SEMS: Self-expanding metal stent.

in 14 patients (25.9%) and sludge/debris in 5 patients (9.3%) (Table 1). There was no difference in the etiology of occlusion between the 3 types of SEMS ($P = 0.773$) (Table 1). The first occlusion presented as cholangitis in 31 (57.4%) and painless jaundice in 23 (42.6%) patients, without any significant difference between the 3 types of SEMS ($P = 0.116$) (Table 1). A plastic stent had been placed before SEMS insertion in 9 patients (Table 1). The overall median (range) duration of the first SEMS patency was 242.5 (74-754) d. More specifically, for Hanaro stents the patency period was 248 (74-582) d, for Wallstents 176 (98-754) d, and for Flexus stents 228.5 (178-398) d, with

no significant difference between them ($P = 0.936$) (Table 1 and Figure 2A).

The outcomes and financial costs of interventions in the course of treatment of first SEMS occlusion are summarized in Table 2. From the 25 patients managed by a second SEMS insertion, 17 (68%) died after a median 116 (62-227) d without requiring further intervention, but 8 (32%) patients presented with re-occluded stents after a median 144 (26-331) d (Table 2). Those with re-occluded stents were treated with either plastic stent insertion (5 patients) or mechanical cleaning (3 patients) and 9 plastic stents were required in total (Table 2). From the 24

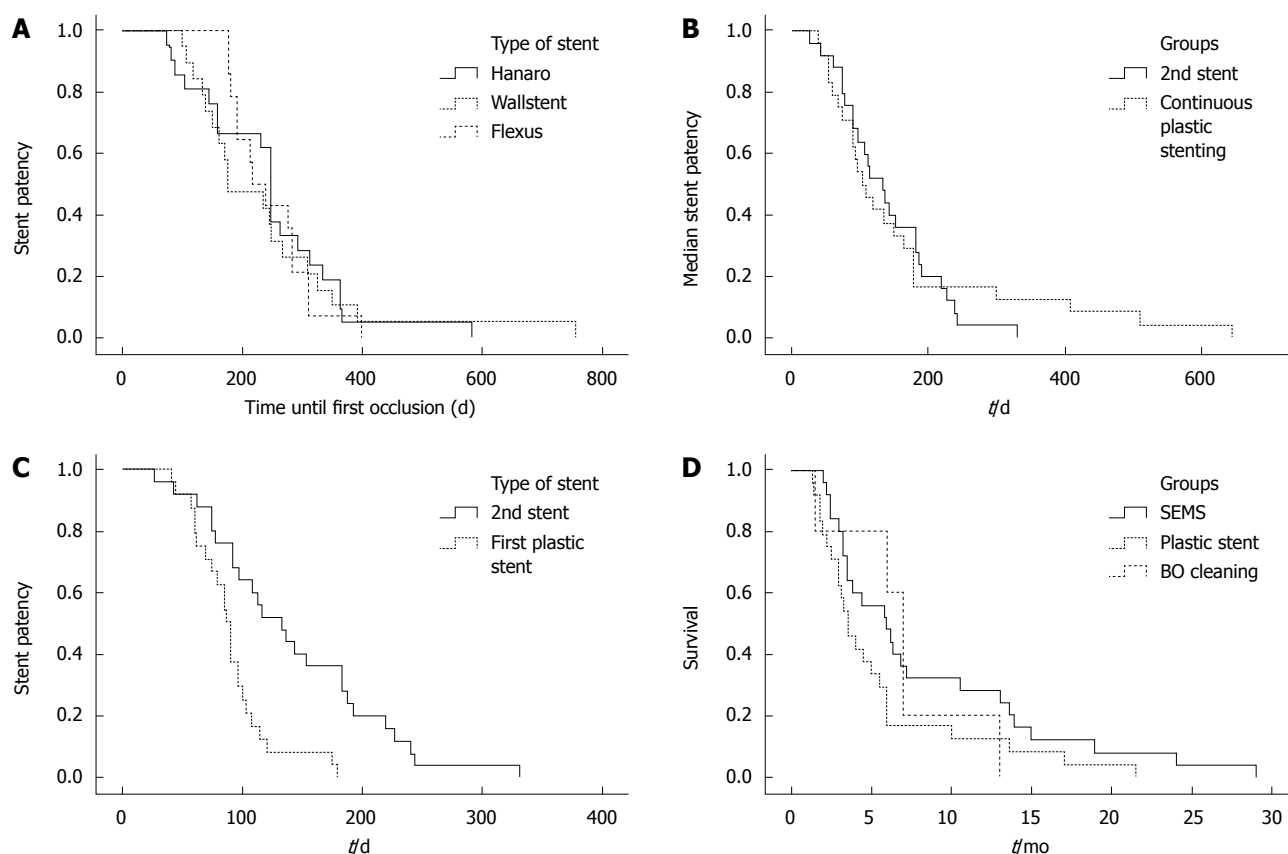


Figure 2 Patency of different stent (A-C) and survival after first self-expanding metal stent occlusion (D). A: Patency of 3 self-expanding metal stent (SEMS) until first occlusion; B: Patency of second SEMS vs overall plastic stent patency; C: Patency of second SEMS vs first plastic stent; D: Survival after first SEMS occlusion.

patients managed by plastic stent insertion, 13 patients (54.2%) died after a median 84 (40-179) d without requiring further intervention but 11 (45.8%) patients presented with re-occluded stents after a median 91 (60-74) d and were treated with plastic stent exchanges as many times as required, resulting in the insertion of 20 additional plastic stents (Table 2). In 2 patients, both with papillary tumors, stent exchange was unsuccessful on the third and fourth ERCP due to duodenal invasion by the tumor. They refused further intervention and died. Of the 5 patients who were treated by mechanical cleaning alone, only one (20%) required no further interventions and died 39 d later, while the other 4 (80%) presented with a second obstruction after a median 112 (21-180) d, treated by plastic stent insertion in 3 patients and SEMS insertion in one (Table 2). Two patients required an additional plastic stent. Overall, 31 patients (57.4%) required no further interventions until their death after a median 97 (39-227) d and 23 patients (42.6%) presented with a second obstruction after a median 96 (21-331) d (Table 2).

Apart from cholangitis, other complications including post-endoscopic retrograde biliary drainage bleeding, cholecystitis, pancreatitis or stent migration were not observed and there was no complication-related mortality.

Median overall patency of the second SEMS was 133 (range, 26-331) d and for successive plastic stenting 106 (range, 39-645) d ($P = 0.856$) (Figure 2B). However, when compared with the patency of the first plastic stent, which

was 88 (range, 40-179) d, the patency of the second SEMS was significantly longer ($P < 0.001$) (Figure 2C). Survival after the first occlusion was 142 (range, 39-870) d: 106 (range, 40-645) d for plastic stent placement, 177 (range, 60-870) d for a second SEMS, and 210 (range, 39-870) d for mechanical cleaning ($P = 0.180$) (Figure 2D).

Overall, the average cost was 1685 (380-3620) Euros per patient: 2170 (1870-3620) Euros per patient for insertion of a second SEMS at initial obstruction, 590 (380-2550) Euros per patient for insertion of a plastic stent at the same setting and 1020 (380-2550) Euros per patient treated by mechanical cleaning ($P < 0.001$) (Table 2).

DISCUSSION

Although endoscopic biliary stenting is accepted as the treatment of choice in patients with inoperable malignant biliary obstruction, and has been associated with reduced morbidity and short hospital stay, the major problem faced after endoscopic stent insertion is stent occlusion. Relative data on the optimal management of occluded SEMSs, are very limited to case reports and small retrospective studies^[12-15]. Our retrospective multicenter series includes the largest number of patients with occluded SEMSs managed endoscopically and is the second study to include different types of occluded SEMSs, thereby better depicting the daily practice worldwide. The initial occlusion rate of SEMSs in our series was 28.8%, consistent with that

of previous studies^[12,13]. Moreover, the occlusion rate was similar between the 3 types of SEMS (Hanaro 35.1%, Wallstent 24.7%, Flexus 26.6%), irrespective of the different design and material. The major cause of SEMSs occlusion was tumor ingrowth (64.8%), confirming the previous observations^[12-15]. This is thought to have been secondary to the growth of tumors within the interstices of the uncovered SEMSs. It is interesting that the rate of ingrowth was similar among the 3 types of SEMS (Hanaro 76.2%, Wallstent 63.2%, Flexus 50%) despite the larger interstices of mesh in Hanaro and Flexus stents.

Our finding that mechanical cleaning of sludge/debris is an ineffective treatment for occluded SEMSs, presenting with high re-occlusion rates in a short time (median: 94 d, range: 21-180) is compatible with other series^[12-14]. Notably, sludge is accrued because the stent surface allows for the adherence of proteins, glycoproteins, or bacteria and the bile flow is insufficient to clean the surface; stent clogging may be caused by microbiological adhesion and biliary stasis. In this regard, treatment with antibiotics and/or ursodeoxycholic acid to prevent clogging of biliary stents in patients with malignant stricture of the biliary tract, however, cannot be recommended routinely on the basis of the existing randomized clinical trials^[16].

Placement of either a second SEMS, independent of its type, or a plastic stent inside the occluded SEMS, was equally effective in resolving the jaundice or the symptoms of cholangitis, in accordance with other series^[12-15,17]. We found that the overall stent patency between second SEMSs and plastic stents for resolution of the first obstruction was not statistically different (133 d *vs* 106 d, $P = 0.856$) (Figure 2B). However, the patency of the second SEMS was significantly longer than that of the first plastic stent [133 (26-331) d *vs* 88 (40-179) d, $P < 0.001$] (Figure 2C), confirming the previous series. There was no difference on second SEMS patency between the 3 types of SEMS [Hanaro 144 (range, 42-331) d, Wallstent 97 (range, 26-243) days, Flexus 133 (range, 92-184) d] ($P = 0.943$). SEMS patency before the first occlusion was not predictive of the duration of the patency of the SEMS placed within the initial stent. This is in contrast to the observation of Katsinelos *et al*^[11] of a positive correlation between the patency of the first Wallstent and the period of patency of the second Wallstent, but in accordance with Bueno *et al*^[3] and Rogart *et al*^[13] studies.

Kaplan-Meier analysis in our study revealed that survival of patients with a second SEMSs was similar to that with plastic stents (Figure 2D). Interestingly, patients who underwent a second SEMS placement had fewer subsequent interventions compared with patients who had plastic stents inserted, but the difference was not statistically significant ($P = 0.33$).

Several studies^[3-10,12-15] have attempted to address the cost-effective management of occluded SEMS for malignant biliary obstruction. In our series, incremental cost analysis showed that the most cost-effective method appeared to be plastic stent insertion ($P < 0.001$) unlike the previous studies^[3,12,13], but similar to the study by Abraham *et al*^[11]. However, based on the special characteristics of

our health system, our financial analysis is disproportionately influenced by the direct cost of stents, either SEMS or plastic, over the cost of ERCPs and the indirect cost of hospitalizations related to stent occlusion. Therefore, the cost-effectiveness data from our study may not apply directly to countries with different health systems.

Our study has limitations similar to those of prior studies, with retrospective analysis and a relatively small number of patients having a variety of causes for SEMS placement and expected survival. Because the subjects were not randomly allocated, firm conclusions cannot be drawn until a prospective randomized and stratified study in larger numbers confirms our findings. However, data from such a study are unlikely to be available in the near future.

In conclusion, our findings support the use of plastic stents as the main intervention in patients with occluded SEMSs despite the increased number of subsequent ERCPs, because it is cost-effective, especially in health systems where the cost of expendables markedly exceeds that of the medical services.

COMMENTS

Background

Self-expandable metal stents (SEMSs) remain the treatment of choice for patients with unresectable malignant biliary stricture and survival time beyond 6 mo. However, despite their large lumen, they are prone to occlusion. The reported experience on the management of occluded SEMSs of different types is limited.

Research frontiers

Experience and consensus on the optimal management of occlusion of different types of SEMSs, which are used more frequently worldwide is lacking. However, such data are useful in clinical decisions about the treatment of occluded biliary uncovered SEMSs.

Innovations and breakthroughs

The present retrospective multicenter study investigated the diverse endoscopic interventions in the management of different types of occluded SEMSs. The findings support the use of a cost-effective plastic stent as the main intervention in patients with occluded uncovered SEMSs.

Applications

These findings are of potential financial importance especially in healthy systems where the cost of SEMSs markedly exceeds the medical service costs.

Terminology

Uncovered SEMSs are occluded by tissue ingrowth or overgrowth and biliary sludge/debris, resulting in recurrent jaundice or cholangitis.

Peer review

In this good retrospective study, the authors found that a cheap plastic biliary stent was as good as an expensive SEMS for the treatment of occluded SEMSs.

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S- Editor Tian L L- Editor Cant MR E- Editor Ma WH

Effect of ginger on gastric motility and symptoms of functional dyspepsia

Ming-Luen Hu, Christophan K Rayner, Keng-Liang Wu, Seng-Kee Chuah, Wei-Chen Tai, Yeh-Pin Chou, Yi-Chun Chiu, King-Wah Chiu, Tsung-Hui Hu

Ming-Luen Hu, Keng-Liang Wu, Seng-Kee Chuah, Wei-Chen Tai, Yeh-Pin Chou, Yi-Chun Chiu, King-Wah Chiu, Tsung-Hui Hu, Division of Hepatogastroenterology, Department of Internal Medicine, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan

Christophan K Rayner, Discipline of Medicine, University of Adelaide, Royal Adelaide Hospital, Adelaide SA 5000, Australia

Author contributions: Wu KL, Rayner CK and Hu TH designed the research; Hu ML, Wu KL, Tai WC, Chou YP and Chuah SK performed the research; Wu KL, Chiu YC and Chuah SK collected and analyzed the data; Hu ML and Chiu KW wrote the paper; Rayner CK and Wu KL revised the paper.

Supported by Grant from National Research Program from National Science Council (NMRP870071)

Correspondence to: Dr. Keng-Liang Wu, Division of Hepatogastroenterology, Department of Internal Medicine, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan. kengliang_wu@yahoo.com.tw

Telephone: +886-7-7317123 Fax: +886-7-7322402

Received: July 21, 2010 Revised: August 16, 2010

Accepted: August 23, 2010

Published online: January 7, 2011

Abstract

AIM: To evaluate the effects of ginger on gastric motility and emptying, abdominal symptoms, and hormones that influence motility in dyspepsia.

METHODS: Eleven patients with functional dyspepsia were studied twice in a randomized double-blind manner. After an 8-h fast, the patients ingested three capsules that contained ginger (total 1.2 g) or placebo, followed after 1 h by 500 mL low-nutrient soup. Antral area, fundus area and diameter, and the frequency of antral contractions were measured using ultrasound at frequent intervals, and the gastric half-emptying time was calculated from the change in antral area. Gastrointestinal sensations and appetite were scored using

visual analog questionnaires, and blood was taken for measurement of plasma glucagon-like peptide-1 (GLP-1), motilin and ghrelin concentrations, at intervals throughout the study.

RESULTS: Gastric emptying was more rapid after ginger than placebo [median (range) half-emptying time 12.3 (8.5-17.0) min after ginger, 16.1 (8.3-22.6) min after placebo, $P \leq 0.05$]. There was a trend for more antral contractions ($P = 0.06$), but fundus dimensions and gastrointestinal symptoms did not differ, nor did serum concentrations of GLP-1, motilin and ghrelin.

CONCLUSION: Ginger stimulated gastric emptying and antral contractions in patients with functional dyspepsia, but had no impact on gastrointestinal symptoms or gut peptides.

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Key words: Ginger (*Zinger officinale*); Functional dyspepsia; Gastric emptying; Antral contraction; Abdominal ultrasound; Ghrelin; Glucagon-like peptide-1; Motilin

Peer reviewer: Ted Dinan, Professor, Department of Psychiatry, Cork University Hospital, Wilton, Cork, C1, Ireland

Hu ML, Rayner CK, Wu KL, Chuah SK, Tai WC, Chou YP, Chiu YC, Chiu KW, Hu TH. Effect of ginger on gastric motility and symptoms of functional dyspepsia. *World J Gastroenterol* 2011; 17(1): 105-110 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/105.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.105>

INTRODUCTION

Functional dyspepsia is a clinical syndrome that is characterized by chronic or recurrent upper abdominal pain or discomfort in the absence of underlying organic disease

that can explain the symptoms^[1]. Pharmacological therapy for patients with functional dyspepsia remains unsatisfactory^[2]. The results of controlled trials have generally been disappointing, and only small benefits relative to placebo have been found with histamine H₂-receptor antagonists^[3], proton-pump inhibitors^[4], and *Helicobacter pylori* eradication^[5]. In addition to poor efficacy, pharmacological agents (e.g. cisapride) are associated with a risk of adverse effects.

Herbal medicine might be an attractive alternative based on the perception of its natural approach and low risk of adverse effects. However, the lack of standardization of herbal ingredients has limited the number of rigorous clinical studies available.

Ginger (*Zingiber officinale*) has been used to treat a number of medical conditions, including those affecting the digestive tract^[6,7], such as dyspepsia, flatulence, nausea and abdominal pain. However, the mechanisms responsible for its beneficial effects are not well understood. Yamahara *et al.*^[8] and Micklefield *et al.*^[9] have reported that gastrointestinal motility is enhanced by ginger and its active constituents, but they did not measure gastric emptying. We previously have shown that ginger increases the frequency of antral contractions and accelerates gastric emptying of a low-nutrient liquid in healthy volunteers^[10].

There have been few studies on the effects of ginger in patients with functional dyspepsia. We hypothesized that acceleration of gastric emptying in patients with functional dyspepsia might be accompanied by a reduction in upper gastrointestinal symptoms, and that its action on gastric motility could be mediated *via* increased secretion of ghrelin^[11] or motilin^[12], or by suppression of glucagon-like peptide-1 (GLP-1)^[13].

MATERIALS AND METHODS

Patients

Eleven patients diagnosed with functional dyspepsia on the basis of Rome III criteria were invited to take part. Patients had persistent or recurrent upper abdominal pain or discomfort, which was characterized by the presence of one or more of early satiety, postprandial fullness, bloating, and nausea. Symptoms had been present for at least 6 wk within the preceding 6 mo, without an identifiable structural or biochemical abnormality to which they could be attributed^[1]. Symptoms of retrosternal pain, burning, and regurgitation were considered features of gastroesophageal reflux disease, rather than of functional dyspepsia. Therefore, patients who had predominantly reflux-related symptoms were excluded. Patients were screened by physical examination, laboratory tests (blood picture, fasting glucose, and liver-function tests), abdominal ultrasonography, and upper gastrointestinal endoscopy to exclude other causes of dyspepsia, and none was taking any medication known to affect gastric motility.

Each subject was studied on two afternoons, separated by at least 7 d, in double-blind randomized order. Following a fast of 8 h for solids and liquids, the patients ingested three capsules that contained a total of 1.2 g ginger root powder (Ginger Root; Nature's Way Products

Inc., Springville, UT, USA), or three identical placebo capsules that contained starch, together with 50 mL water. One hour later, they consumed 500 mL chicken and corn soup (United Kanboo, Taipei, Taiwan), which contained 118.6 kcal (2.6 g protein, 2.6 g fat, 21.2 g carbohydrate). The soup was boiled and subsequently cooled to 37°, and was consumed over 5 min ($t = -5$ to 0 min). All patients underwent trans-abdominal ultrasound to measure antral area, fundic area and diameter^[14], and antral contractions at intervals using an Aloka SSD-2000 CL Ultrasound Machine (Aloka, Tokyo, Japan) with a 3.5-MHz annular array probe. Antral contractions were defined as > 50% change in antral area compared to the relaxed area ($\Delta A/A$)^[15], and their frequency as the number of contractions during 5-min periods beginning at 5, 30, 60 and 120 min after soup ingestion. A questionnaire with visual analogue scales (VASs)^[16] for symptoms pain, nausea, abdominal discomfort, bloating and abdominal fullness, was administered at 10-min intervals between $t = -10$ and 90 min. Grading was made on a 100-mm unmarked line between "no symptoms" at one end and "excruciating symptoms" at the other. Venous blood was sampled at $t = -10$, 30, 60 and 90 min for measurement of blood glucose and plasma peptides. Blood glucose concentrations were determined immediately using a portable blood glucose meter (MediSense Companion 2 meter; MediSense Inc., Waltham, MA, USA). The accuracy of this method has been confirmed using the hexokinase technique^[17]. The remainder of the samples was collected into ice-chilled EDTA-treated tubes that contained 400 KIU/mL aprotinin. Plasma was separated and samples stored at -70°C for subsequent analysis of GLP-1, ghrelin and motilin concentrations, using ELISA. Ghrelin was measured by a commercial ELISA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA); intra- and interassay coefficients of variation (CV) were < 5% and < 9%, respectively; motilin and GLP-1 were also measured by a commercial ELISA kit from Phoenix Pharmaceuticals.

Statistical analysis

Gastric half-emptying time (T₅₀) was defined as the time for antral area to decrease to half the maximum increase above baseline, and was calculated by linear interpolation between time points^[10]. The values on the two study days were compared using the Wilcoxon signed rank test. Antral area, fundic area and diameter, frequency of antral contractions and gastrointestinal sensation scores were compared using repeated measures ANOVA. Results are shown as median and range for T₅₀, and mean \pm SD for other variables. $P < 0.05$ was considered significant.

RESULTS

All subjects tolerated the study well.

Antral area and gastric emptying

Gastric emptying was more rapid after ginger than placebo [T₅₀: 12.3 (8.5-17.0) min *vs* 16.1 (8.3-22.6) min, P

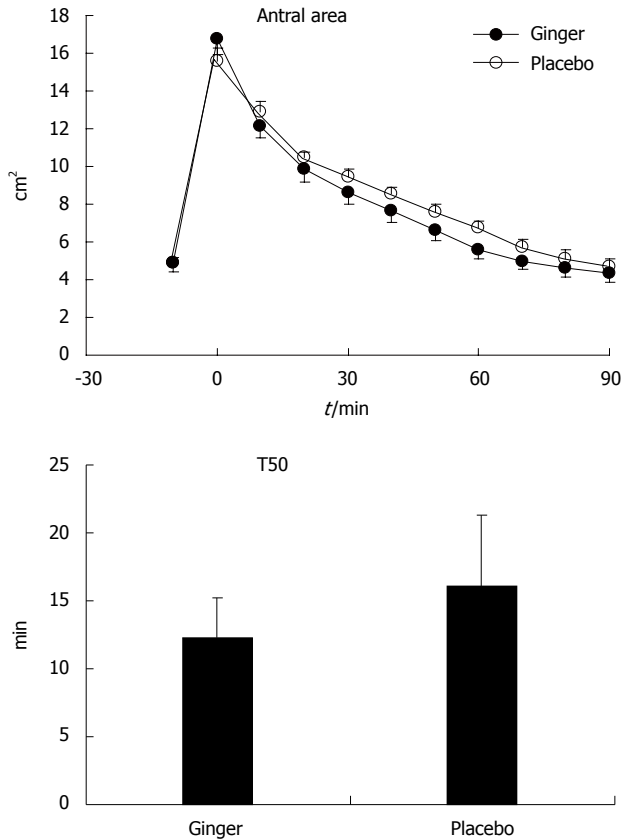


Figure 1 Antral area after ginger and placebo in patients with functional dyspepsia who consumed 500 mL low-nutrient soup between -5 and 0 min. Gastric emptying was more rapid after ginger than with placebo [T50: 12.3 (8.5-17.0) min vs 16.1 (8.3-22.6) min, $P \leq 0.05$]. There was a trend for smaller antral area after ginger ($P = 0.13$); data are means \pm SE, $n = 11$.

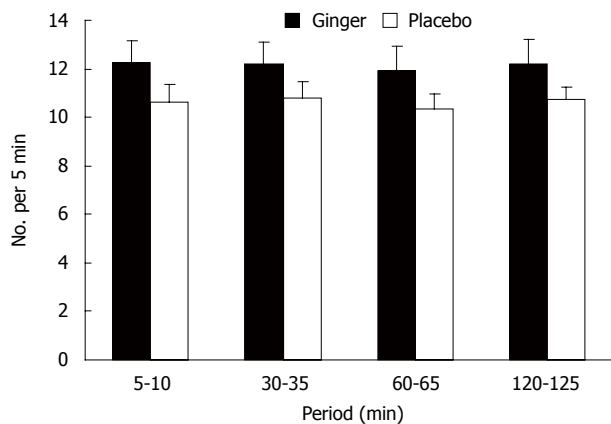


Figure 2 Frequency of antral contractions after ginger and placebo in patients with functional dyspepsia. There was a trend for a higher frequency of antral contractions after ginger ($P = 0.06$). Data are means \pm SE, $n = 11$.

≤ 0.05]. There was a trend for smaller antral area after ginger, although this did not reach statistical significance ($P = 0.13$) (Figure 1).

Antral contractions

There was a trend for more antral contractions after ginger compared to placebo ($P = 0.06$) (Figure 2).

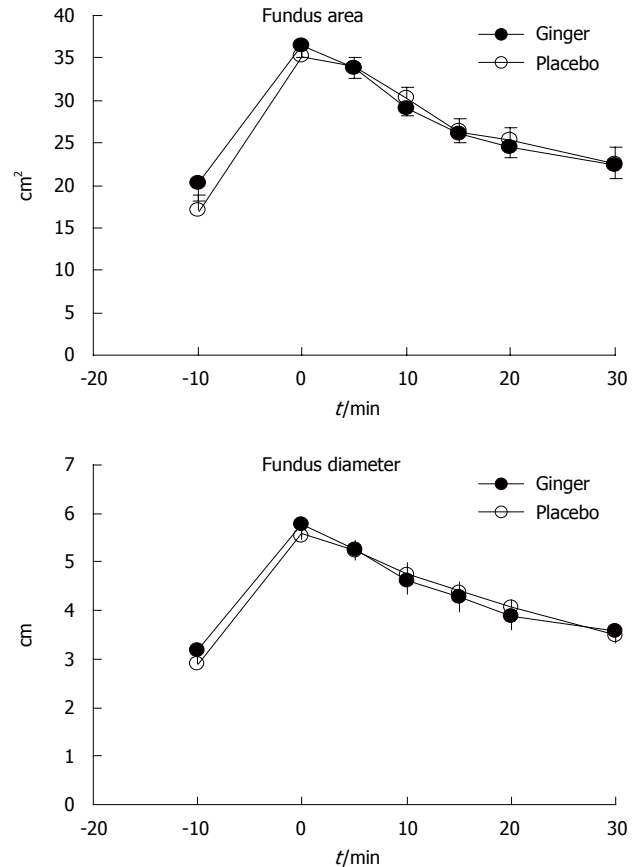


Figure 3 Fundus area and diameter in patients with functional dyspepsia. There was no difference in either measure between ginger and placebo. Data are means \pm SE, $n = 11$.

Fundic area and diameter

Fundus dimensions did not differ between the two study days (Figure 3).

Gastrointestinal sensations

Soup ingestion was associated with increased fullness and bloating, and decreased hunger and appetite scores, but without any difference between ginger and placebo. There were no significant changes in nausea or abdominal discomfort from baseline, or any differences in these sensations between study days (Figure 4).

Gastrointestinal peptide concentrations

There were no differences in plasma concentrations of motilin, ghrelin, or GLP-1 between the two study days (Figure 5).

DISCUSSION

In this study, we demonstrated that ginger increased the rate of gastric emptying in patients with functional dyspepsia when compared to placebo, and that this was associated with a trend for an increased frequency of gastric antral contractions, but no change in dimensions of the fundus. This was consistent with our previous study in healthy volunteers^[10]. Despite more rapid emptying, ginger

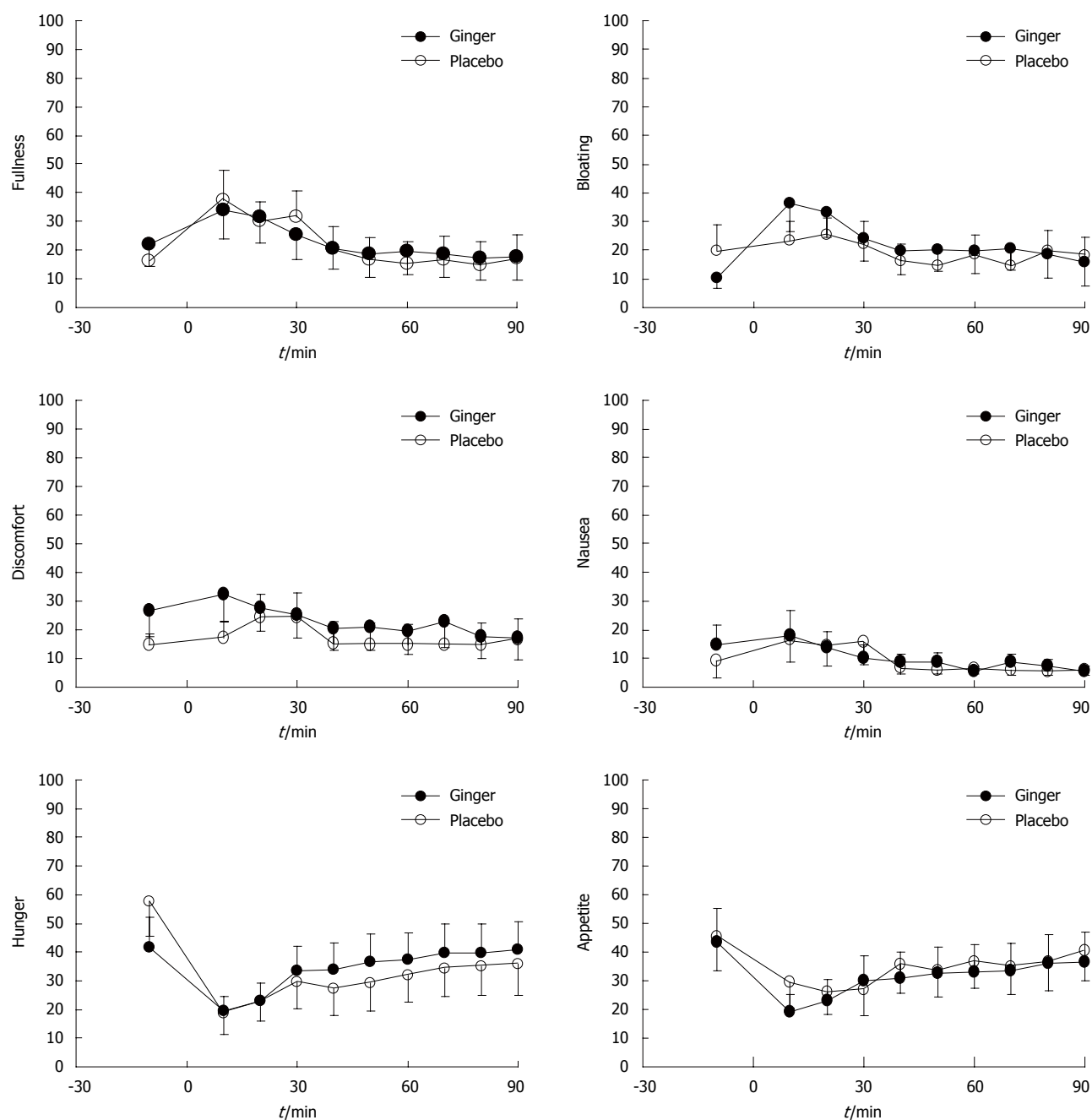


Figure 4 Visual analogue scale scores for gastrointestinal symptoms after ginger and placebo in patients with functional dyspepsia who consumed 500 mL low-nutrient soup between -5 and 0 min. There was no difference in any sensation between the two study days. Data are means \pm SE, $n = 11$.

had no effect on gastrointestinal symptom scores in our patients.

About 40% of patients with functional dyspepsia have abnormally delayed gastric emptying^[18], and prokinetic medications have often been used in the treatment of this condition^[19]. We did not select our dyspeptic patients on the basis of delayed gastric emptying, and as a group, their rate of gastric emptying was comparable to the healthy volunteers that we had studied previously using the same technique^[10]. It is possible that ginger could have improved symptoms in a more selected group of patients who had delayed emptying, and in particular, those with an abnormally wide antrum, a feature that has been associated particularly with bloating^[20].

It is also possible that the lack of symptomatic improvement with ginger was related to the low-nutrient nature of the soup meal. Although this meal was associated with increases in fullness and bloating, the changes were modest. A meal with a higher caloric load, particularly one that contained more lipid^[21], might have provoked more symptoms, from which it would be possible to demonstrate an improvement with ginger. Similarly, although we used the same dose of ginger as Lien *et al.*^[22], who reported a reduction in nausea induced by circularvection, our subjects reported low ratings for nausea throughout the study, thus, it would be difficult to demonstrate an effect of ginger if one existed. Stadelmann *et al.*^[23] have reported that a combination of peppermint oil and ginger extract

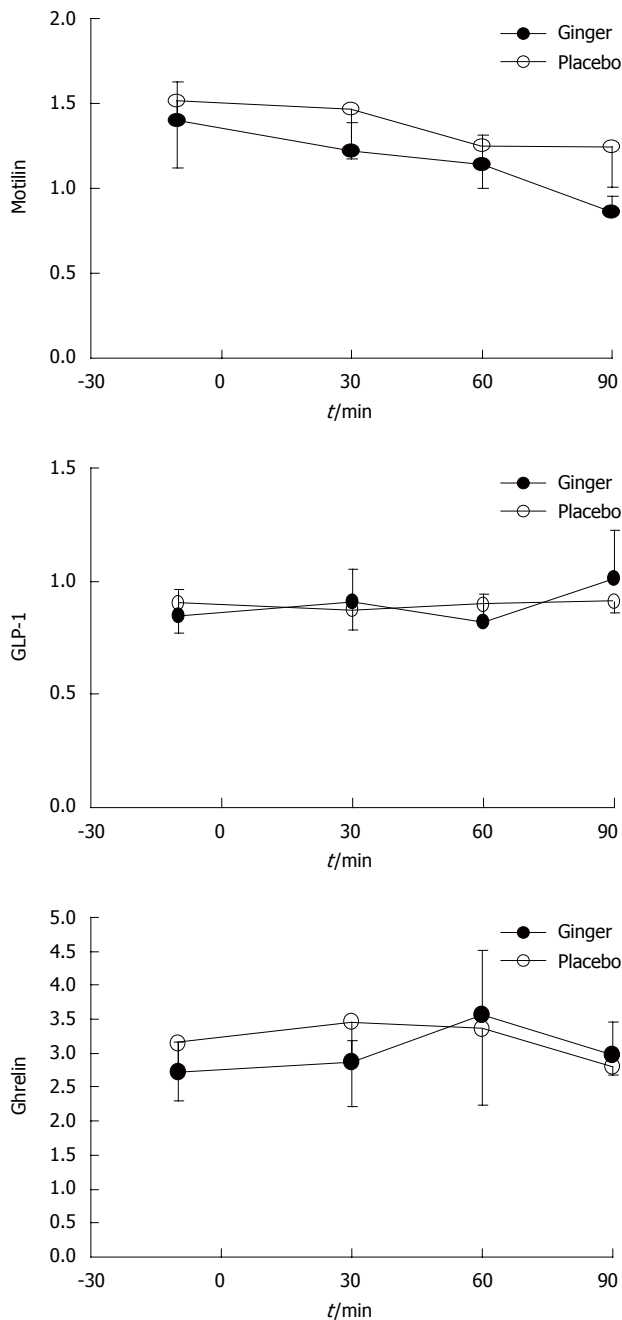


Figure 5 Plasma concentrations of glucagon-like peptide-1, motilin and ghrelin in patients with functional dyspepsia. There was no difference in the concentrations of any hormone between the ginger and placebo groups. Data are means \pm SE, $n = 11$. GLP-1: Glucagon-like peptide-1.

for 4 wk improved gastrointestinal symptom scores when compared to placebo, but the relative contribution of each active agent is unclear.

The mechanism by which ginger could enhance antral contractions and gastric emptying is not clear. We could not demonstrate any modulation of gut-derived hormones that are known to affect gastric motility, including motilin, ghrelin or GLP-1. It would be of interest to examine whether ginger affects the plasma concentrations or the actions of cholecystokinin, because this hormone is reported to be elevated in patients with functional dyspep-

sia^[21]. Abdel-Aziz *et al*^[24] have reported the potential for ginger to act on the 5-HT₃ receptor ion-channel complex, by binding the serotonin binding site, and Shibata *et al*^[25] have reported that a component of Dai-Kenchu-Tou (which contains ginger) stimulated gastric motility through cholinergic and 5-HT₃ receptors in dogs. The limitation of this study is that our observation was limited to 90 min of gastric emptying, and a single dose of ginger would not have been adequate for treatment of dyspepsia symptoms in patients with functional dyspepsia, especially as this disease is chronic and recurrent. Therefore, it is difficult to draw any clear conclusion. Further proper clinical trials of several weeks' treatment with ginger capsules seem to be necessary before starting trials in subgroups of patients with functional dyspepsia.

In summary, we confirmed that the acceleration of gastric emptying by ginger that we initially demonstrated in healthy volunteers extended to patients with functional dyspepsia. Further studies could be indicated in specific subgroups of patients (e.g. those with predominant bloating or nausea, or those with known delayed gastric emptying), to determine whether this can be a useful therapeutic approach.

ACKNOWLEDGMENTS

The authors thank Miss Ya-Hui Huang, Division of Hepatogastroenterology, Department of Internal Medicine, Chang Gung Memorial Hospital-Kaohsiung Medical Center for her assistance for blood sample collection and ELISA study.

COMMENTS

Background

Pharmacological therapy for patients with functional dyspepsia remains unsatisfactory. There have been few studies on the effects of ginger in patients with functional dyspepsia.

Research frontiers

Ginger (*Zingiber officinale*) has been used to treat a number of medical conditions, including those that affect the digestive tract. In this study, the authors demonstrated that the effect of ginger could be a potential mechanism for mediating gastric motility.

Innovations and breakthroughs

The authors had previously shown that ginger increases the frequency of antral contractions and accelerates gastric emptying of a low-nutrient liquid in healthy volunteers. However, the actual effect of ginger on patients with functional dyspepsia is still unknown, and this is believed to be the first study to explore this issue. Furthermore, current *in vivo* studies suggest that ginger is an effective therapy for patients with functional dyspepsia.

Applications

By understanding how ginger works on gastric motility, this study might represent a future strategy for therapeutic intervention in patients with delayed gastric emptying.

Terminology

The acceleration of gastric emptying by ginger that we initially demonstrated in healthy volunteers extends to patients with functional dyspepsia.

Peer review

Overall, this preliminary study of 11 patients with functional dyspepsia seems well designed. The data were appropriately analyzed and indicate that ginger has an impact on symptoms and gastric emptying. A large study needs to be undertaken to demonstrate its efficacy convincingly.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Protection of the liver against CCl₄-induced injury by intramuscular electrotransfer of a kallistatin-encoding plasmid

Yong Diao, Xiao-Feng Zhao, Jun-Sheng Lin, Qi-Zhao Wang, Rui-An Xu

Yong Diao, Xiao-Feng Zhao, Qi-Zhao Wang, Rui-An Xu, Institute of Molecular Medicine, Huaqiao University, Quanzhou 362021, Fujian Province, China

Jun-Sheng Lin, Centre for Reproduction and Genomics, AgResearch, Invermay, Mosgiel 9053, New Zealand

Author contributions: Diao Y, Zhao XF and Wang QZ performed the majority of experiments; Lin JS provided analytical tools and was also involved in editing the manuscript; Diao Y and Xu RA designed the study and wrote the manuscript.

Supported by The State High Technology Research and Development Program of China (863 Program), No. 2008AA02Z135; the Important National Science and Technology Specific Projects, No. 2009ZX09103-643; and the Natural Science Foundation of China, No. 30973591

Correspondence to: Yong Diao, Professor, Institute of Molecular Medicine, Huaqiao University, Quanzhou 362021, Fujian Province, China. diaoyong@hqu.edu.cn

Telephone: +86-595-22692516 Fax: +86-595-22690516

Received: July 9, 2010 Revised: October 16, 2010

Accepted: October 23, 2010

Published online: January 7, 2011

ferred mice, protection against CCl₄-induced liver injury was reflected by significantly decreased serum ALT, AST, MDA and TNF- α levels compared to those in control mice ($P < 0.01$ to 0.05 in a dose-dependent manner). Histological observations also revealed that hepatocyte necrosis, hemorrhage, vacuolar change and hydropic degeneration were apparent in mice after CCl₄ administration. In contrast, the damage was markedly attenuated in the Kal gene-transferred mice. The expression of hepatic fibrogenesis marker transforming growth factor- β 1 was also reduced in the pKal transferred mice.

CONCLUSION: Intramuscular electrotransfer of plasmid pKal which was formulated with PLG significantly alleviated the CCl₄-induced oxidative stress and inflammatory response, and reduced the liver damage in a mouse model.

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Key words: Kallistatin; Gene delivery systems; Electroporation; Drug formulation; Liver injury

Peer reviewers: Dr. Juan Carlos Laguna Egea, Professor of Pharmacology, Unitat de Farmacologia, Facultat de Farmàcia, Universitat de Barcelona, Avenida Diagonal 643, Barcelona 08028, Spain; Valentina Medici, MD, Assistant Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of California Davis, 4150 V Street, Suite 3500, Sacramento, CA 95817, United States

Abstract

AIM: To investigate the effect of transgenic expression of kallistatin (Kal) on carbon tetrachloride (CCl₄)-induced liver injury by intramuscular (im) electrotransfer of a Kal-encoding plasmid formulated with poly-L-glutamate (PLG).

METHODS: The pKal plasmid encoding Kal gene was formulated with PLG and electrotransferred into mice skeletal muscle before the administration of CCl₄. The expression level of Kal was measured. The serum biomarker levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), malonyldialdehyde (MDA), and tumor necrosis factor (TNF)- α were monitored. The extent of CCl₄-induced liver injury was analyzed histopathologically.

RESULTS: The transgene of Kal was sufficiently expressed after an im injection of plasmid formulated with PLG followed by electroporation. In the Kal gene-trans-

Diao Y, Zhao XF, Lin JS, Wang QZ, Xu RA. Protection of the liver against CCl₄-induced injury by intramuscular electrotransfer of a kallistatin-encoding plasmid. *World J Gastroenterol* 2011; 17(1): 111-117 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/111.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.111>

INTRODUCTION

Gene therapy refers to a therapeutic approach by introduction and expression of genetic material in particular cells

or tissues. Over the past two decades, great efforts and abundant resources have been invested into the development of an effective, safe and relatively long-lasting gene therapy strategy. Great success has been made recently so that gene therapy was elected as one of the breakthroughs of 2009 by Science^[1]. In order to achieve the goal of therapy, a carrier, or “vector”, is the key required to deliver the genetic material into the target cells. Whilst exciting progress in the development of gene therapy have been made, a safe and efficient method of gene delivery has been elusive thus far. Initially, a number of viral vectors were chosen, owing to their high efficiency in gene transfer. Unfortunately, they also bring added risks to the patients, even though their virulence was genetically disabled. Non-viral vectors avoid the risks. However, lower transferring efficiency has limited their clinical use thus far. To boost efficacy of non-viral gene therapies several delivery methods have been under investigation. Electro-gene therapy is one of the most efficient non-viral approaches for gene therapy^[2-4]. It has already been considered a safe method because naked DNA is not immunogenic and plasmid DNA does not have the risk of insertional mutagenesis^[5]. Moreover, DNA electrotransfer can be accomplished using devices that have already been used on human cancer patients to deliver cytotoxic molecules such as bleomycin and cisplatin to solid tumors by electrochemotherapy^[6,7]. Thus all elements are available for safe and widespread use of this efficient technology and for implementing the use of genes for medical purposes^[8]. To further improve the transfer efficiency, plasmids were formulated in poly-L-glutamate (PLG) prior to intramuscular (im) injection and electroporation. Such a combined approach significantly elevated transgene expression in myofibers^[9].

Kallistatin (Kal) that belongs to the serine protease inhibitor family is widely expressed in organs such as the liver, kidneys, and blood vessels. Previous studies have demonstrated kallistatin to be a potent anti-inflammatory agent. Kallistatin administration by gene delivery attenuates oxidative stress, apoptosis, inflammation, and organ damage in animal models^[10-13]. These findings indicate that kallistatin may play an important role as an antioxidant in maintaining oxidative balance and preventing oxidative endothelial and tissue injury.

Oxidative stress is a state of redox imbalance caused by increased reactive oxygen species (ROS) generation and decreased antioxidant capacity. Administration of carbon tetrachloride (CCl₄) is an established experimental model of severe toxic liver injury involving generation of oxidative stress and is frequently used for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs^[14]. Antioxidants and anti-inflammatory agents can play a role in liver protection by scavenging active oxygen and free radicals and neutralizing lipid peroxides.

In the present study, we first investigated the feasibility of improving the expression of the transgene after introducing the PLG formulated plasmid into mouse skeletal muscle by electroporation, then investigated the therapeutic efficacy of Kal expression in the circulation on CCl₄-induced liver injury. After CCl₄ had been injected into mice to induce liver injury, evaluations of liver marker

enzymes, the extent of oxidative stress and liver histology were performed, revealing that elevated levels of human Kal were effective in alleviating oxidative stress and protecting liver against CCl₄-induced liver damage.

MATERIALS AND METHODS

Materials

Sodium salt of PLG (15-50 kDa) was purchased from Stsien Co (Nanjing, China); EndoFree plasmid Giga kit was purchased from Qiagen GmbH (Hilden, Germany); DNA Delivery Device was purchased from TERESA Healthcare Sci-Tech Company (Shanghai, China); reporter lysis buffer and β -galactosidase enzyme assay system was purchased from Promega (Madison, USA); BCA Protein Assay Kit was purchased from Pierce (Rockford, USA); Quantikine kit was purchased from R&D Systems Inc (Minneapolis, USA).

Experimental animals

Balb/c mice (6- to 8-wk-old females, Slaccas Company, Shanghai) were used throughout this study. The animals were maintained on a 12/12 h day/night cycle at room temperature 18-24°C. Food and water were provided *ad libitum*. The Animal Studies Ethics Committee of Huaqiao University approved all the experiments reported here.

Plasmid DNA

The expression plasmid pLac encoding β -galactosidase under the control of the cytomegalovirus immediate-early promoter, and the pKal under the control of the same promoter and driving a coding sequence for the human Kal, were constructed as reported previously^[15] and kept in our laboratory. The integrity of the sequence was determined by DNA sequencing. Plasmids were transformed and expanded into *E. coli* strain JM-109 and purified with the EndoFree plasmid Giga kit in accordance with the supplier's protocol. DNA was dissolved in Endofree TE buffer and kept frozen in aliquots at a concentration of 2 mg/mL.

Formulation of plasmid DNA

Formulation was made by mixing the plasmid DNA with the sodium salt of PLG before adjusting the NaCl concentration to 0.15 mol/L with a 5 mol/L stock solution. The plasmid and polymers were allowed to incubate at room temperature for 15 min prior to adding NaCl for tonicity adjustment. The pH of the formulation was adjusted using dilute hydrochloric acid or sodium hydroxide to 7.0. Sterility of the formulation was achieved by sterile filtration through sterile 0.22 μ m pore size filters, and was injected immediately after preparation.

Im injection

Mice were anesthetized by intraperitoneal (ip) injection of pentobarbital sodium at a dose of 30 mg/kg body weight. The plasmid was injected directly into the tibialis anterior (TA) muscles of the mice using a 500 μ L syringe. The injected volumes were 50 μ L for each side of the TA muscles.

In vivo electroporation

Two minutes after the im injection of plasmid DNA, an electrical field was applied to the area around the injection. Two silver needle electrodes were inserted 3 mm apart into the TA muscles and 6 electric pulses were applied using the TERESA DNA delivery device. The electric pulses were 50 ms in duration at a voltage of 60 V.

β-galactosidase assay

The TA muscles treated with plasmid pLac were excised at different time points post delivery, and were analyzed for β-galactosidase expression. The samples were collected immediately after euthanizing the animals. The samples were frozen by keeping in liquid nitrogen and then stored at -70°C until further use. These samples were weighed, minced into small pieces and homogenized with reporter lysis buffer using a homogenizer. The tissue homogenate was then centrifuged at 15000 g and 4°C for 15 min and the clear supernatant was separated for further analysis. The β-galactosidase activity was measured using the β-galactosidase enzyme assay system. The total protein content of the samples was measured with the BCA protein assay kit. Finally, the enzyme activity in the samples was expressed as milliunits/mg of protein.

CCl₄ treatment

The mice were divided into the following groups: (1) control; (2) CCl₄; (3) pLac (50 μg) + CCl₄; (4) pKal (100 μg) + CCl₄; (5) pKal (50 μg) + CCl₄; and (6) pKal (25 μg) + CCl₄. Each group was composed of 8 mice. CCl₄ was dissolved in corn oil vehicle (1:1, v/v). The mice received a single ip injection with the CCl₄ preparation at a dose of 0.8 mL/kg body weight to induce liver injury 6 d after plasmid administration. Control mice received an ip injection of an equal volume of corn oil alone. The mice were sacrificed and the blood samples were collected *via* the inferior vena cava 24 h after CCl₄ treatment. The livers were sampled immediately afterwards.

Measurement of serum biomarker levels

The serum concentration of kallistatin and tumor necrosis factor (TNF)-α were measured with the Quantikine Immunoassay kit following the manufacturer's instructions. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using a Hitachi 7020 biochemical analyzer. Lipid peroxidation was detected by measuring the serum level of malonyldialdehyde (MDA) spectrophotometrically, the end product derived from the breakdown of polyunsaturated fatty acids and related esters, with the classical thiobarbituric acid method^[16].

Histological analysis

The excised liver was fixed in buffered formalin, embedded in paraffin, cut into 5 μm-thick sections, and examined with hematoxylin eosin (HE) staining. Pathology was scored in a blinded manner by a trained pathologist by counting the number of necrotic or inflammatory foci per microscopic field^[17]. Five fields were checked at 200 × magnification as follows: 0 (absent), 1 (< 2foci), 2

(2-4 foci), and 3 (> 4 foci). The inflammatory response and muscle damage arising from plasmid injection and electroporation was evaluated by analysis of HE-stained slides of TA muscles.

Immunohistochemical analysis

After deparaffinization in xylene and rehydration in graded ethanols, endogenous peroxidase activity of the paraffin-embedded slides was blocked by incubation with 3% hydrogen peroxide (H₂O₂) at 37°C for 10 min. The slides were then treated twice in a microwave oven for 5 min in citrate buffer (pH 6.0) at high power to retrieve antigens. After blocking with goat serum at 37°C for 30 min, the specimens were incubated with the rabbit polyclonal antibodies (primary antibody) against transforming growth factor (TGF)-β1 overnight at 4°C, followed by incubation with biotinylated anti-rabbit antibody at 37°C for 30 min and then horseradish peroxidase conjugated streptavidin at 37°C for 10 min. The slides were stained using 3,3'-diaminobenzidine-H₂O₂, counterstained with hematoxylin, and examined under a light microscope.

Statistical analysis

Data are given as means ± SD. Analysis of variance was performed to test the significance; *P*-values were considered significant when less than 0.05.

RESULTS

Transgene expression

The transgene expression in the TA muscle was enhanced by the PLG formulation (Figure 1). The expression of β-galactosidase in the animals that received plasmid formulated in 6 mg/mL PLG reached its peak level at 5 d post-delivery and was 5-fold higher than that in the mice who received naked plasmid. Thus PLG could enhance the expression of an electroporated plasmid injected im in muscles.

The electroporation of an increasing dose of plasmid pKal (25-100 μg) into TA muscle led to a dose-dependent increase in human Kal plasma concentration, detected on the 7th day after plasmid injection (Figure 2). The level of serum Kal in the 50 μg group was almost 2-fold higher than that in the 25 μg group, but was only slightly lower than in the 100 μg group, suggesting there was a saturation of expression such that the relationship of plasmid dose and transgene expression level was not linear.

Protective effects on CCl₄-induced liver damage

The mice induced with a single dose of CCl₄ developed hepatic damage as compared with the normal control group (*P* < 0.01), as shown by marked changes in ALT and AST activities in the serum (Table 1). Injection of pKal resulted in a significant reduction in ALT and AST activities with each of the 3 doses compared with the CCl₄ alone group (*P* < 0.01-0.05).

Effects on serum TNF-α level

TNF-α is one of the pro-inflammatory cytokines which

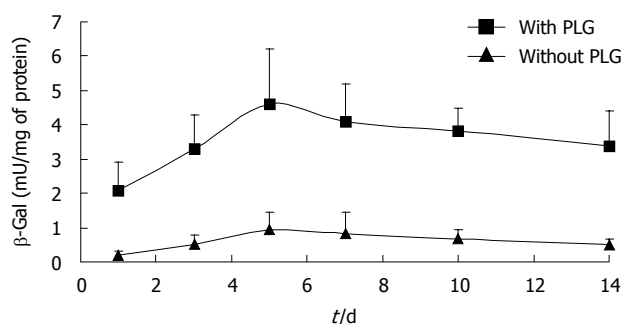


Figure 1 Time-course study of the effect of the poly-L-glutamate formulation on transgene expression of pLac in the tibialis anterior muscle after electroporation transfer. The plasmids were transferred with (square) and without (triangle) poly-L-glutamate (PLG) formulation. The values shown are mean \pm SD, $n = 8$.

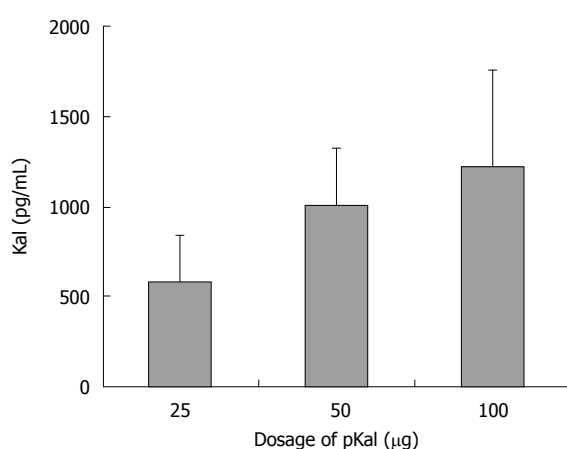


Figure 2 Dose-dependent kallistatin concentrations in the serum responded to intramuscular electrotransfer of a plasmid pKal in poly-L-glutamate formulation. The values shown are mean \pm SD, $n = 8$. Kal: Kallistatin.

are early mediators of tissue damage and repair. Mouse serum TNF- α concentration was measured to evaluate the influence of Kal on CCl₄-induced inflammatory responses (Table 1). CCl₄ exposure markedly stimulated TNF- α releasing in comparison with the control group ($P < 0.01$). The serum TNF- α levels in the 3 pKal + CCl₄ groups were significantly lower than that in the CCl₄ group ($P < 0.01-0.05$) in a dose-dependent manner. These results clearly suggested that the CCl₄-induced inflammatory response could be suppressed by pretreatment with pKal.

Effects on hepatic oxidative stress

The generation of ROS and increase of hepatic lipid peroxidation are important features of chronic liver diseases. To examine the effects of Kal on hepatic oxidative stress, liver tissue was homogenized to determine the hepatic MDA level 24 h after CCl₄ administration (Table 1). MDA is the end product of lipid peroxidation; its level indirectly reflects the degree of oxidative stress. The hepatic MDA concentration in the group treated with CCl₄ was only significantly higher than in the control group ($P < 0.01$). The 3 pKal pretreatment groups significantly attenuated the elevated MDA compared with CCl₄ and pLac + CCl₄ groups ($P < 0.01-0.05$).

Table 1 Serum biomarker levels affected by transgene of kallistatin on carbon tetrachloride-induced liver injury ($n = 8$, mean \pm SD)

Group	ALT (U/L)	AST (U/L)	MDA (nmol/mg protein)	TNF- α (pg/mL)
Control	37 \pm 7	48 \pm 7	0.46 \pm 0.10	2.9 \pm 2.1
CCl ₄	134 \pm 26 ^b	183 \pm 20 ^b	1.15 \pm 0.29 ^b	28.1 \pm 11.8 ^b
pLac + CCl ₄	123 \pm 18 ^b	181 \pm 28 ^b	1.07 \pm 0.33 ^b	28.8 \pm 9.4 ^b
pKal (100 μ g) + CCl ₄	95 \pm 24 ^d	121 \pm 35 ^d	0.48 \pm 0.27 ^d	12.4 \pm 4.0 ^d
pKal (50 μ g) + CCl ₄	98 \pm 28 ^c	143 \pm 37 ^c	0.65 \pm 0.25 ^d	14.5 \pm 3.5 ^d
pKal (25 μ g) + CCl ₄	101 \pm 32 ^c	151 \pm 37 ^c	0.82 \pm 0.21 ^c	17.3 \pm 4.8 ^c

^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs CCl₄ group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MDA: Malonyldialdehyde; TNF: Tumor necrosis factor; CCl₄: Carbon tetrachloride.

Histological analysis

To analyze the extent of liver injury, liver sections were stained with hematoxylin and eosin (Figure 3). No apparent damage was found in the liver sections from the control mice (Figure 3A). In contrast, extensive damage was detected in the sections from CCl₄ and pLac + CCl₄ groups (Figure 3B and C). Hepatocyte necrosis was the predominant histopathologic lesion, and the affected livers displayed hemorrhage, vacuolar change, hydropic degeneration of hepatocytes and infiltration of inflammatory cells. All these lesions in the pKal + CCl₄ groups were significantly attenuated (Figure 3D-G).

Immunohistochemical detection of TGF- β 1

In liver tissue from the control group, the expression of TGF- β 1 was negative in liver cells and weakly positive in stromal cells of the portal area (Figure 4A). In the CCl₄ and pLac + CCl₄ groups, the expression of TGF- β 1 was strongly positive in the cytoplasm of both hepatic parenchymal cells and stromal cells (Figure 4B and C). Compared with the CCl₄ group, the expression of TGF- β 1 was significantly reduced in the pKal treatment groups (Figure 4D-F).

Treatment-associated muscle damage

The safety of plasmid injection and electroporation was evaluated by im injection of 50 μ g pLac followed by electroporation. Fifty μ L saline injections were used as a control. The injected TA muscles were harvested on day 7, fixed, dehydrated, and analyzed by HE staining. The representative images of the muscle samples in various groups are shown in Figure 5. The treatment induced low inflammatory responses at the injection site. No necrosis was observed for any of the groups.

DISCUSSION

The transfer ability of the recombinant plasmid can be improved by the combination of proper formulation, im injection, and electroporation. To improve the efficacy and reproducibility of plasmid delivery, protective, interactive, non-condensing polymers and non-ionic block co-polymers, consisting of ethylene oxide and propylene

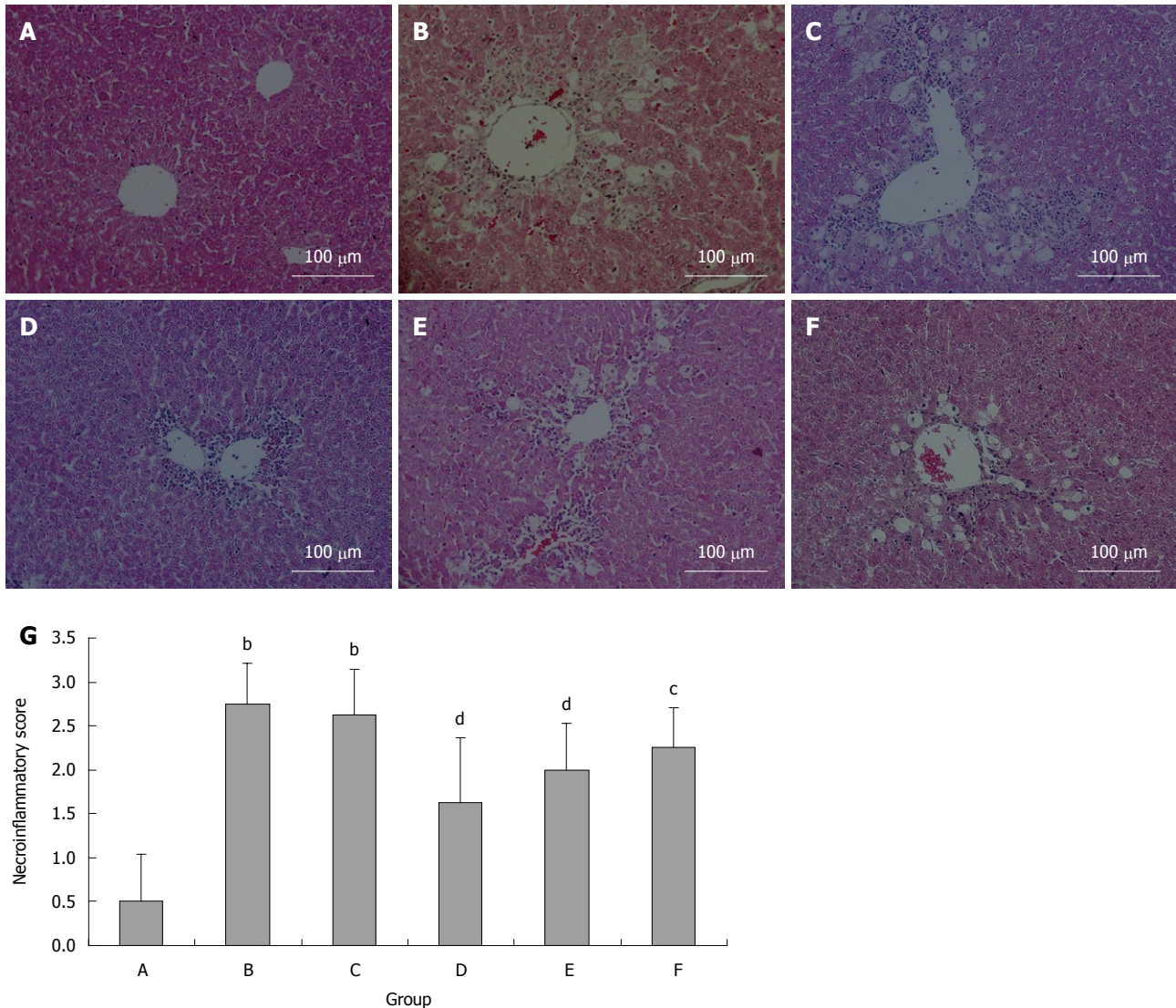


Figure 3 Protective effects of kallistatin expression against carbon tetrachloride-induced liver injury in mice. HE staining was performed on paraffin embedded sections of the liver tissues. Representative sections are shown for each group. A: Control; B: Carbon tetrachloride (CCl₄); C: pLac + CCl₄; D: pKal (100 μg) + CCl₄; E: pKal (50 μg) + CCl₄; F: pKal (25 μg) + CCl₄; G: Necroinflammatory scores. ^b*P* < 0.01 vs control group; ^c*P* < 0.05, ^d*P* < 0.01 vs CCl₄ group.

oxide monomers, have been developed^[18,19]. Both systems have increased distribution of DNA within the muscle tissue and augmented transgene expression compared with DNA in saline. Negatively charged polymers, such as PLG, could also improve the expression of genes delivered by im injection and electroporation, and elevated levels of secreted gene products in multiple *in vivo* models have been reported^[18,20]. Although the mechanism by which PLG enhances transgene expression is unknown, based on our data we concur with other authors' findings that the formulation of PLG can (1) disperse plasmids throughout the electrical field at the time of electroporation; (2) protect plasmids from nuclease degradation; and (3) facilitate intracellular uptake and trafficking of transcriptional active plasmid in muscle cells^[9].

The technique of electroporation has been used for nearly 30 years as a means of introducing DNA into cells *in vitro*, and is now widely used for transfection of plasmids into different tissues *in vivo*. More recently, electroporation has been used for the treatment of cutaneous and

subcutaneous tumors in humans and its safety was proved clinically^[21]. Our results also showed that electroporation treatment-associated muscle damage was minimal. These experiences have paved the way for the clinical use of gene electroporation in humans. The DNA electroporation is reaching the clinical stage as several clinical trials to transfer genes in tumors and in muscle are ongoing^[22,23].

Using an intramuscular electrotransfer method, we demonstrated that the PLG formulation of pKal provided a satisfactory expression level of Kal. The expressed Kal possesses antioxidant properties and the secreted Kal could contribute to protection of liver against CCl₄-induced damage in a mouse model, as indicated by both histological observation and biochemical evaluation. The Kal gene-transferred mice demonstrated a significant reduction in the level of serum peroxidation marker MDA, with a concomitant improvement in the activities of the hepatic antioxidative defense system. This suggests that Kal is able to protect against hepatic cellular membrane oxidative damage *via* a free radical scavenging property.

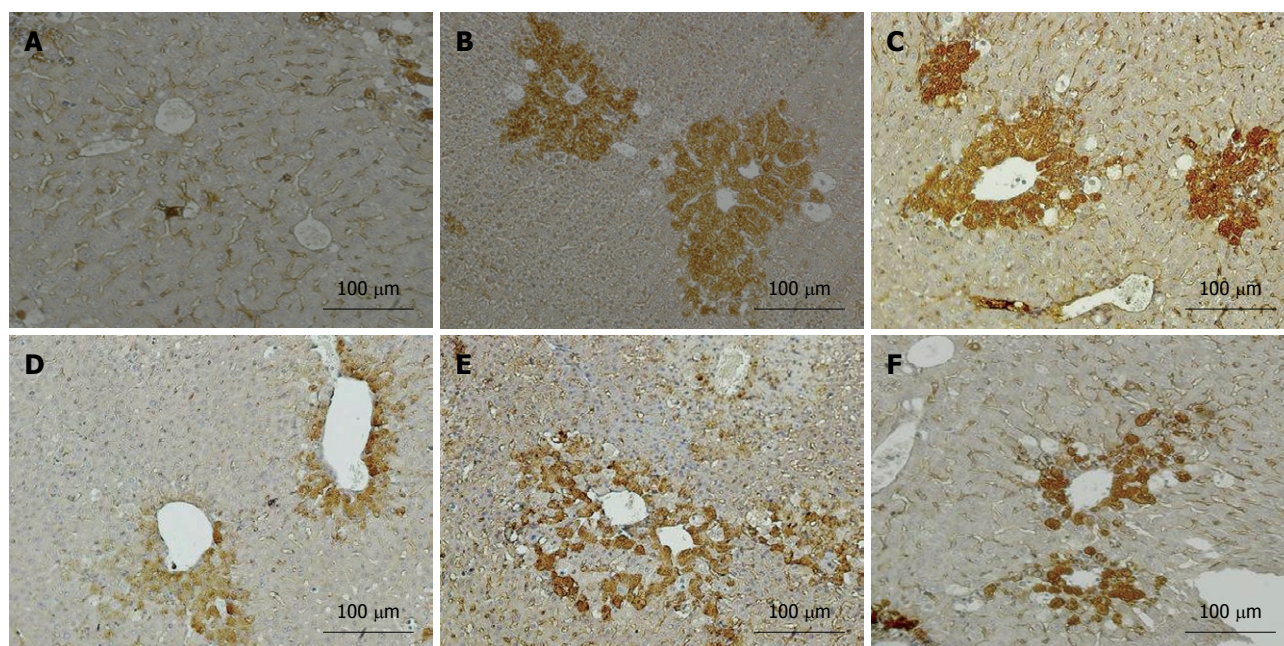


Figure 4 Immunohistological analysis of transforming growth factor- β 1 (brown) in the mice livers treated with carbon tetrachloride. A: Control; B: Carbon tetrachloride (CCl₄); C: pLac + CCl₄; D: pKal (100 μ g) + CCl₄; E: pKal (50 μ g) + CCl₄; F: pKal (25 μ g) + CCl₄.

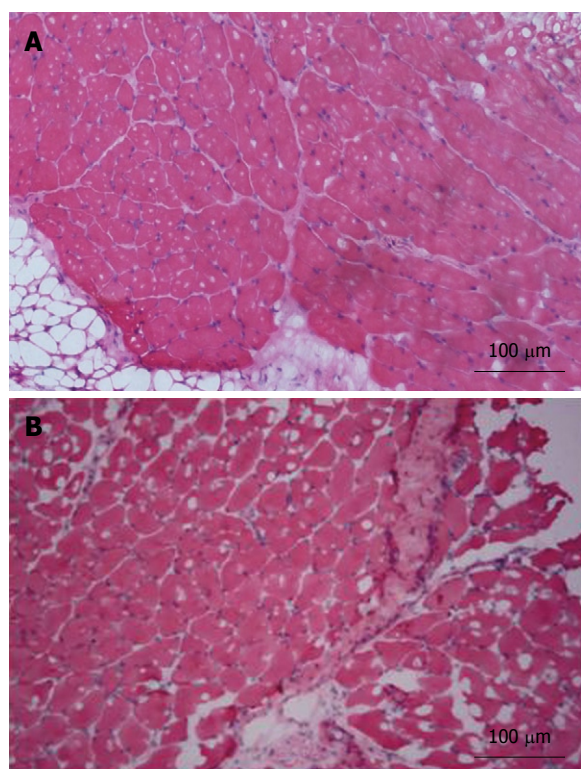


Figure 5 Inflammatory response and muscle damage arising from plasmid injection and electroporation. A: Saline; B: pLac.

Our results also exhibited the anti-inflammatory activity of Kal. TNF- α is a proinflammatory cytokine produced predominantly by macrophages and plays a key role in the host defense response to injury and infection. The expression of Kal in the circulation inhibited the increase in TNF- α production from Kupffer cells following CCl₄ injection, due to its anti-inflammatory activity.

The efficiency of most protein drugs, whose half-life *in vivo* is generally shorter than chemical drugs, markedly depends on their plasma kinetics. Taking the advantages of gene transfer, a steady-state therapeutic level of the recombinant protein in the circulation could be maintained for a long time. This is especially important in the treatment of chronic disease or the prevention of disease. Our results showed that the constant expression level of Kal in the pKal group with the lowest dosage was already sufficient to prevent CCl₄-induced liver damage. These data provided compelling and mechanistic evidences for the importance of Kal in regulation of liver injury.

TGF- β 1 in the liver is secreted by hepatocytes, Kupffer cells, stellate cells (HSC), endothelial cells and infiltrating mononuclear cells, and plays a pivotal role in hepatic fibrogenesis. Among many inflammatory cytokines involved in liver fibrosis, TGF- β 1 appears to be the most important, because (1) there is higher TGF- β 1 expression in activated HSC; (2) TGF- β 1 has potency in upregulating extracellular matrix expression; (3) there is higher expression of TGF- β receptors on HSC; and (4) TGF- β 1 increases the expression of tissue inhibitor of metalloproteinases-1. Therefore, many antifibrosis strategies focus on reducing the secretion of TGF- β 1 and blocking the TGF- β signal transduction pathway to reduce TGF- β 1-induced HSC proliferation. Our results clearly demonstrated the inhibitory effect of Kal expression on the hepatic TGF- β 1 level in an experimental animal model of liver damage induced by CCl₄.

In conclusion, the data of this study provided the evidence that the combination of PLG formulation, im injection and electroporation of plasmid encoding the Kal gene is an effective gene therapeutic method in a CCl₄-induced liver damage model. The beneficial effects of this technique to reduce the expression of TGF- β 1 also make

it a promising strategy for the treatment of hepatic fibrosis in the future.

COMMENTS

Background

Acute and chronic hepatic injuries cause high morbidity and mortality worldwide. Although the pathogenesis is not fully understood, it is clear that reactive oxygen species play a key function in the pathological changes in the liver. Kallistatin is a member of the serine proteinase inhibitor superfamily and was shown to have pleiotropic effects, including anti-oxidative stress, anti-inflammation and angiogenesis. Studies show it can attenuate oxidative stress, apoptosis, inflammation, and organ damage. However, the short half-life of kallistatin protein *in vivo* limits its efficiency. In contrast, long-term transgene expression can be achieved by gene therapy.

Research frontiers

Gene therapy has already proven to be a novel and promise modality in reducing liver injury, but is still in its infancy, and ideal gene delivery systems for gene transfer with high and prolonged gene expression, as well as less cytotoxicity or immunogenicity remain to be developed. Viral vectors are so far the most efficient tools for delivery of genes into mammalian cells. Drawbacks such as cost, immunogenicity, and difficulties in manufacture have shifted the interest towards nonviral vectors. However, the low efficiency of gene transfer hampers the development of nonviral vectors severely.

Innovations and breakthroughs

In order to boost the efficacy of non-viral gene therapy delivery, DNA electrotransfer technology was applied and the plasmid formulation was also optimized. The kallistatin expression *in vivo* was increased greatly; the animal model showed that the gene therapy strategy was effective in alleviating oxidative stress and protecting the liver against carbon tetrachloride (CCl₄)-induced liver damage.

Applications

Although additional studies are necessary to translate this technology into clinical trials in humans, the results provide a rationale to develop new pharmacological strategies in the clinical management of patients with acute and chronic liver injury.

Terminology

Electro-gene therapy: a method involving injection of a naked plasmid encoding a marker gene or a therapeutic gene, followed by *in vivo* electroporation, where short electrical pulses are applied to the injected tissue. The gene expression of the injected plasmid was reported to increase more than 100-fold.

Peer review

The paper described the improvement of the intramuscular electrotransfer of a kallistatin-encoding plasmid by using poly-L-glutamate in plasmid formulation. The ensuing increased presence of kallistatin in plasma protected those transfected mice from the hepatotoxic effects of a single dosage of CCl₄. In general, results are sound and their interpretation is correct.

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S- Editor Sun H L- Editor Cant MR E- Editor Lin YP

Risk factors and gene polymorphisms of inflammatory bowel disease in population of Zhejiang, China

Zi-Wei Wang, Feng Ji, Wei-Jun Teng, Xiao-Gang Yuan, Xiao-Ming Ye

Zi-Wei Wang, Feng Ji, Wei-Jun Teng, Xiao-Gang Yuan, Xiao-Ming Ye, Department of Digestive Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Author contributions: Wang ZW and Ji F designed the research and wrote the paper; Teng WJ, Yuan XG and Ye XM performed the research and analyzed the data.

Correspondence to: Feng Ji, Professor, Department of Digestive Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. jifeng1126@sina.com

Telephone: +86-571-87236532 Fax: +86-571-87236611

Received: July 12, 2010

Revised: September 20, 2010

Accepted: September 27, 2010

Published online: January 7, 2011

tients with IBD and healthy controls had heterozygous or homozygous SNPs variants.

CONCLUSION: Milk, fried food and stress are associated with increased risk of IBD. The common variants in *NOD2/CARD15* gene are not associated with IBD in China's Zhejiang population.

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Key words: Inflammatory bowel disease; Risk factors; Epidemiology; Gene polymorphism; *NOD2/CARD15* gene

Peer reviewer: Dr. John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent, ME20 7NH, United Kingdom

Abstract

AIM: To identify the risk factors and three single nucleotide polymorphisms (SNPs) of *NOD2/CARD15* gene in inflammatory bowel disease (IBD) of the population in Zhejiang, China.

METHODS: A case-control study was conducted using recall questionnaire to collect data on demographic, socioeconomic, lifestyle characteristics and dietary behaviors from 136 determined IBD patients and 136 paired healthy controls. COX regression method was used to screen the statistically significant risk factors for IBD. The polymorphisms of *NOD2/CARD15* gene *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC* were genotyped and further compared between 60 patients with IBD and 60 healthy controls by polymerase chain reaction and restriction fragment length polymorphism.

RESULTS: IBD occurred primarily in young and middle-aged people. The mean age for IBD patients was 42.6 years. The ratio of males to females was 1.23:1. COX regression indicated a higher statistical significance in milk, fried food and stress compared with the other postulated risk factors for IBD. None of the pa-

Wang ZW, Ji F, Teng WJ, Yuan XG, Ye XM. Risk factors and gene polymorphisms of inflammatory bowel disease in population of Zhejiang, China. *World J Gastroenterol* 2011; 17(1): 118-122 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/118.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.118>

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of idiopathic inflammatory bowel disease (IBD) whose etiology is multifactorial and still vague. Currently, the development of IBD is considered to have a close relationship with immunology, genetics, environment and infection. The incidence of IBD in Western populations increased during the past few decades with an estimated incidence of 0.35%-1.00% for CD and 0.10%-1.00% for UC^[1]. *NOD2/CARD15* is the first verified predisposing gene of CD where three *NOD2* variants *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC* were found to be associated with CD in the Caucasian populations^[2,3]. Nevertheless, these single nucleotide polymorphisms

(SNPs) were not found to predispose to CD in Japanese and Hong Kong populations^[4,5], leaving controversies on their exact role in CD. The number of patients with IBD has been increasing in China, but only a few studies have investigated the risk factors in IBD. Moreover, association between *NOD2* gene and the development of IBD has seldom been evaluated in the Chinese population^[6]. Therefore, the purpose of this study was to identify the risk factors by case-control studies and determine whether the *NOD2* variants are associated with IBD in the population of Zhejiang, China.

MATERIALS AND METHODS

Subjects

One hundred and thirty six patients with IBD and 136 age and sex-matched healthy controls were recruited from the First Affiliated Hospital of Zhejiang University, Jinhua Central Hospital, Ningbo Medical Treatment Center, Lihuili Hospital and Taizhou Hospital of Zhejiang Province between January 2005 and December 2008. The age of IBD patients (84 UC and 52 CD) ranged from 18 to 85 years. Written informed consent was obtained from all the cases and controls. Blood samples were collected from 60 patients (32 UC and 28 CD) and 60 healthy controls randomly. IBD was diagnosed based on the clinical, radiographic, endoscopic and histologic criteria.

Questionnaire

Each subject received a questionnaire to obtain demographic data. The questionnaire also contained items specifically related to IBD: education background, heredity, occupation condition (occupation classification and stress), habitat condition during the past 5 years (drinking water and toilet), infection, appendectomy, measles, oral contraceptive use, estrogen replacement, dietary habits (vegetarian diet or carnivorous diet), smoking history, tea drinking, and alcohol, milk, fried food and spicy food intake. All questionnaires were checked for completeness, and doubtful responses from both patients and healthy controls were confirmed upon return of the questionnaire.

Genotyping

DNA was isolated from peripheral blood using the Genomic DNA Isolation Kit (Sangon, Shanghai, China). All polymerase chain reaction (PCR) assays were performed in a 25 μ L volume reaction. Three single nucleotide polymorphisms (SNPs) of *NOD2/CARD15* gene were amplified by specific primers (Table 1)^[7] under the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturing at 94°C for 20 s, annealing at 60°C (*Arg702Trp*), 55°C (*Gly908Arg*) or 58°C (*Leu1007fsinsC*) for 30 s and extension at 72°C for 1 min, and final incubation at 72°C for 7 min. PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

Genotyping for *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC* was performed using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP):

Table 1 The primers for *NOD2/CARD15* gene

SNPs	Primers	Length of products (bp)
<i>Arg702Trp</i>	Forward 5'-CTTCCTG-GCAGGGCTGTGTC3' Reverse 5'-CATGCAC-GCTCTTGGCCTCAC3'	176
<i>Gly908Arg</i>	Forward 5'-AAGTCTGTAAT-GTAAAGCCAC3' Reverse 5'-CCCAGCTCCTCCCTCTTC3'	380
<i>Leu1007fsinsC</i>	Forward 5'-CCTG-CAGTCTCTTTAACTGG3' Reverse 5'-CTTAC-CAGACTTCCAGGATG3'	168

SNPs: Single nucleotide polymorphisms.

a 10 μ L aliquot of the product was digested with an appropriate restriction enzyme. The tubes were incubated at 37°C for 4 h, and then transferred to 65°C for 20 min. After digestion, fragment sizes for carriers of the polymorphic alleles decreased, resulting in the presence of different fragments (Table 2). Products were electrophoresed in a 15% polyacrylamide gel and visualized by silver nitrate staining.

Statistical analysis

Semi-quantitative data were analyzed using COX regression to calculate relative risk (RR) and their 95% confidence interval. Frequencies and susceptibilities of mutations among CD, UC and controls were compared based on χ^2 or Fisher exact test. All data were analyzed in SPSS (version 13.0), where *P* value of 0.05 or less was considered statistically significant in all cases.

RESULTS

A total of 272 subjects were enrolled in the study who all completed the questionnaires. The mean age for IBD patients was 42.6 years and the ratio of males to females was 1.23:1. The result showed that milk, fried food intake and stress were risk factors for IBD in both univariate and multivariate logistic regression analysis (Tables 3 and 4). The number of cases of infection, appendectomy, oral contraceptive use, and estrogen replacement was too small for statistical analysis, so the data of these variables were not shown in the tables.

Electropherogram of the amplified DNA fragments is shown in Figure 1. PCR-PFLP analyses showed that *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC* alleles of 60 IBD patients and 60 healthy controls were all wild type. None of the patients with IBD and healthy controls had heterozygous or homozygous SNPs variants.

DISCUSSION

The etiology and pathogenesis of IBD have been and continue to be intensely investigated. Accumulating evi-

Table 2 The single nucleotide polymorphisms, restriction enzymes and products of *NOD2* gene

SNPs	Polymorphic alleles	Restriction enzymes	Wild-type alleles (bp)	Mutant alleles (bp)
Arg702Trp	C2104T	<i>Msp</i> I	76 + 54 + 24 + 22	130 + 24 + 22
Gly908Arg	G2722C	<i>Hha</i> I	380	242 + 138
Leu1007fsinsC	3020insC	<i>Nla</i> IV	168	128 + 40

SNPs: Single nucleotide polymorphisms.

Table 3 Univariate logistic regression analysis of risk factors for inflammatory bowel disease

Variables	χ^2	<i>P</i>	RR	95% CI	
				Lower bound	Upper bound
Habitat condition during the past 5 yr	1.192	0.274	0.653	0.303	1.404
Educational background	1.250	0.265	0.724	0.284	1.528
Occupation classification	0.894	0.293	0.615	0.314	1.412
Alcoholic drinking	0.987	0.361	0.712	0.194	1.512
Cigarette smoking	1.215	0.194	0.843	0.247	1.384
Tea drinking	1.523	0.165	0.631	0.315	1.423
Stress	18.452	< 0.001	1.295	1.151	1.457
Milk intake	25.425	< 0.001	1.279	1.162	1.407
Fried food intake	24.378	< 0.002	1.286	1.154	1.417

RR: Risk ratio; CI: Confidence interval.

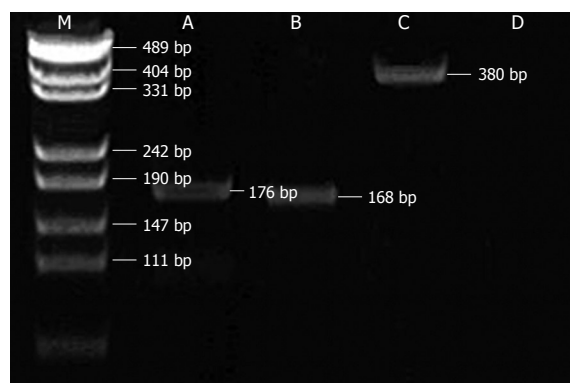
Table 4 Multivariate logistic regression analysis of risk factors for inflammatory bowel disease

Variables	χ^2	<i>P</i>	RR	95% CI	
				Lower bound	Upper bound
Milk intake	10.713	0.0011	1.243	1.091	1.415
Fried food intake	14.267	0.0002	1.238	1.108	1.383
Stress	13.377	0.0003	1.241	1.102	1.394

RR: Risk ratio; CI: Confidence interval.

dences strongly suggest that it is mediated immunologically and that the inflammatory process is influenced by environmental and host factors. In this study, we found a higher prevalence of IBD in young and middle-aged people which may be associated with the strong gastrointestinal immune function and intense immune response. The prevalence of CD was higher in males (36 cases) than in females (16 cases), but the prevalence of UC was similar among males (39 cases) and females (45 cases), which is consistent with other studies.

Many environmental factors may be involved in the pathogenesis of IBD, including: dietary habits, cigarette smoking, appendectomy, oral contraceptive use, infection and so on. However, the epidemiologic evidence for an etiologic role of these potential risk factors in IBD is inconsistent. In this study, there was a strong evidence for an increased risk of developing IBD associated with stress, milk intake and fried food. Stress increases gut muscle tone and intestinal transit time. Several studies reported that people with functional gastrointestinal disorders had significantly more behavioral and emotional symptoms than

**Figure 1** Amplified fragments of *NOD2/CARD15* gene. M: DNA marker; A: Amplified fragment of Arg702Trp; B: Amplified fragment of Leu1007fsinsC; C: Amplified fragment of Gly908Arg; D: Negative control.

healthy people^[8]. UC patients are most likely to experience the problems like mood and anxiety^[9]. Some hormones released during stress (e.g. corticotrophin-releasing factor) can promote intestinal inflammation and alter visceral sensitivity when released locally in the gut^[10]. Epidemiological studies showed that the prevalence of IBD varies among populations. The immigrants had a different incidence of IBD within a generation. It has been suggested that different food antigens play a significant role. The present study confirms an increased risk of developing IBD associated with milk intake. IgE-mediated allergy to cow's milk proteins is common in the children and adults who experience repeated gastrointestinal symptoms, and often is the first manifestation of food allergy. Cow's milk protein allergy may induce the abnormal immune response of digestive tract that increases the relative risk of IBD. In epidemio-

logic studies, high-temperature cooking methods have been associated with the formation of carcinogenic substances such as heterocyclic amines, acrylamide and polycyclic aromatic hydrocarbons^[11]. These products have been associated with endothelial dysfunction and inappropriate immune responses, which results in the development of IBD and intestinal cancer.

Cigarette smoking that has been reported as a risk factor for IBD in many studies might even have beneficial effects on the course of UC, but exacerbates the course of CD. The potential mechanisms involved in this dual relationship may include the effects of nicotine administration on inflammatory cytokine, changes in blood flow and gut permeability^[12]. In this study, cigarette smoking was not significantly associated with the development of IBD. Studies showed that IBD patients were more likely to be white collar and urban residents with high educational background. Infection and oral contraceptive use were considered to increase the risk of developing IBD, whereas vegetarian diet, tea drinking, spicy food intake and appendectomy decreased the risk. In this study, no significant association was found between these factors and IBD.

Monozygotic twin concordance, familial predisposition, and segregation analyses have shown that genetic factors confer susceptibility to IBD. *NOD2/CARD15* gene locates on chromosome 16q12, encoding a member of the Apat-1/Ced-4 superfamily of apoptosis regulator that is expressed in monocytes. *NOD2/CARD15* gene is involved in the recognition of lipopolysaccharide and subsequent activation of necrosis factor- κ B, and disturbs the activation of the innate immune system by bacterial antigens. *NOD2/CARD15* gene mutation or deletion induces the abnormal innate immune response, which is important for immunological protection against intestinal microbes and may contribute to the development of IBD. Increased *CARD15* was detected in mononuclear and epithelial cells of colon in CD patients^[13,14]. Hugot *et al.*^[2] showed that a frame shift variant and two missense variants were associated with CD. This result is in accordance with the studies in different populations^[3,7]. In our study, common *NOD2* variants associated with increased susceptibility to CD in Caucasian populations were not verified in the Zhejiang population. It is conceivable that the *NOD2* variants present in Caucasian patients are rare or nonexistent in the Zhejiang population and not detected in our limited population sample. Our results are in agreement with those studies in Asian patients^[4,5]. This diversity of linkage analyses may arise from the heterogeneity of the disease and differences in genetic background of the population studied.

Human epidemiologic studies combine both parts of the typical risk assessment process into a single study by assessing the degree of exposure and the association with the risk of disease in a single study. The present study supports that intestinal environmental and genetic factors are vital for the pathogenesis of IBD. However, the heterogeneity among the small number of studies limited the ability to draw conclusions. Further studies using a larger cohort of patients with IBD are warranted to identify the risk factors and gene susceptibility to IBD.

COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of idiopathic inflammatory bowel disease (IBD) whose etiology is multifactorial and still vague. The development of IBD is considered to be closely related to immunology, genetics, environment and infection. *NOD2/CARD15* is the first verified predisposing gene of CD in the Caucasian populations.

Research frontiers

The etiology and pathogenesis of IBD have been and continue to be intensely investigated. Three *NOD2* variants *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC* were found to be associated with CD in the Caucasian populations, but not in Japanese and Hong Kong populations. In this study, the authors identified the risk factors by case-control study and determined whether the *NOD2* variants are associated with IBD in China's Zhejiang population.

Innovations and breakthroughs

Only a few studies have investigated the risk factors of IBD in China. Moreover, the association between *NOD2* gene and the development of IBD has seldom been evaluated in the Chinese population. This study demonstrated that milk, fried food and stress are associated with increased risk of IBD, and the common variants in *NOD2/CARD15* gene are not associated with IBD in the Zhejiang population.

Applications

NOD2 variants present in Caucasian patients may be rare or nonexistent in the Zhejiang population. Milk, fried food and stress are the potential risk factors for IBD.

Terminology

NOD2/CARD15 gene locates on chromosome 16q12, encoding a member of the Apat-1/Ced-4 superfamily of apoptosis regulator that is expressed in monocytes. *NOD2/CARD15* gene is involved in the recognition of lipopolysaccharide and subsequent activation of necrosis factor- κ B, and disturbs the activation of the innate immune system by bacterial antigens. *NOD2/CARD15* gene mutation or deletion can induce the abnormal innate immune response.

Peer review

This is an interesting paper looking at 136 IBD patients and paired healthy controls from the Zhejiang population comparing risk factors, and SNP analysis of *NOD2/CARD15* in 60 patients and paired controls. No patients had variants in the *NOD2/CARD15* gene by the methods used. Milk, fried food and stress were cited as potential risk factors. This work adds to the existing literature on *NOD2*, and supports the finding of lack of association with IBD in oriental populations.

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S- Editor Sun H L- Editor Ma JY E- Editor Ma WH

Surgical vs percutaneous radiofrequency ablation for hepatocellular carcinoma in dangerous locations

Ji-Wei Huang, Roberto Hernandez-Alejandro, Kristopher P Croome, Lu-Nan Yan, Hong Wu, Zhe-Yu Chen, Pankaj Prason, Yong Zeng

Ji-Wei Huang, Lu-Nan Yan, Hong Wu, Zhe-Yu Chen, Pankaj Prason, Yong Zeng, Department of Hepato-Biliary-Pancreatic Surgery, Division of Liver Transplantation, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Roberto Hernandez-Alejandro, Kristopher P Croome, Department of Surgery, University of Western Ontario, Hepato-biliary and Liver Transplant Surgery, London Health Sciences Centre, London Ontario, N6A 4S2, Canada

Author contributions: Hernandez-Alejandro R and Croome KP designed the study; Huang JW, Yan LN, Wu H, Chen ZY, Prason P and Zeng Y performed the research; Huang JW, Zeng Y, Hernandez-Alejandro R and Croome KP made the analyses and wrote the article.

Correspondence to: Yong Zeng, MD, Department of Hepato-Biliary-Pancreatic Surgery, Division of Liver Transplantation, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. zengyongmd@gmail.com

Telephone: +86-28-85422475 Fax: +86-28-85422475

Received: August 2, 2010 Revised: October 11, 2010

Accepted: October 18, 2010

Published online: January 7, 2011

Abstract

AIM: To compare the long-term outcome of percutaneous vs surgical radiofrequency ablation (RFA) for hepatocellular carcinoma (HCC) in dangerous locations.

METHODS: One hundred and sixty-two patients with HCC in dangerous locations treated with percutaneous or surgical RFA were enrolled in this study. The patients were divided into percutaneous RFA group and surgical RFA group. After the patients were regularly followed up for a long time, their curative rate, hospital stay time, postoperative complications and 5-year local tumor progression were compared and analyzed.

RESULTS: No significant difference was observed in curative rate between the two groups (91.3% vs 96.8%, $P = 0.841$). The hospital stay time was longer

and more analgesics were required while the incidence of bile duct injury and RFA-related hemorrhage was lower in surgical RFA group than in percutaneous RFA group ($P < 0.05$). The local progression rate of HCC in dangerous locations was significantly lower in surgical RFA group than in percutaneous RFA group ($P = 0.05$). The relative risk of local tumor progression was 14.315 in percutaneous RFA group.

CONCLUSION: The incidence of severe postoperative complications and local tumor progression is lower after surgical RFA than after percutaneous RFA.

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Key words: Hepatocellular carcinoma; Radiofrequency ablation; Liver cirrhosis; Recurrence; Local therapy

Peer reviewer: Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euro-clinic, 19 Mavromateon Street, Athens 10 34, Greece

Huang JW, Hernandez-Alejandro R, Croome KP, Yan LN, Wu H, Chen ZY, Prason P, Zeng Y. Surgical vs percutaneous radiofrequency ablation for hepatocellular carcinoma in dangerous locations. *World J Gastroenterol* 2011; 17(1): 123-129 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/123.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.123>

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common malignant neoplasm in the world^[1], causing more than 500 000 deaths every year^[2]. HCC is prevalent in Asia and Africa and its incidence has steadily increased in European and American populations^[3,4]. Theoretically, the best treatment of HCC is orthotopic liver transplantation (OLT) which provides the opportunity for its cure^[5], but the scarcity of donors limits this treatment.

In the last two decades, local ablative therapy has become a safe and effective procedure for small HCC, of which radiofrequency ablation (RFA) is considered the most promising one^[6]. It was reported that RFA for small HCC provides a comparable survival time and local tumor control after surgical resection^[7,8], and may also be used as a bridge therapy for liver transplantation^[9-11]. RFA is minimally invasive with a lower complication rate and a shorter hospital stay time than hepatectomy^[12,13].

Although the indication of RFA is much wider than that of surgical resection for HCC, tumors in some circumstances are reported^[14-18] not quite suitable for RFA, such as a central nodule near the porta hepatis due to the risk of injuring major bile ducts, a nodule near large vessels due to a heat sink effect-induced incomplete ablation, a peripheral nodule near extrahepatic organs due to the risk of alimentary tract perforation or pleural effusion caused by heat injury.

In our institute, tumor location is not simply regarded as a contraindication of RFA. This retrospective study was designed to compare the long-term outcome of percutaneous and surgical RFA for HCC in these so-called dangerous locations. To the best of our knowledge, it is the first study comparing the efficacy of surgical and percutaneous RFA for HCC in dangerous locations of the liver.

MATERIALS AND METHODS

Diagnostic criteria

Diagnosis of HCC was made according to the diagnostic criteria for HCC recommended by the European Association for the Study of the Liver^[19], which was based on ultrasound-guided biopsy, or the concordant classical dynamic radiological features of HCC in two radiologic techniques, or one radiologic technique showing typical features of HCC together with an elevated α fetoprotein (AFP) level over 400 ng/mL.

Definition

Tumor in dangerous locations^[18] was defined as a lesion (\leq 0.5 cm in diameter) near large vessels such as a primary or secondary branch of the portal vein, the base of hepatic veins, or the inferior vena cava (IVC), or as a lesion (less than 0.5 cm in diameter) near extrahepatic organs measured on radiological images.

A curative treatment^[19] was defined as no residual viable tumor tissue within the treatment zone confirmed by a 4-wk-afterward-performed spiral triphasic enhanced CT after a complete ablation of the lesion assessed by intraoperative ultrasonography (IOUS).

Local tumor progression^[20] was defined as the appearance of viable tumor tissue that was contiguous with the area completely ablated during follow-up.

Inclusion criteria and enrollment

In our institute, a curative RFA is usually expected for patients conforming to the Milan criteria for liver transplantation. The inclusion criteria in this study included patients with a confirmed diagnosis of HCC or a solitary HCC (\leq

5 cm in diameter) or up to 3 nodules ($<$ 3 cm in diameter), liver function of Child-Pugh class A or B, a prothrombin time of less than 5 s, a HBV-DNA-PCR quantitation of less than 10^5 copies/mL, but without extrahepatic metastasis or obvious vascular invasion, previous or simultaneous malignancies or evident bleeding tendency (a platelet count $> 50 \times 10^9$ /L or correctable by transfusion, no previous treatment of HCC, and those suitable and willing to be treated with RFA).

The study was performed according to the guidelines of the Helsinki Declaration. A written informed consent was obtained from each patient before intervention. Between February 2003 and February 2007, RFA was performed for 794 consecutive HCC patients in West China Hospital. Of these patients, 513 were diagnosed as primary HCC, 484 of them met the inclusion criteria. Of these 484 patients, 162 had at least one nodule in dangerous locations.

Follow-up

Patients were followed up at a three month interval after treatment. Abdominal ultrasonography and helical computer tomography (CT), serum AFP measurement and liver function tests were performed during each visit. When intrahepatic recurrence was suspected, spiral CT or magnetic resonance imaging (MRI) was performed. When extrahepatic metastases were suspected, thoracic CT and bone scintigraphy were performed. Local tumor progression was specifically noticed as the endpoint in this study.

Statistical analysis

Differences in the surgical and percutaneous RFA groups were analyzed by the unpaired *t* test for continuous variables and by the χ^2 test or continuity correction method for categorical variables. Local tumor progression curves were plotted with the Kaplan-Meier method and compared by the log-rank test. Relative prognostic significance of the variables in predicting local tumor progression was assessed with univariate and multivariate Cox proportional hazards regression models. All variables with their $P < 0.05$ by univariate comparison were subjected to multivariate analysis. Results of multivariate analysis were presented as relative risk (RR) with corresponding 95% confidence intervals (CI). Statistical analysis was performed using the SPSS 13.0 statistical software (SPSS Company, Chicago, Illinois, USA). All statistical tests were two-sided and differences were considered when $P < 0.05$.

RFA procedure

Equipments: All RFA procedures were performed on an inpatient basis by surgeons from the Department of Hepato-biliary-pancreatic Surgery using a commercially available system (Radionics, Cool-Tip System, Burlington, MA, USA), single/clustered needle electrode(s) with a 2 cm or 3 cm exposed tip and ultrasound guidance (Vivid4, GE, USA; iU22, Philips, USA). Clustered electrodes were used systematically for lesions ($>$ 3 cm in diameter).

Percutaneous RFA: General anesthesia was employed, 2-4 grounding pads were attached to the thighs of pa-

tients and the electrode was inserted into the lesion according to a route assessment via ultrasound. The needle tip was inserted to the bottom of the tumor (i.e. the most distal border from the skin puncture site) in the first session to avoid gas formation between non-ablated lesion and ultrasound transducer. At the time of subsequent RFA sessions, the electrode position was determined via IOUS scrutiny. The ablation subsequence was always from “bottom” to “top” to provide a clear, real-time ultrasound image. The electrode was inserted at different sites and overlapping ablations were performed until the entire lesion was ablated as determined by IOUS.

Assessment of ablation: After measurement of the baseline impedance, generator output power was gradually increased from 80 W to 200 W, with a peristaltic pump infusing cold saline into the electrode lumen to maintain the tip temperature below 20°C. The timer was usually set to 12 min for each session. Impedance was synchronously monitored with the system. Session in the same site was repeated until the impedance increased at least 10 Ohms over baseline and became stable. The electrode was heated to 90-100°C before it was drawn back in order to eliminate seeding cancer cells and prevent bleeding. Treatment was continued until complete ablation features were achieved in IOUS.

RFA in dangerous locations

Percutaneous RFA: The route of electrode insertion should be carefully considered on ultrasound scrutiny. When the tumor was in segment VII, close to the diaphragm, the electrode was inserted through the right pleural cavity of patients. Saline was infused into the right pleural cavity to compress the right lobe of the lung, then the electrode reached the target under the ultrasound-guidance and percutaneous RFA was achieved through an artificial serothorax. A thoracic close drainage was needed for 2 d after therapy.

Surgical RFA: A right subcostal incision with a midline extension was chosen. Extensive dissociation of the liver was usually performed from the ligaments and adhesions to other organs, such stomach, colon or kidneys and large vessels. The route of surgical RFA was assessed by IOUS on the liver surface. The distance between the tumor and other vulnerable organs or vessels could be enlarged when the operator rotated the liver.

Ablation timing: The time of RFA was usually irregular in the dangerous locations, RFA was stopped as soon as the ultrasound detected microbubbles generated by RFA reaching the distal border of the assumed area. An experienced operator managed most injuries to adjacent organs and structures as well as the heat sink effect from large vessels with RFA.

Assessment of response

Response was assessed according to the modified European Association for the Study of the Liver criteria^[19].

Table 1 Demographic parameters of patients undergoing percutaneous and surgical radiofrequency ablation

Parameters	Patients undergoing percutaneous RFA (n = 63)	Patients undergoing surgical RFA (n = 93)	P value
Age (yr)	57.8 ± 16.1	52.4 ± 11.7	0.531
Gender (M/F)	55/14	81/12	0.205
HBV infected	58	91	0.006
HCV infected	1	0	
None-HBV&HCV	10	2	
Liver cirrhosis	46	71	0.174
AST (IU/L)	47.3 ± 36.2	44.6 ± 33.8	0.311
ALT (IU/L)	42.3 ± 31.4	44.1 ± 19.6	0.354
TB (μmol/L)	15.4 ± 3.4	14.2 ± 5.6	0.601
ALB (g/L)	39.1 ± 9.8	41.7 ± 5.4	0.852
Child A/B	61/8	93/0	0.001
PLT (< 10 ¹¹)	13	9	0.092
PT (> 15')	6	14	0.224
Tumor number, 1/2/3	51/16/2	79/13/1	0.095
Tumor size (cm), > 3/≤ 3	11/78	23/85	0.099
Solitary HCC (cm), ≤ 3	39	62	0.188
Tumor in dangerous locations	73	95	0.242
AFP (ng/mL), ≤ 400/> 400/> 1210	13/41/15	17/60/16	0.000

Non-hepatitis B virus (HBV) & hepatitis C virus (HCV): Patients who were negative for HBV and HCV antibody but not for anti-HBs. RFA: Radiofrequency ablation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: α fetoprotein; TB: Total bilirubin; PLT: Platelet; PT: Prothrombin time; ALB: Albumin.

Spiral triphasic enhanced CT was performed one month after RFA. Residual viable tumor was diagnosed if an enhanced area was noted within the treatment zone. If RFA was repeated, another CT was performed four weeks later to assess the response to RFA. If residual viable tissue of the tumor still existed, RFA was considered a failure and the patient was treated with transcatheter hepatic arterial chemoembolization (TACE).

Potential conflict of interest

This study did not receive any support from industry or private corporations.

RESULTS

Of the 482 patients, 162 had at least one nodule in the dangerous locations (156 had a lesion and 6 had 2 lesions in the dangerous locations) and 320 had HCC in the ordinary location. Of the 162 patients with HCC in dangerous locations, 34 had their diagnosis made by biopsy and 128 were diagnosed non-invasively, 69 received percutaneous RFA and 93 underwent surgical RFA. The demographic parameters of patients who underwent percutaneous and surgical RFA are listed in Table 1. A significant difference was found in HBV/HCV-infection and serum AFP level ($P < 0.05$). The tumor locations and adjacent vessels and organs in patients who underwent percutaneous and surgical RFA are shown in Table 2. The mean follow-up time of patients who underwent percutaneous and surgical RFA was 28.4 ± 14.7 mo (range 3-81 mo) and 31.6 ± 24.1 mo

Table 2 Locations of lesions in patients undergoing percutaneous and surgical radiofrequency ablation

	RFA	
	Percutaneous (<i>n</i> = 89)	Surgical (<i>n</i> = 108)
Segment location		
I	0	0
II	7	12
III	13	17
IV	9	12
V	10	14
VI	6	11
VII	12	20
VIII	12	7
Adjacent vessels or organs		
PH	8	12
RHV	17	15
MHV	8	12
LHV	11	16
IVC	6	8
Heart	3	5
Stomach	9	17
Lung	15	2
R.Kidney	4	7
Colon	5	12
GB	3	2

$P = 0.640$ by Pearson χ^2 test for segment location and $P = 0.054$ by Pearson χ^2 test for adjacent vessels or organs. Lesion between segments was registered at the major location. *n*: Lesion number; RFA: Radiofrequency ablation; PH: Porta hepatis; RHV: Right hepatic vein; MHV: Middle hepatic vein; LHV: Left hepatic vein; IVC: Inferior vena cava; GB: Gall bladder.

(range 6–78 mo), respectively ($P > 0.05$). Censored patients included 17 out of the 69 patients who underwent percutaneous RFA and 22 out of the 93 patients who underwent surgical RFA ($P = 0.885$).

Patients who underwent percutaneous RFA

Eighty-nine lesions were found in 69 patients who underwent percutaneous RFA (Table 1). The mean treatment session was 2.0 ± 1.2 /lesion for the 78 nodules (≤ 3 cm in diameter) and 3.4 ± 0.8 /lesion for the 7 nodules (larger than 3 cm but smaller than 5 cm in diameter). Of the 89 lesions, 73 nodules were found in the dangerous locations, the mean tumor size was 1.7 ± 1.1 cm, and the mean treatment session was 3.7 ± 2.1 /lesion. The complete RFA rate was 98.6% (68/69) assessed intraoperatively, and the curative rate was 91.3% (63/69) assessed by CT 4 wk thereafter. The RFA failure rate was 4.3% (3/69). Two patients failed to achieve a curative outcome after 2 times of percutaneous RFA. The last patient had one nodule (1 cm in diameter) in 2 tumors very close to the pericardium. RFA was aborted due to the concern of malpositioning the electrode byIOUS. These three patients were later treated with TACE.

Patients who underwent surgical RFA

One hundred and eight lesions were found in 93 patients who underwent surgical RFA (Table 1). The mean treatment session was 1.2 ± 0.5 /lesion for the 85 nodules (≤ 3 cm in diameter) and 2.8 ± 0.9 /lesion for the 23 nodules

Table 3 Major complications of radiofrequency ablation

Classification of complications	Percutaneous RFA (<i>n</i> = 69)	Surgical RFA (<i>n</i> = 93)	<i>P</i> value
Grade I			
Analgesics requirement	17	58	0.000
Fever above 38.5°C	23	45	0.055
Grade II			
Ascites	4	11	0.190
Persistent jaundice	2	0	0.315
Gastric hemorrhage	0	3	0.132
Grade III			
Hydrothorax requiring drainage	5	9	0.586
Skin burn	1	0	0.244
Encapsulated effusion needing drainage	3	1	0.184
Grade IV			
Partial hepatic infarction	1	3	0.471
Gastric perforation	1	0	0.244
Bile duct injury	5	1	0.040
Procedure-related hemorrhage	6	1	0.018
Malignant seeding	2	0	0.315

RFA: Radiofrequency ablation.

(larger than 3 cm but smaller than 5 cm in diameter). The mean tumor size and mean treatment session were 1.8 ± 1.0 cm and 2.9 ± 2.0 /lesion, respectively, for the 95 nodules in the dangerous locations. The complete RFA rate was 100% (93/93) assessed intraoperatively, and the curative rate was 96.8% (90/93) assessed by CT 4 wk afterward. The RFA failure rate was 1.1% (1/93). A nodule (4 cm in diameter) in a patient who failed to RFA compressed the right hepatic duct. To avoid the bile duct injury, a stent was inserted into the compressed bile duct, and ethanol was injected into the adjacent tumor border to the bile duct before RFA. Unfortunately, bile fistula still occurred on day 25 after operation, and CT showed an incomplete ablation of the tumor. A T-tube drainage was placed *via* laparotomy later. Tumor encroaching on the right hepatic duct wall was highly suspected, and treated with palliative therapy due to poor liver function.

Hospital stay time and mortality of patients, and complications of RFA

The hospital stay time of HCC patients was significantly longer after surgical RFA than after percutaneous RFA (6.1 ± 3.1 d *vs* 3.5 ± 2.9 d, $P < 0.001$).

No patient died within 30 d after surgical and percutaneous RFA with a mortality of 0%.

According to the accorcion severity grading system of surgical complications^[21], the complications of percutaneous and surgical RFA are shown in Table 3. The number of patients requiring analgesics was significantly greater after surgical RFA than after percutaneous RFA ($P < 0.001$). However, the incidence of bile duct injury and RFA-related hemorrhage was higher in patients after percutaneous RFA than after surgical RFA ($P = 0.05$).

Local tumor progression

During the 5-year study period after treatment, the local

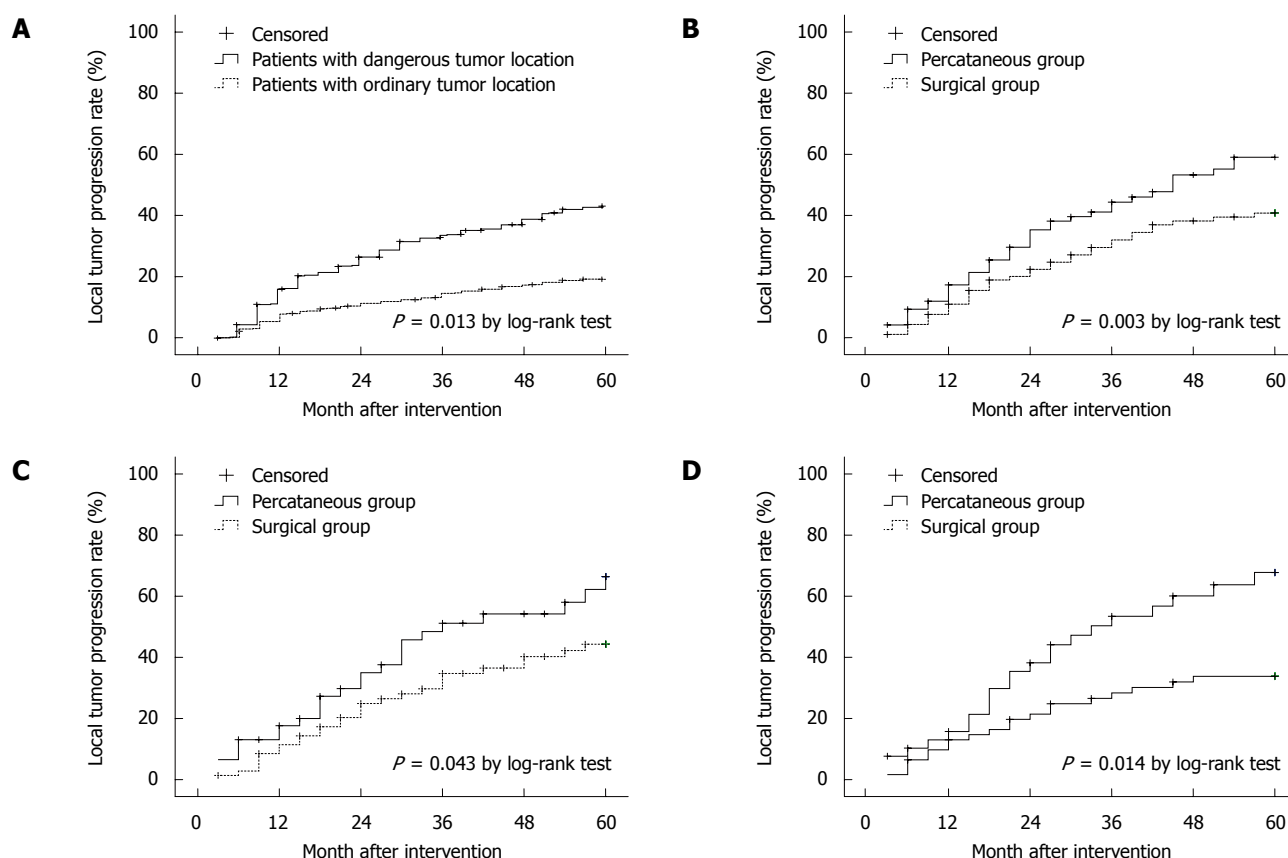


Figure 1 Local tumor progression in patients with hepatocellular carcinoma at common and dangerous locations (A), and hepatocellular carcinoma (B), cirrhosis (C), solitary hepatocellular carcinoma with its diameter ≥ 3 cm (D) after percutaneous and surgical radiofrequency ablation.

tumor progression was observed in 75 (46.3%) out of the 162 patients with HCC in the dangerous locations and in 71 (22.2%) out of the 320 patients with HCC in the general locations. The local tumor progression was more severe in patients with HCC in the dangerous locations than in those with HCC in the general locations ($P < 0.015$, Figure 1A).

Of the 162 patients with HCC in the dangerous locations, 69 and 93 were treated with percutaneous RFA and surgical RFA, respectively. Local tumor progression was observed in 40 out of the 69 patients with HCC after percutaneous RFA and in 35 out of the 93 patients after surgical RFA. The 1-, 2-, 3-, 4-, 5-year local tumor progression rate was 17.4%, 36.2%, 46.4%, 53.6%, 57.6%, respectively, for patients after percutaneous RFA, and 9.9%, 21.5%, 30.1%, 35.5%, 37.6%, respectively, for those after surgical RFA. The local tumor progression was more severe in patients after percutaneous RFA than after surgical RFA ($P < 0.003$, Figure 1B).

Forty-six out of the 63 cirrhotic patients underwent percutaneous RFA and 71 out of the 93 cirrhotic patients underwent surgical RFA (Table 1). The local tumor progression was more severe in patients after percutaneous RFA than after surgical RFA ($P < 0.05$, Figure 1C).

Thirty-nine out of the 69 patients with solitary HCC (≤ 3 cm in diameter) underwent percutaneous RFA and 62 out of the 93 patients with HCC underwent surgical RFA (Table 1). The local tumor progression was more

severe in patients after percutaneous RFA than after surgical RFA ($P < 0.05$, Figure 1D).

Univariate analysis revealed that 5 out of the 10 variables (RFA approach, Child-Pugh class, total bilirubin level, serum AFP level and tumor size) were related to local tumor progression. Multivariate Cox proportional hazards regression analysis showed that percutaneous RFA, total bilirubin level > 10 ng/L and tumor size > 3 cm were the related risk factors for HCC. The corresponding relative risks were 14.315 (95% CI: 4.857-25.412), 8.124 (95% CI: 2.325-101.587), and 11.741 (95% CI: 3.754-21.665), respectively (Table 4).

DISCUSSION

One important advantage of RFA for liver tumors is micro-invasive when compared with partial hepatectomy^[12,13,22-25]. Some institutes have reported RFA on an out-patients basis^[26]. However, even though the morbidity of malignant seeding in the needle tract is low, it is hard to avoid^[27,28]. Moreover, hemorrhage after the electrode is drawn out appears undetectable in a short time by ultrasonography.

In this study, the hospital stay time of patients with HCC was significantly longer with more analgesics required after surgical RFA than after percutaneous RFA. Surgical RFA seemed more invasive than percutaneous RFA. However, the incidence of more severe complica-

Table 4 Univariate and multivariate analysis of relative risks for local tumor progression

Variable	Univariate analysis	Multivariate analysis	
	P value	Relative risk (95% CI)	P value
Percutaneous vs surgical RFA	0.000	14.315 (4.857-25.412)	0.000
Age (yr) (> 60 vs ≤ 60)	0.402		
HBV- infected (Y vs N)	0.455		
Child-Pugh (B vs A)	0.038		
Albumin (IU/L), ≤ 35 vs > 35	0.233		
Total bilirubin (mg/L), > 10 vs ≤ 10	0.010	8.124 (2.325-101.587)	0.012
Serum AFP (ng/mL), ≥ 400 vs < 400	0.019		
Prothrombin time, ≤ 15' vs > 15'	0.512		
Tumor size (cm), > 3 vs ≤ 3	0.003	11.741 (3.754-21.665)	0.005
Tumor number, multiple vs single	0.111		

RFA: Radiofrequency ablation; HBV: Hepatitis B virus; AFP: α fetoprotein.

tions, such as bile duct injury and procedure-related hemorrhage, was lower in patients after surgical RFA than after percutaneous RFA.

It was reported that a lesion in dangerous locations of the liver is treated with artificial hydrothorax and ascites to achieve percutaneous RFA^[18]. A curative RFA was achieved with artificial hydrothorax in 3 patients in this study. However, artificial ascites was not applied when HCC near extrahepatic organs was treated, because the local ascites was not always capable of dividing a safety zone, the fluidity of liquid made the ascites lack of tension to support a safety zone, membrane adhesions unusually existed between organs and liver, the lesion was often located very close to the surface of the liver when the artificial ascites was needed, and ascites decreased the temperature at the outer part of the lesion when RFA was performed. Thus viable tumor cells could survive.

Compared with surgical RFA, IIOUS of percutaneous RFA is indirect (through abdominal wall), and the choice of route to the lesion is restricted. Injury of important structures, such as bile ducts or extrahepatic organs, should be avoided and RFA should eliminate the viable tumor cells in the assumed area as complete as possible. RFA should be stopped as soon as the ultrasonography shows microbubbles generated by RFA reaching the assumed distal border. In this study, the mean session for each lesion in the dangerous locations was 3.7 ± 2.1 /lesion in percutaneous RFA group and 2.9 ± 2.0 /lesion in surgical RFA group. The patients undergoing percutaneous RFA needed significantly more sessions than those undergoing surgical RFA to ablate a lesion ($P < 0.05$). The effect of percutaneous RFA mainly depends on the experience of operators. On the contrary, surgical RFA may provide a direct ultrasonography monitoring the liver surface, even a visual contact during the procedure. A mobilized liver could offer more choices of route for the electrode and a reliable safety zone in extrahepatic organs or IVC. Thus more attention should be paid to tumor elimination, even the routine impedance-dependent assessment technique can be applied in some surgical RFA procedures and in evaluation by IIOUS, which may be more accurate than that used in percutaneous RFA procedures

and can at least in part explain why more severe local tumor progression was found in patients after percutaneous RFA than after surgical RFA.

This study has the following limitations. First, it was a retrospective study and therefore had inherent defects due to the nature of the method. Second, the rate of censor patients was relatively high in patients undergoing percutaneous and surgical RFA. Third, physicians with diverse experiences might achieve different outcomes of percutaneous RFA. Finally, there was a significant difference in proportion of HBV/HCV-infection and serum AFP level between the patients who underwent surgical or percutaneous RFA.

In conclusion, surgical RFA seems more invasive than percutaneous RFA and the incidence of severe postoperative complications and local tumor progression is lower after surgical RFA than after percutaneous RFA for HCC in dangerous locations.

COMMENTS

Background

The efficacy of radiofrequency ablation (RFA) on hepatocellular carcinoma (HCC) has been debated for a long time. Whether RFA is effective against HCC both in dangerous location and in common location remains controversial.

Research frontiers

The corona of micro-invasion makes lots of colleagues concentrate on percutaneous RFA. Although laparoscopic RFA has been applied recently in some major institutes, laparoscopic ultrasonography needs a long time to be evaluated. Surgical RFA seems more suitable to be employed in most hospitals when difficult circumstances are encountered.

Innovations and breakthroughs

This study was a retrospective study assessing the value of surgical and percutaneous RFA for local or regional HCC in difficult anatomical positions with a large number of patients and a long follow-up time.

Applications

This study may help clinicians to choose RFA when encountering HCC in difficult anatomical positions.

Terminology

Tumor in the dangerous location is defined as a lesion (≤ 0.5 cm in diameter) near large vessels, such as a primary or secondary branch of the portal vein, the base of hepatic veins, or the inferior vena cava (IVC), or a lesion near extrahepatic organs (less than 0.5 cm in diameter) measured on radiological images.

Peer review

This article is a multinational collaborative study, assessing the value of percu-

taneous vs surgical RFA for local or regional HCC in difficult anatomical positions. It is a very interesting and clinically useful study with a large number of patients who were followed up for a long time, thus permitting evaluation of the final outcome of respective treatment modalities.

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S- Editor Sun H L- Editor Wang XL E- Editor Ma WH

Foregut duplication cysts: A report of two cases with emphasis on embryogenesis

Thaer Khoury, Louis Rivera

Thaer Khoury, Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY 14263, United States

Louis Rivera, Surgical Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263, United States

Author contributions: Khoury T and Rivera L contributed equally to this work; Khoury T designed the research and provided discussion of the pathology; Rivera L provided the discussion of the clinical features; both authors contributed equally to the writing of the manuscript.

Correspondence to: Thaer Khoury, MD, Department of Pathology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, United States. thaer.khoury@roswellpark.org
 Telephone: +1-716-8454178 Fax: +1-716-8452370

Received: April 15, 2010 Revised: May 24, 2010

Accepted: May 31, 2010

Published online: January 7, 2011

Abstract

Duplication cyst of the stomach with a pseudostratified columnar ciliated epithelium is extremely rare. We describe two cases of these cysts, with emphasis on their immunophenotype and embryogenesis. The first patient was a 29-year-old man who presented with cramping abdominal pain in his left lower quadrant. The second patient was a 26-year-old woman who had a history, over several years, of chronic epigastric abdominal pain radiating to her back. Both lesions were surgically removed. They showed the same histomorphology. The cysts were lined by a pseudostratified respiratory epithelium with ciliated cells. The first cyst was connected to the stomach, while the second cyst was not connected. Both cysts expressed thyroid transcription factor-1 (TTF-1) and surfactant. In this report, we explore the possible embryogenesis of these lesions in the light of TTF-1 and surfactant expression.

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Key words: Duplication cyst; Stomach; Thyroid transcription factor-1; Surfactant; Embryogenesis

Peer reviewer: Alexander Becker, MD, Department of Surgery, Haemek Medical Center, Afula 18000, Israel

Khoury T, Rivera L. Foregut duplication cysts: A report of two cases with emphasis on embryogenesis. *World J Gastroenterol* 2011; 17(1): 130-134 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i1/130.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.130>

INTRODUCTION

A gastrointestinal duplication is defined as a spherical hollow structure with a smooth muscle coat, lined by a mucous membrane, and attached to any part of the gastrointestinal tract, from the base of the tongue to the anus. However, foregut duplication cyst of the stomach is rare^[1,2]. Foregut duplications may or may not communicate with the gastrointestinal tract, and are usually diagnosed at a young age^[2]. There have been relatively few case reports describing this entity^[3-12]. Adenocarcinoma has been reported in four cases of gastric duplication cyst, but not in cysts that have a ciliated epithelium^[13-16]. Controversy exists concerning the embryological origin of these anomalies^[11,17,18]. Here, we present two cases of gastric ciliated duplication cyst with emphasis on immunophenotype and embryogenesis.

CASE REPORT

Case 1

Case history: The patient was a 29-year-old Caucasian man who presented to his local emergency department for evaluation of acute abdominal pain. The pain started in the lower abdomen and then localized to the left lower quadrant. He had mild nausea, but no vomiting. He had a history of gastroesophageal reflux disease. Physical examination was notable only for left lower quadrant tenderness to palpation. A computed tomography scan (CT-scan) of the abdomen and pelvis was performed and revealed a



Figure 1 A computed tomography scan of the abdomen and pelvis was performed utilizing intravenous and oral contrast. A 4 cm × 5 cm cystic mass (arrow) appears along the greater curvature of the stomach adjacent to the spleen.

mass at the greater curvature of the stomach (Figure 1). An esophagogastroduodenoscopy was performed, revealing a submucosal mass in the fundus of the stomach, approximately 2 cm from the gastroesophageal junction (Figure 2), which was soft on compression. Endoscopic ultrasound revealed a cystic mass in the submucosa of the fundus of the stomach. The imaging and endoscopic findings were most consistent with a gastric duplication cyst. To secure the diagnosis and prevent possible malignant degeneration, the patient was advised to undergo a partial gastrectomy.

Pathological findings: Macroscopically, a sack-like lesion (8.5 cm × 5.5 cm × 4.8 cm) was found with a smooth capsulated wall. The inner wall was also smooth. Microscopically, the cyst was lined by an antrum-type gastric mucosa and a respiratory epithelium with ciliated cells (Figure 3A and B). No intestinal-type epithelium was present. The outside layer consisted of a circular and a longitudinal muscle wall, with a myenteric plexus. The gastric epithelium immunophenotype was cytokeratin 20+, MUC5a/c+, cytokeratin 7+, MUC1-, MUC2-, CDX-2-, TTF-1-, and surfactant-, while the ciliated epithelium had the following immunophenotype: cytokeratin 20-, MUC5a/c-, cytokeratin 7+, MUC1-, MUC2-, CDX-2-, TTF-1+, and surfactant+ (Figure 3C-F).

Case 2

Case history: The patient was a 26-year-old woman with history of chronic epigastric pain for eight years following her first childbirth. The pain was intermittent, and colicky, without identifiable aggravating factors. More recently, the pain had become severe in intensity and required narcotics for relief. The pain was not associated with nausea, vomiting, abdominal distention, fevers, or chills. She admitted to a normal appetite, but had had an unintentional 50-pound weight loss over the last year. She underwent an esophagogastroduodenoscopy, which revealed a normal esophagus, a small hiatal hernia, and diffuse moderate inflammation of the stomach. A CT-scan of the abdomen revealed a cystic mass near the gastroesophageal junction along the lesser curvature of the stomach, which extended to the

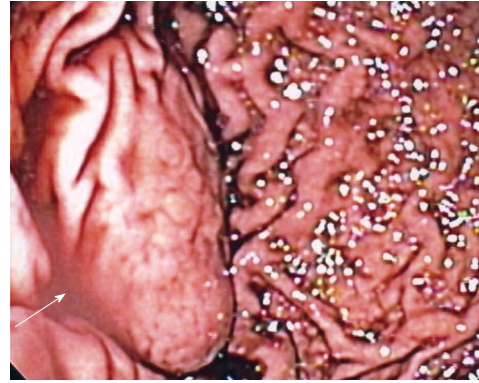


Figure 2 Retroflexed endoscopic view of a gastric mass arising in the fundus (arrow). Overlying rugae are effaced, suggestive of a submucosal process.

superior margin of the pancreas and abutted the liver and inferior vena cava (Figure 4). The mass was nonenhancing and cystic in appearance. The primary suspicion was that this represented a congenital gastrointestinal duplication cyst. A partial gastrectomy was performed for further evaluation and treatment.

Pathological findings: Macroscopically, the cyst was an ovoid-shaped structure (5 cm × 2.2 cm × 2 cm). The external surface was covered with tan, shiny serosa. The cystic cavity was filled with a tan-white mucoid material. The cyst inner surface was unremarkable, tan, and smooth. Microscopically, the cyst was lined by a respiratory epithelium with ciliated cells (Figure 5A and B). No intestinal-type epithelium was present. The outside layer consisted of a circular and a longitudinal muscle wall, with a myenteric plexus. The epithelium had the following immunophenotype: cytokeratin 20-, MUC5a/c-, cytokeratin 7+, MUC1-, MUC2-, CDX-2, TTF-1+, and surfactant+ (Figure 5C and D).

DISCUSSION

Gastrointestinal duplication can occur in any region of the gastrointestinal tract; however, a foregut duplication cyst of the stomach is rare. The stomach is the site of only 24% of all alimentary tract duplication cysts, which is the least common site after the ileum, esophagus, and colon. Of reported gastric duplication cysts, most occur in females, with 80% presenting in patients younger than 12 years of age. They are characteristically located on the distal greater curvature, and the majority are cystic and non-communicating. Histologically, they are usually lined with a typical gastric mucosa^[15]. As in the present two cases, a ciliated pseudostratified epithelium has been reported^[3-12].

We present two foregut duplication cysts of the stomach, one connected and one separated, in a 29-year old woman and a 26-year old man, respectively. These two cases showed distinct morphological and immunoprofile findings. Both cysts were lined with pseudostratified ciliated epithelia, and expressed TTF-1 and surfactant.

The pathogenesis of alimentary tract duplications is

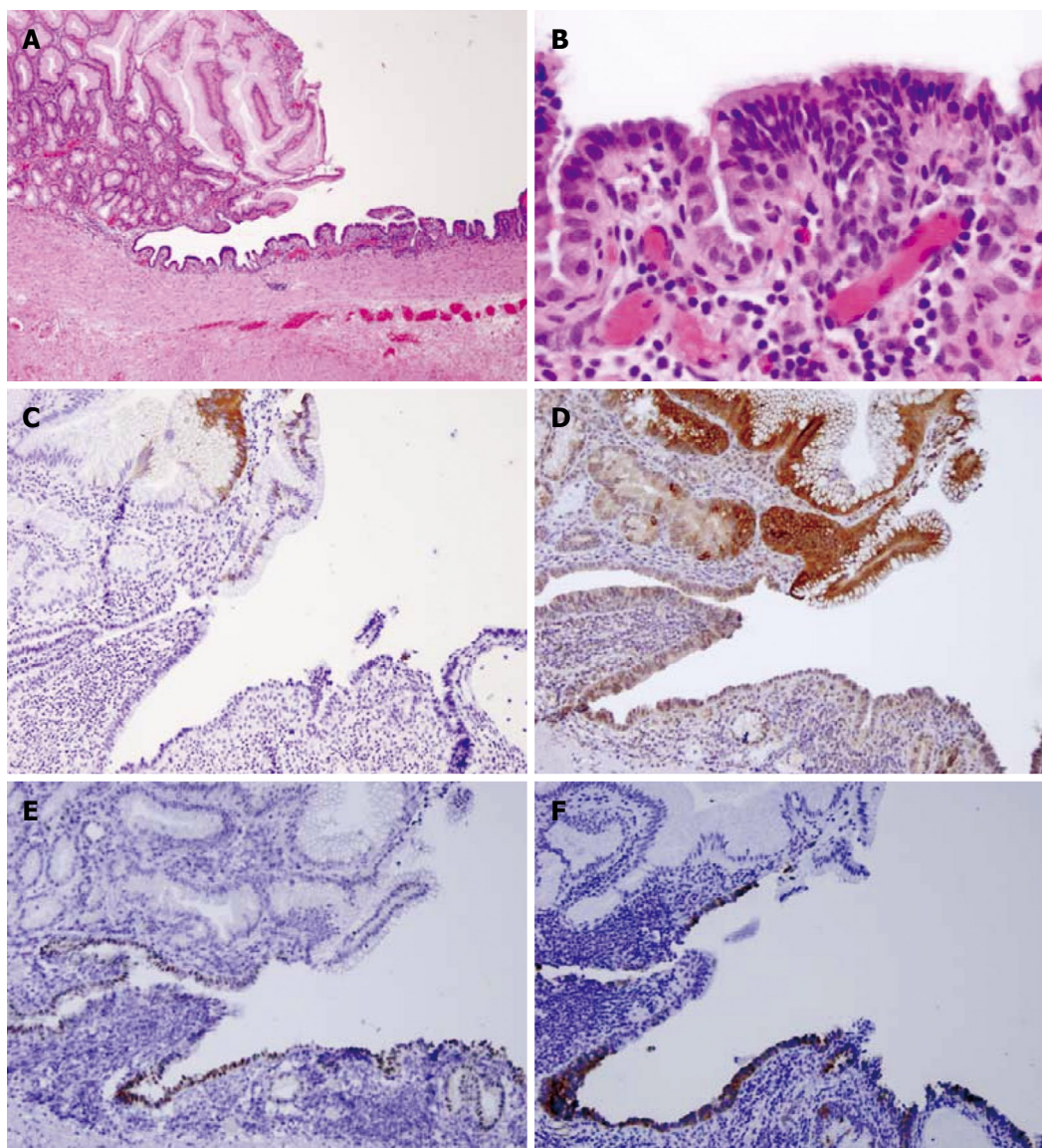


Figure 3 Case 1. A: Hematoxylin and eosin stain of gastric/ciliated epithelium (4 ×); B: High power view of the ciliated epithelium (40 ×); C: CK20 showing staining in the surface gastric epithelium, but not in the ciliated epithelium (10 ×); D: MUC5a/c staining showing positive expression in the gastric epithelium but not in the ciliated epithelium (10 ×); E: Thyroid transcription factor-1 nuclear staining in the ciliated epithelium only (10 ×); F: Surfactant staining in the ciliated epithelium only (10 ×).



Figure 4 Case 2. Computed tomography-scan of the abdomen and pelvis with intravenous and oral contrast showing a non-enhancing cystic structure (arrow) abutting the lesser curvature of the stomach and liver along the ligamentum venosum.

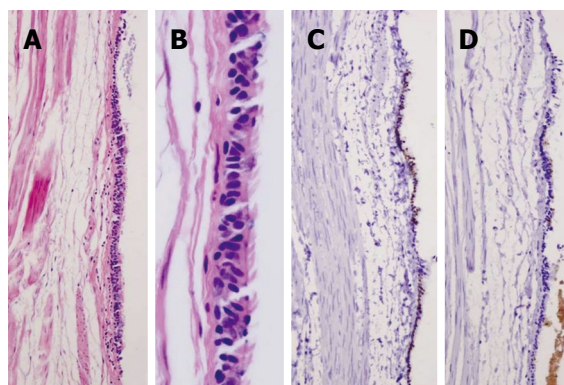


Figure 5 Case 2. A, B: Hematoxylin and eosin staining of pseudostratified ciliated epithelium (A: 4 ×, B: 40 ×); C: Thyroid transcription factor-1 nuclear staining in the ciliated epithelium (10 ×); D: Surfactant staining in the ciliated epithelium (10 ×).

controversial. There has been no suggestion of an explanation based on embryological development^[17]. However, others have suggested theory of embryological origin from supernumerary foregut buds^[11]. Others have proposed that abnormal recanalization after the solid epithelial stage of embryonic bowel development is the underlying cause of these lesions^[18].

We found that these cysts are lined with a ciliated pseudostratified epithelium and express TTF-1 and surfactant. These two factors are involved in lung embryogenesis. These findings might partially explain the embryogenesis of these lesions. When the embryo is approximately four weeks old, the respiratory diverticulum appears along the ventral wall of the pharyngeal gut. The esophagotracheal septum gradually partitions this diverticulum from the dorsal part of the foregut. In this manner, the foregut divides into the ventral portion, the respiratory primordium, and a dorsal portion, which becomes the esophagus and stomach^[19]. The middle branch of the tracheal trifurcation is grossly and histologically identical to the other two branches of the trifurcation, including expression of TTF1, which become the lungs^[20]. Early in the development of the esophagus, it is lined by a pseudostratified columnar epithelium. This is a characteristic of the respiratory epithelium, but the mucosa of the esophagus apparently undergoes subsequent metaplasia and squamous differentiation^[1,8]. Based on the presence of the ciliated epithelium, we propose that a branch coming off the trifurcation gives rise to the duplication cyst. Later, the cyst either remains connected to the esophagus/stomach or becomes separated.

TTF-1 is a homeobox transcription factor whose expression in the developing foregut is specifically limited to the respiratory tract^[21-23]. It is particularly important for normal lung development^[24-28]. TTF-1 enhances branching morphogenesis of embryonic lung explants *in vitro*^[29]. A *TTF-1* knockout mouse developed no distal lung parenchyma^[30]. Furthermore, TTF-1 is thought to be important for terminal respiratory epithelial cytodifferentiation. It has the capacity to regulate expression of surfactant proteins, A, B, and C, as well as the Clara cell secretory protein, all markers of respiratory cell specific differentiation^[25,26,28,31-33]. Thus, the presence of TTF-1 and surfactant expression is a function of lung differentiation. The presence of these two factors in the described cysts might explain why these cysts maintain their phenotype of a respiratory epithelium with no squamous metaplasia. As with tracheo-esophageal fistulae^[20], TTF-1 might have lost its normal patterning role in the developing lung, preventing induction of branching morphogenesis in the duplication cyst. The nonbranching pattern of growth of the duplication cyst might be attributable to local mesenchymal-epithelial interactions that override TTF-1 patterning activity.

In conclusion, gastric duplication cysts are rare, and their origin remains uncertain. The two cases presented here, and the resulting histological findings, suggest a novel origin. In both cases, the cysts are lined with a pseudostratified respiratory epithelium with ciliated cells, which express TTF-1 and surfactant. This suggests an origin from

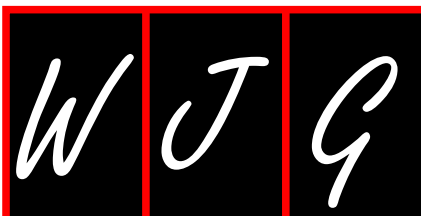
the respiratory diverticulum, which arises from the ventral foregut and could also explain why these cysts do or do not maintain their connection to the gastrointestinal tract.

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S- Editor Wang JL L- Editor Stewart GJ E- Editor Ma WH



Duplicated appendix complicated by appendiceal cancer

Hugh J Freeman

Hugh J Freeman, Department of Medicine (Gastroenterology), University of British Columbia Hospital, Vancouver, BC, V6T 1W5, Canada

Author contributions: Freeman HJ contributed to all of this work.

Correspondence to: Hugh J Freeman, MD, Professor, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver, BC, V6T 1W5, Canada. hugfree@shaw.ca

Telephone: +1-604-8227216 Fax: +1-604-8227236

Received: October 15, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: January 7, 2011

Abstract

A 37-year old male presented with an acute abdomen suggestive of an appendiceal perforation. Urgent laparotomy showed a duplicated appendix with one of the lumens involved with appendicitis and a focal peri-appendicular abscess while the other lumen had a localized appendiceal cancer. Recognition of congenital intestinal duplications in adults is important to avoid serious clinical consequences.

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Key words: Duplicated appendix; Bifid appendix; Appendiceal cancer; Congenital duplication.

Peer reviewer: Dr. Benjamin Perakath, Professor, Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India

Freeman HJ. Duplicated appendix complicated by appendiceal cancer. *World J Gastroenterol* 2011; 17(1): 135-136 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i1/135.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i1.135>

TO THE EDITOR

An article recently published in the *World Journal of Gas-*

troenterology concerned a duplicated vermiform appendix in an adult^[1]. The authors emphasized the need for surgical intervention to avoid any future complication, in particular, the disconcerting possibility of malignant degeneration within the duplication^[2,3]. Duplication of the appendix, fortunately, is distinctly rare, reportedly occurring in only 1/25 000 patients (0.04%) operated on for acute appendicitis^[4]. Clinical features may depend on the type of appendiceal duplication as a number of variants have been described, specifically, type A with incomplete duplication and a common base (so-called bifid appendix), and types B or C, both forms having complete duplication with independent bases^[5,6].

We had a similar, but unusual experience in a 37-year old adult male in August 1996. He presented with an acute abdomen thought to be a perforated appendix. An urgent laparotomy showed a type A duplicated or bifid appendix with an acute appendicitis involving only one lumen associated with a focal periappendiceal abscess and localized peritonitis. The other lumen showed a well-differentiated carcinoma extending into, but not through the muscularis propria. No vascular or lymphatic invasion was identified. Imaging studies and carcinoembryonic antigen testing were negative. Two weeks later, a further ileocecal resection was done. The resected intestine and 17 mesenteric lymph nodes showed no malignancy. Family history revealed that his father had synchronous mucinous colon cancers at the age of 45 years and his brother was diagnosed with colon cancer at the age of 26 years. A paternal uncle died of colon cancer at the age of 35 years. No other congenital abnormalities were recognized and there was no known contact with a toxic or noxious agent *in utero*. Colonoscopy screening at the ages of 20, 24 and 30 years was completed in other centers because of his family history of colon cancer and no abnormalities were detected. A colonoscopy in November 1996 in our hospital revealed no other abnormalities, similar to colonoscopies in 2000, 2005 and 2010. Because of his family history, genetic evaluation to exclude a HNPCC was negative including gene testing for MLH1 and MSH2.

Carcinoma in the intestinal tract developing with co-existent duplication has been rarely reported and appears

limited to only a small number of cases^[3,7]. Appendiceal cancer has been associated with a duplication thought to represent a Meckel's diverticulum^[8] as well as in a single prior report of a duplicated appendix^[9]. While this presentation may also mimic colon cancer^[10], missing a second duplicated appendix in this setting could lead to serious clinical consequences. In the present case, appendiceal duplication was fortunately recognized and an early stage carcinoma was successfully resected.

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S- Editor Tian L L- Editor Wang XL E- Editor Ma WH



ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Philip Abraham, Dr., Professor, Consultant Gastroenterologist and Hepatologist, P. D. Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma - Sapienza, Viale del Policlinico 155, Roma 00161, Italy

John Beynon, BSc, MB BS, MS, FRCS (ENG.), Consultant Colorectal Surgeon, Singleton Hospital, Sketty Lane, Swansea, SA2 8QA, United Kingdom

Erwin Biecker, MD, PhD, Department of Gastroenterology and Hepatology, Helios Klinikum Siegburg, Siegburg 53343, Germany

Mauro Bortolotti, MD, Professor, Department of Internal Medicine and Gastroenterology, University of Bologna, Via Massarenti 48 Bologna, 40138, Italy

Lisardo Bosca, Dr., Professor, Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain

Luis Bujanda, PhD, Professor, Departament of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Giuseppe Currò, MD, University of Messina, Via Panoramica, 30/A, 98168 Messina, Italy

Uday C Ghoshal, Dr., MD, DNB, DM, FACC, Additional Professor, Department of Gastroenterology, Sanjay Gandhi Postgraduate

Institute of Medical Science, Lucknow 226014, India

Maria Concepción Gutiérrez-Ruiz, PhD, Department of Health Sciences, Universidad Autónoma Metropolitana-Iztapalapa, DCBS, Av San Rafael Atlixco 186, Colonia Vicentina, México, DF 09340, México

Serdar Karakose, Dr., Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Sang Geon Kim, Dr., PhD, MS, BS, Professor, Chairman, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, South Korea

Marshall John Marshall, MD, Professor of Medicine, Division of Gastroenterology, University of Missouri School of Medicine, Columbia, MO 65201, United States

Seung Woon Paik, MD, PhD, Professor, Division of Gastroenterology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Mihaela Petrova, Dr., MD, PhD, Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia 1606, Bulgaria

Jorgen Rask-Madsen, MD, FRCP, Professor of Gastroenterology, Department of Gastroenterology, Herlev Hospital, Skodsborg Strandvej 280A, 2942, Herlev, DK-2730, Denmark

Ekihiro Seki, MD, PhD, Department of Medicine, University of California San Diego, Leichag Biomedical Research Building Rm 349H, 9500 Gilman Drive MC#0702, La Jolla, CA 92093-0702, United States

Hiroyuki Uehara, MD, PhD, Chief, Division of Pancreatology, Department of Gastroenterology, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari, Osaka 537-8511, Japan

Julian RF Walters, Dr., PhD, MD, BSc, MBBS, Department of Gastroenterology, Imperial College London, Hammersmith Hospital, Du Cane Road, London, W12 0HS, United Kingdom



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 04-05, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 07-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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Name of journal

World Journal of Gastroenterology

CSSN

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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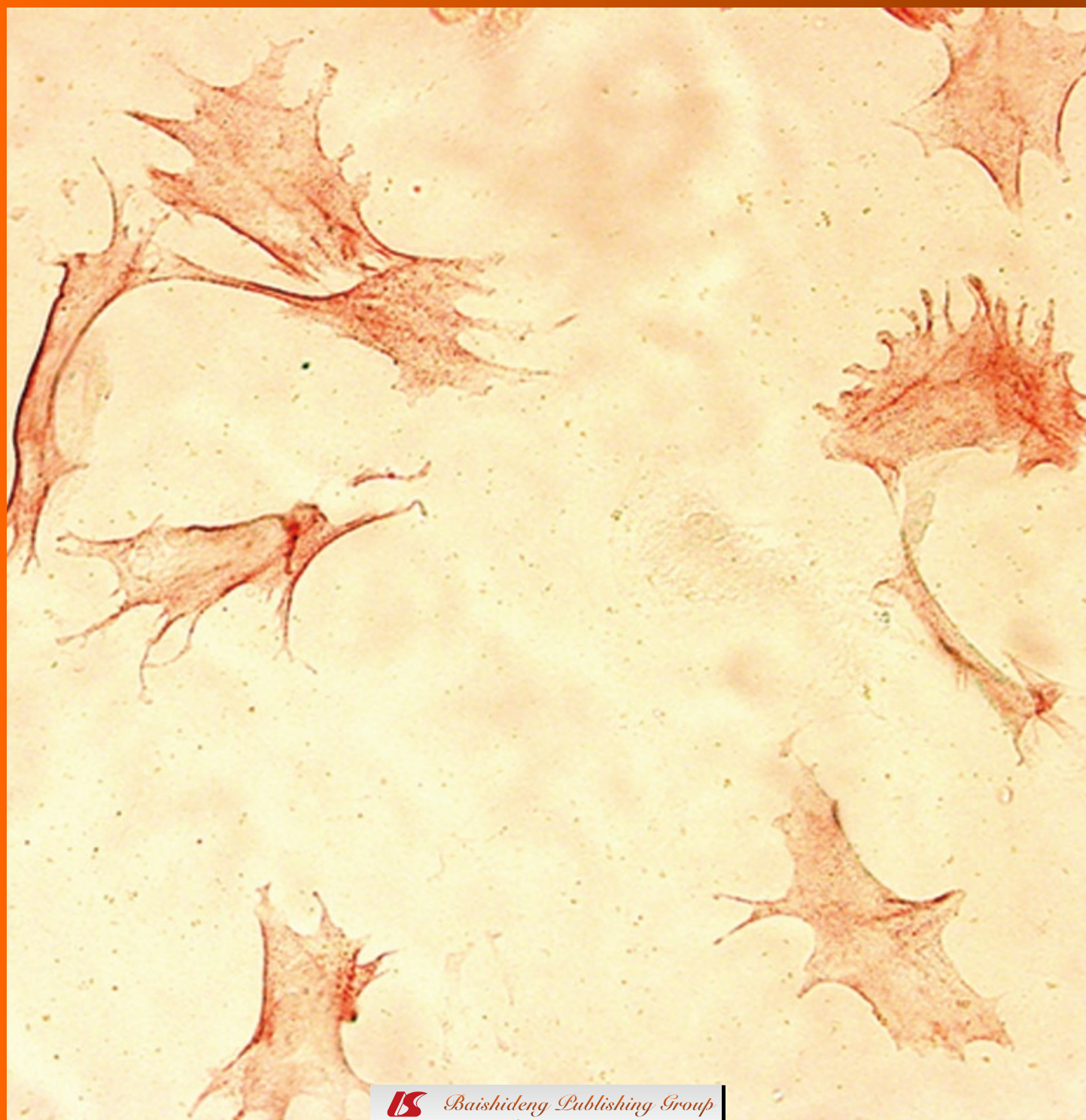
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Switching-on of serotonergic calcium signaling in activated hepatic stellate cells.
World J Gastroenterol 2011; 17(2): 164-173
<http://www.wjgnet.com/1007-9327/full/v17/i2/164.htm>

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NAME OF JOURNAL
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LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
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EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
January 14, 2011

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

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Pathologic pancreatic endocrine cell hyperplasia

Debra Ouyang, Deepti Dhall, Run Yu

Debra Ouyang, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Deepti Dhall, Department of Pathology, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Run Yu, Division of Endocrinology and Carcinoid and Neuroendocrine Tumor Center, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Author contributions: All authors contributed equally to this paper.

Correspondence to: Run Yu, MD, PhD, Division of Endocrinology and Carcinoid and Neuroendocrine Tumor Center, Cedars-Sinai Medical Center, B-131, 8700 Beverly Blvd, Los Angeles, CA 90048, United States. run.yu@cshs.org

Telephone: +1-310-4234774 Fax: +1-310-4230440

Received: October 8, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: January 14, 2011

likely yield insights into the pathogenesis and treatment of diabetes and pancreatic neuroendocrine tumors.

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Key words: Glucagon receptor; Hyperplasia; Nesidioblastosis; Islet; Pancreatic endocrine cell; Neuroendocrine tumor

Peer reviewer: Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma - Sapienza, Viale del Policlinico 155, Rome 00161, Italy

Ouyang D, Dhall D, Yu R. Pathologic pancreatic endocrine cell hyperplasia. *World J Gastroenterol* 2011; 17(2): 137-143
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/137.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.137>

Abstract

Pathologic hyperplasia of various pancreatic endocrine cells is rare but has been long known. β cell hyperplasia contributes to persistent hyperinsulinemic hypoglycemia of infancy, which is commonly caused by mutations in the islet ATP-sensitive potassium channel, and to non-insulinoma pancreatogenous hypoglycemia in adults, which may or may not be associated with bariatric surgery. α cell hyperplasia may cause glucagonoma syndrome or induce pancreatic neuroendocrine tumors. An inactivating mutation of the glucagon receptor causes α cell hyperplasia and asymptomatic hyperglucagonemia. Pancreatic polypeptide cell hyperplasia has been described without a clearly-characterized clinical syndrome and hyperplasia of other endocrine cells inside the pancreas has not been reported to our knowledge. Based on morphological evidence, the main pathogenetic mechanism for pancreatic endocrine cell hyperplasia is increased endocrine cell neogenesis from exocrine ductal epithelium. Pancreatic endocrine cell hyperplasia should be considered in the diagnosis and management of hypoglycemia, elevated islet hormone levels, and pancreatic neuroendocrine tumors. Further studies of pathologic pancreatic endocrine cell hyperplasia will

INTRODUCTION

The pancreas, a key regulator of nutrient digestion, absorption, and utilization, can be divided into two major components, the endocrine and exocrine pancreas. The endocrine pancreas consists of five distinct cell types, α , β , δ , ϵ , and pancreatic polypeptide (PP) cells, that produce glucagon, insulin, somatostatin, ghrelin, and PP, respectively^[1-3]. The pancreatic endocrine cells may give rise to distinct neuroendocrine tumors such as insulinoma, gastrinoma, glucagonoma, VIPoma, and non-functioning tumors^[4-6]. In contrast, pancreatic endocrine cell hyperplasia as a group of diseases is a relatively unexplored area. From the 1960s to the present day, there have been various reports regarding pancreatic endocrine cell hyperplasia. Much of the literature has focused on β cell hyperplasia in particular, but hyperplasia of other pancreatic endocrine cells has also been described, some in great detail. In this review we summarize the body of literature on pathologic pancreatic endocrine cell hyperplasia.

Hyperplasia refers to an increased number of a certain type of cells in a given organ or tissue than is ordinarily observed. Mechanisms regulating pancreatic endocrine cell

number include proliferation (division of existing cells), apoptosis (controlled cell death), and neogenesis (differentiation of endocrine cells from the exocrine epithelium), and abnormalities in each could result in hyperplasia^[7-9]. The diagnostic criteria of pancreatic endocrine cell hyperplasia are not universally agreed upon. Rindi *et al.*^[10] defines pancreatic endocrine cell hyperplasia as an expansion of the endocrine cell mass to more than 2% (in adults) or 10% (in infants) of the total pancreas mass. As it is impractical to do detailed pancreatic morphometry in a clinical specimen, the diagnosis of pancreatic endocrine cell hyperplasia is often subjective. Most would regard an islet size large than 250 μm in diameter and an increase in islet numbers as evidence of pancreatic endocrine cell hyperplasia^[11-14].

Pancreatic endocrine cell hyperplasia can be non-specific and involve most or all types of islet cells or specific and involve predominantly one cell type. Non-specific, focal endocrine hyperplasia and microadenoma are not uncommon incidental pathological findings in the pancreas; if carefully screened, up to 10% of adults harbor these lesions at autopsy^[15]. In those patients, all types of pancreatic endocrine cells could be focally hyperplastic. Most of those lesions probably do not indicate clinical significance. Diffuse pancreatic endocrine cell hyperplasia and microadenoma are a feature of multiple endocrine neoplasia type 1 (MEN1), and to a less extent, von Hippel-Lindau (VHL) disease^[16-19]. All types of endocrine cells can be hyperplastic, but β and α cells are more often so, probably because these cells are normally more numerous than other types. In this article, we will focus on diffuse and specific pancreatic endocrine cell hyperplasia as a group of diseases. We define it pathologically as an overwhelming increase in islet size and/or number in all the pancreatic sections examined so that it is reasonable to assume that the remaining pancreas or unexamined pancreas blocks should exhibit similar changes. Moreover, the hyperplastic endocrine cells should be mainly limited to one type of islet cells which have apparently similar cell lineage supported by consistent hormone production profile and other cellular markers. Finally we will only discuss the literature on pathologic pancreatic endocrine cell hyperplasia in humans.

β CELL HYPERPLASIA

The pancreatic β cells are the only source of insulin, the hormone that decreases blood glucose levels by increasing glucose uptake and decreasing hepatic glucose output. Physiological hyperplasia of β cells is commonly seen in patients with insulin resistance and early-stage type 2 diabetes, and is intensely studied for diabetes treatment^[20,21]. Except for postprandial hypoglycemia, we are not aware of any reports that physiological β cell hyperplasia causes clinical syndromes^[22,23]. The physiological β cell hyperplasia appears to be tightly regulated and has not been reported to give rise to pancreatic neuroendocrine tumors. We thus consider it part of insulin resistance syndromes and will not address it further in this article.

Admittedly, pathologic β cell hyperplasia is a controversial term in both infants and adults in the absence of endocrine tumor syndromes. Although β cell hyperplasia has been recorded in non-insulinoma hyperinsulinemic hypoglycemia, it is rather moderate in most cases and may be even non-existent in some cases^[24]. “Nesidioblastosis” has been used by some authors to denote the same pathologic changes^[25-28]. The term nesidioblastosis was coined in the first half of the 20th century initially to describe islet neogenesis from pancreatic ductal epithelium in neonates with hyperinsulinemic hypoglycemia^[29]. β cell hyperplasia and hypertrophy often accompany nesidioblastosis^[24,30-32]. Although islet neogenesis is also observed in normal infants, by the 1970s, nesidioblastosis was used to describe all forms of persistent congenital hyperinsulinism in infants, whether the hyperinsulinemic states were associated with hyperplasia or not^[25,26]. In recent years, nesidioblastosis has also been used to describe acquired hyperinsulinism with β cell hyperplasia in adults^[27,28]. As the use of this term is not consistent, we use nesidioblastosis strictly as a morphological term in describing any endocrine cells (not limited to β cells) budding from the ductal epithelium and use “persistent hyperinsulinemic hypoglycemia of infancy (PHHI)” to describe the various forms of similar such diseases in neonates or infants and “non-insulinoma pancreatogenous hypoglycemia (NIPH)” to describe hypoglycemia syndromes without evidence of insulinoma in adults^[24,30-41]. Both PHHI and NIPH are associated with pathologic β cell hyperplasia in most cases.

Clinically characterized by hyperinsulinemic hypoglycemia in infants and neonates, PHHI is not a single disease entity but a group of related diseases^[33-35]. In the majority of patients, insulinoma is not identified but the pancreas exhibits focal or diffuse β cell abnormalities commonly associated with genetic mutations affecting β cells^[35,42]. In about a third of cases, focal β cell hypertrophy and hyperplasia is observed^[33,34]. The endocrine cells are arranged in huge islet-like structures separated by acinar cells or connective tissue, and some harbor large nuclei. The endocrine cell proliferation rate is generally increased. All types of endocrine cells are represented in the islet-like structures with normal spatial distribution. The percentage of β cells is higher (70%-90%) than normal (50%). The diffuse form is found in about two-thirds of cases. Although the β cell mass is only mildly increased compared with normal control, islet size varies and some islets are very large while others are poorly defined and irregularly shaped small endocrine cell clusters. As in the focal form, all types of endocrine cells are represented in the islet-like structures with normal spatial distribution, and some endocrine cells have large hyperchromatic nuclei. The endocrine cell proliferation rate, however, is not increased in the diffuse form^[43]. Clinically it is important to differentiate the focal from the diffuse form as partial pancreatectomy is sufficient for the former while near-total or total pancreatectomy is required for the latter. There is a correlation between the underlying genetic abnormalities and the pancreas pathology. The focal hypertrophy and

Table 1 Summary of 9 cases of α cell hyperplasia

Study	Toda <i>et al.</i> ^[49]	Brown <i>et al.</i> ^[50]	Martignoni <i>et al.</i> ^[51]	Chen <i>et al.</i> ^[52]	Yu <i>et al.</i> ^[53]	Henopp <i>et al.</i> ^[54]
Location	Japan	USA	Germany	Taiwan	USA	Germany
Ethnicity	Japanese	ND	ND	Chinese	Persian	ND
Age (yr)	74	48	54	45	60	25-44
Sex	F	M	M	M	F	2F/2M
Clinical	Diabetes	Diabetes	Mild diabetes	Mild diabetes	Nonspecific	Various
Glucagon (pg/mL) Pre-op	ND	4200	Elevated	ND	59284	4-25-fold
Glucagon (pg/mL) Post-op	ND	5700	ND	ND	Elevated	ND
Imaging	Negative	Mass in body	Negative	Diffusely enlarged	Mass on uncinata	ND
Octreotide scan	ND	ND	Negative	ND	Negative	ND
Pathology	Numerous micro-glucagonoma	Glucagonoma, α cell hyperplasia	Glucagonoma, α cell hyperplasia	α cell hyperplasia	NF-PNET, α cell hyperplasia	α cell hyperplasia

ND: Not described; NF-PNET: Non-functioning pancreatic neuroendocrine tumor.

hyperplasia is associated with a paternally inherited ATP-sensitive potassium channel defect with loss of maternal heterozygosity on the 11p chromosome, and is thus sporadic^[35,42]. The diffuse form is most commonly associated with traditional mutations leading to defects in the same potassium channel, and can be either sporadic or familial. It is still unclear how the genetic abnormalities lead to the unique islet structure and β cell morphology.

In adults, NIPH is a rare cause of hyperinsulinemic hypoglycemia^[36-41]. NIPH is characterized by mostly postprandial hypoglycemia rather than fasting hypoglycemia which is usually seen in patients with insulinoma^[38-40]. This syndrome is distinguished from reactive hypoglycemia in that in some patients, the development of severe neuroglycopenic symptoms including diplopia, dysarthria, confusion, disorientation, and even convulsions and coma, may occur in addition to adrenergic symptoms that predominate in reactive hypoglycemia^[22,23,38-40]. This syndrome is also distinguished from PHHI in that these patients do not have mutations of the KIR6.2 (KCNJ11) and SUR1 (ABCC8) genes, which encode the subunits of the pancreatic ATP-sensitive potassium channel^[38]. Evidence of β cell hyperplasia is present in every patient studied^[36-41]. The islets exhibit normal structure but diffusely are more numerous and larger than those in normal control, and nesidioblastosis is pervasive. All types of islet cells are normally distributed throughout the islets but the predominant cell type is the β cell. Partial pancreatectomy often resolves hypoglycemia^[36-41]. The association of NIPH and bariatric surgery is controversial. Hypoglycemia after bariatric surgery is common and is mostly caused by dumping syndrome^[44,45]. In a minority of patients, hypoglycemia is associated with hyperinsulinemia and can only be controlled by partial pancreatectomy^[44,46]. It was initially reported that the endocrine pancreas essentially exhibits the same changes as described above for patients without a history of obesity and bariatric surgery^[44,46]; later studies, however, failed to demonstrate β cell hyperplasia when obese patients without bariatric surgery were used as controls^[47]. Thus bariatric surgery may not by itself cause β cell hyperplasia.

In summary, pathologic β cell hyperplasia causes

hypoglycemia and requires pancreatectomy in most patients. Pancreatic neuroendocrine tumor pathogenesis is a concern but has not been observed clinically. Increased neogenesis (morphologically as nesidioblastosis) appears to be the main mechanism responsible for β cell hyperplasia. The etiology of β cell hyperplasia commonly is mutations in the ATP-sensitive potassium channel in infants; in adults, it is still unknown but is probably related to unknown genetic changes. Further study of β cell hyperplasia will undoubtedly provide insights into fundamental β cell biology and suggest novel therapies for diabetes.

α CELL HYPERPLASIA

Although much research has focused on β cell hyperplasia, α cell hyperplasia also occurs and recent studies have shed light on its mechanism. The α cells are the second most common cells in an islet^[1-3]. The first case of diffuse α cell hyperplasia and hyperglucagonemia was published more than 40 years ago in a 69-year-old man with hyperparathyroidism and calcific pancreatitis^[48]. The cause of α cell hyperplasia and hyperglucagonemia in that case was not clear but the pancreatitis may have contributed to α cell hyperplasia or the patient may have had multiple endocrine neoplasia type 1 which encompasses primary hyperparathyroidism and pancreatic endocrine cell hyperplasia^[16,17]. To our knowledge, nine more cases of α cell hyperplasia have been reported afterwards (Table 1)^[49-54]. Glucagon levels were elevated in all cases where they were measured. The ethnicity of patients included Asian, Iranian, or presumably Caucasians. Both female and male patients were affected and all patients were adults aged from 25 to 74; none had a family history of endocrine tumors. Only one patient presented with typical glucagonoma syndrome in the form of necrolytic migratory erythema, deep vein thrombosis, and weight loss, and the other eight patients presented with non-specific symptoms in whom hyperglucagonemia was identified during the work-up for possible pancreatic neuroendocrine tumors.

Regardless whether the α cell hyperplasia is associated with glucagonoma syndrome, its morphology is remarkably similar in all cases^[49-54]. We have performed detailed

Table 2 Summary of 8 cases of pancreatic polypeptide cell hyperplasia

Study	Tomita <i>et al</i> ^[60]	Farley <i>et al</i> ^[61]	Martella <i>et al</i> ^[62]	Pasieka <i>et al</i> ^[63]	Albazzaz <i>et al</i> ^[64]	Bunning <i>et al</i> ^[65]
Location	USA	USA	Italy	Canada	UK	USA
Ethnicity	ND	ND	ND	ND	ND	ND
Age (yr)	70	66	50-70	37	76	71
Sex	F	M	3F	F	M	M
Clinical	Diarrhea	Diarrhea	ZES	Diarrhea	Bowel obstruction	Nausea ZES
PP (pg/mL) Pre-op	ND	Highly elevated	About 3 fold	ND	ND	ND
PP (pg/mL) Post-op	ND	ND	ND	ND	ND	ND
Imaging	Mass in pancreas head	Mass in pancreas head	Nonspecific	Normal	Mass in pancreas head	Normal
Octreotide scan	ND	ND	ND	ND	ND	Uptake in pancreas head
Pathology	PP cell hyperplasia	PP cell hyperplasia	PP cell hyperplasia	PP cell hyperplasia	PP cell hyperplasia	PP cell hyperplasia

ND: Not described; ZES: Zollinger-Ellison syndrome; PP: Pancreatic polypeptide.

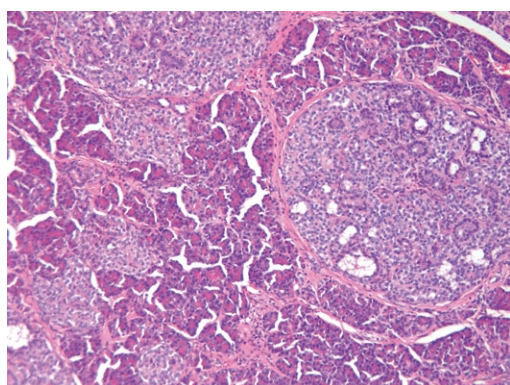


Figure 1 α cell hyperplasia of a patient with homozygous inactivating mutation of the glucagon receptor. Note the large islets with nesidioblastosis. Most of the islets are positive for glucagon but negative for insulin. 100 \times .

histological studies on the α cell hyperplasia of our patient (Figure 1)^[53]. In this patient's pancreas, hyperplastic islets were innumerable and classic nesidioblastosis was commonly seen. Most of these hyperplastic islets (60%-80%) contain endocrine cells positive for glucagon but negative for insulin, but smaller, normal-looking islets exhibit normal insulin and glucagon hormonal expression. Accompanying the α cell hyperplasia, there are a 4-cm non-functioning pancreatic neuroendocrine tumor and multiple microadenomas. Henopp *et al*^[54] further note that it is difficult to distinguish microglucagonomas from hyperplastic islets in α cell hyperplasia, and in some very large islets (> 300-500 μ m), there appears to be an imperceptible transition from α cell hyperplasia to microglucagonoma. Thus the morphological studies suggest that α cell hyperplasia gives rise to glucagonoma and other pancreatic endocrine tumors.

The pathogenesis of α cell hyperplasia has been elucidated in our patient^[55]. As the patient has extremely elevated glucagon levels but without glucagonoma syndrome, which resembles the phenotype of mice without a glucagon receptor^[56,57], we sequenced the patient's glucagon receptor gene and identified a novel homozygous inactivating P86S mutation^[55]. When tested *in vitro*, the P86S mutant glucagon receptor exhibits partial cytoplasmic

localization and decreased glucagon binding. Compared with the wild-type glucagon receptor, the P86S mutant produces less cAMP under physiological concentrations of glucagon. The hyperplastic α cells in our patient also produce glucagon-like peptide 1 and PP, suggesting immature, more embryonic traits. We believe that our patient has a novel disease which we term "Mahvash disease" because it has a distinct etiology (inactivating glucagon receptor mutation), pathology (α cell hyperplasia), and clinical syndrome (hyperglucagonemia and pancreatic neuroendocrine tumors).

Thus clinically, there appear to be at least two types of α cell hyperplasia, functional and reactive. Functional α cell hyperplasia is analogous to adult β cell hyperplasia (which produces non-insulinoma pancreatogenous hyperinsulinemic hypoglycemia, NIPH) and produces non-glucagonoma hyperglucagonemic glucagonoma syndrome. Partial or total pancreatectomy may be a logical treatment. Currently only one case of functional α cell hyperplasia is known^[54]. Our case and possibly a few others represent reactive α cell hyperplasia (equivalent to Mahvash disease) which produces hyperglucagonemia as a result of inactivated glucagon signaling and consequently does not cause glucagonoma syndrome^[49-54]. The clinical significance of reactive α cell hyperplasia is pancreatic neuroendocrine tumors so that clinical, laboratory, and imaging surveillance are required to identify those tumors early. Once identified, these tumors should be treated as a regular pancreatic neuroendocrine tumor.

PP CELL HYPERPLASIA

PP-producing cells represent about 10% of endocrine cells in an islet^[1-3]. The PP cells often take up a peripheral position, mixed with α and δ cells. The physiologic effects of PP are not very clear but include inhibiting gallbladder contraction and pancreatic enzyme secretion and decreasing appetite and food intake^[58,59]. PP cell hyperplasia was first described in 1980 and a total of eight cases of diffuse PP cell hyperplasia have been reported to our knowledge (Table 2)^[60-65]. As in α cell hyperplasia, both sexes were affected and patients were aged from 37 to 76 years; all

patients were without a family history of endocrine tumors. Watery diarrhea was a common symptom and four of the eight patients had simultaneous or a history of gastrinoma and Zollinger-Ellison syndrome. The clinical significance of PP cell hyperplasia and elevated PP levels remain relatively unknown. It is not clear if PP cell hyperplasia indeed causes elevated PP levels. First of all, there does not appear to be a direct relationship between the number of PP cells in the pancreas and the serum levels of circulating PP^[66]. In addition, PP levels were unknown in most patients and were measured in only two of the eight patients; in one of these two patients, the PP levels were comparable to those in asymptomatic patients with pancreatic neuroendocrine tumors. Lastly, the association of PP cell hyperplasia and gastrinoma may confound the clinical presentations of PP cell hyperplasia as diarrhea is a common symptom of gastrinoma. Thus far, a clinical syndrome of elevated PP levels cannot be established and it is not clear whether PP cell hyperplasia causes watery diarrhea. PP cell hyperplasia has also been found to be positive on somatostatin receptor scintigraphy, an imaging modality used to visualize neuroendocrine tumors^[65].

The histology of PP cell hyperplasia is very similar to that of α or β cell hyperplasia but the hyperplastic cells are PP cells^[60-65]. There are numerous PP cell clusters, some of which are very large and dysplastic. The PP cells are the predominant cells found inside or outside the islets. Extensive PP cell neogenesis is inferred from the evident nesidioblastosis. While the association with gastrinoma suggests that it may be secondary to gastrinoma, the etiology of PP cell hyperplasia remains elusive.

HYPERPLASIA OF OTHER PANCREATIC ENDOCRINE CELLS

Somatostatin is secreted by the δ cells of the islets. Islet somatostatin probably plays a paracrine role in inhibiting insulin and glucagon secretion from the neighboring β and α cells^[67]. Somatostatin cell hyperplasia inside the pancreas appears to be extremely rare and only one case of focal δ cell hyperplasia has been reported which is in association with pancreatic cancer^[68]. Hyperplasia of gastrin cells inside the pancreas has not been reported. We could not identify any literature on hyperplasia of ghrelin cells or vasoactive intestinal peptide cells either inside the pancreas or in other organs.

PANCREATIC ENDOCRINE CELL HYPERPLASIA AND PATHOGENESIS OF NEUROENDOCRINE TUMORS

It is not clear if pancreatic endocrine cell hyperplasia represents precursor lesions for pancreatic neuroendocrine tumors^[69,70]. Diffuse endocrine cell hyperplasia, dysplasia, and microadenoma are present in the pancreas of patients with MEN1 and VHL, and are indeed considered as pre-

cursor lesions^[16-19]. The hyperplastic pancreatic endocrine cells in patients with MEN1 and in mice with a heterozygous *menin* mutation are polyclonal and retain the normal *menin* allele, indicating that deletion of one copy of *menin* causes pancreatic endocrine cell to proliferate without tumorigenesis^[71,72]. Loss of heterogeneity (LOH) of the *menin* locus is present in adenomas as small as 0.3 mm in diameter, demonstrating that these microadenomas are true tumors according to Knudson's two-hit hypothesis of tumor development^[73]. Interestingly, the exact pattern of LOH is different between microadenomas, suggesting that these microadenomas arise independently from the hyperplastic background^[71,72]. As only a select number of clinical adenomas eventually develop while there are numerous microadenomas, additional mutations have to accrue to form larger and clinically significant pancreatic neuroendocrine tumors (PNETs). There have been no reports of similar precursor lesions for sporadic PNETs. Endocrine hyperplasia, dysplasia, and microadenoma, however, are not uncommon findings in the pancreas^[27]. It is not known if these lesions are monoclonal. Although most of these lesions probably do not indicate clinical significance, they could represent precursor lesions giving rise to sporadic PNETs, since all clinical PNETs have to pass through a microadenoma stage during their growth^[73]. It is thus plausible that PNETs develop from precursor (pre-malignant) lesions such as hyperplasia and microadenoma in familial PNET syndromes and at least partly in sporadic cases such as the patient we describe with α cell hyperplasia and pancreatic neuroendocrine tumors^[53]. The key question of what additional genetic changes are needed to transform a microadenoma to a clinical PNET remains unanswered.

CONCLUSION

Pathologic pancreatic endocrine cell hyperplasia is a distinct group of diseases with various clinical, histological, and etiological features. β cell hyperplasia in adults causes non-insulinoma pancreatogenous hypoglycemia and probably contributes to persistent hyperinsulinemic hypoglycemia in infants. α cell hyperplasia causes glucagonoma syndrome or pancreatic neuroendocrine tumors. The main pathogenetic mechanism for pancreatic endocrine cell hyperplasia is increased endocrine cell neogenesis rather than proliferation of existing cells. The etiology has only been elucidated in some patients with demonstration of mutations of relevant genes regulating pancreatic endocrine cell phenotype. Although rare, this group of diseases does affect a significant number of patients and should be considered in the diagnosis and management hypoglycemia, elevated islet hormone levels, and pancreatic neuroendocrine tumors. Moreover, as the pancreatic endocrine cells are critical in regulating glucose metabolism and their hyperplasia may result in tumorigenesis, further studies of pathologic pancreatic endocrine cell hyperplasia will likely yield insights into the pathogenesis and treatment of diabetes and pancreatic neuroendocrine tumors.

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S- Editor Tian L L- Editor Cant MR E- Editor Zheng XM

Dr. Marco Scarpa, PhD, Series Editor

Quality of life in patients with esophageal stenting for the palliation of malignant dysphagia

Giorgio Diamantis, Marco Scarpa, Paolo Bocus, Stefano Realdon, Carlo Castoro, Ermanno Ancona, Giorgio Battaglia

Giorgio Diamantis, Marco Scarpa, Paolo Bocus, Stefano Realdon, Carlo Castoro, Giorgio Battaglia, Veneto Oncological Institute (IOV-IRCCS), Via Gattamelata 64, Padua 35128, Italy

Ermanno Ancona, Giorgio Battaglia, Department of Surgical and Gastroenterological Sciences, University of Padua, Via Giustiniani 2, Padua 35128, Italy

Author contributions: Diamantis G, Scarpa M, Castoro C, Ancona E and Battaglia G designed the research; Diamantis G, Scarpa M, Bocus P, Realdon S and Castoro C performed the research; Castoro C, Ancona E and Battaglia G contributed analytic tools; Diamantis G, Scarpa M, Bocus P, Realdon S and Castoro C analyzed the data; Diamantis G and Scarpa M wrote the paper; all authors revised and approved the final version of the manuscript.

Correspondence to: Giorgio Diamantis, MD, Veneto Oncological Institute (IOV-IRCCS), Via Gattamelata 64, Padua 35128, Italy. giodiamantis@gmail.com

Telephone: +39-49-8218840 Fax: +39-49-8211707

Received: September 2, 2010 Revised: October 16, 2010

Accepted: October 23, 2010

Published online: January 14, 2011

or palliative intervention. To date, only a few published studies can be found on Medline examining HRQoL in patients with advanced EC treated with SEMS implantation. The aim of this study was to review the impact on HRQoL of SEMS implantation as palliative treatment in patients with EC. All Medline articles regarding HRQoL in patients with advanced EC, particularly those related to SEMS, were reviewed. In most studies, relief of dysphagia was the only aspect of HRQoL being measured and SEMS implantation was compared with other palliative treatments such as brachytherapy and laser therapy. SEMS insertion provides a swift palliation of dysphagia compared to brachytherapy and no evidence was found to suggest that stent implantation is different to laser treatment in terms of improving dysphagia, recurrent dysphagia and better HRQoL, although SEMS insertion has a better technical success rate and also reduces the number of repeat interventions.

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Key words: Esophageal cancer; Health-related quality of life; Self-expandable metal stents

Peer reviewers: Piero Marco Fisichella, MD, Assistant Professor of Surgery, Medical Director, Swallowing Center, Loyola University Medical Center, Department of Surgery, Stritch School of Medicine, 2160 South First Avenue, Room 3226, Maywood, IL 60153, United States; David Ian Watson, Professor, Head, Flinders University Department of Surgery, Room 3D211, Flinders Medical Center, Bedford Park, South Australia 5042, Australia

Abstract

Incidence of esophageal cancer (EC) is rising more rapidly in the Western world than that of any other cancer. Despite advances in therapy, more than 50% of patients have incurable disease at the time of presentation. This precludes curative treatment and makes palliative treatment a more realistic option for most of these patients. Dysphagia is the predominant symptom in more than 70% of patients with EC and although several management options have been developed in recent years to palliate this symptom, the optimum management is not established. Self-expanding metal stents (SEMS) are a well-established palliation modality for dysphagia in such patients. Health-related quality of life (HRQoL) is becoming a major issue in the evaluation of any therapeutic

Diamantis G, Scarpa M, Bocus P, Realdon S, Castoro C, Ancona E, Battaglia G. Quality of life in patients with esophageal stenting for the palliation of malignant dysphagia. *World J Gastroenterol* 2011; 17(2): 144-150 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/144.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.144>

INTRODUCTION

Esophageal cancer (EC) is a devastating disease with an incidence that is rising more rapidly in the Western world than that of any other cancer^[1]. Despite advances in therapy, more than 50% of patients have incurable disease at the time of presentation and only 5%-10% of patients survive 5 years later^[2]. The incidence rates of EC are highly variable across geographic areas. Whereas rates are relatively low in many parts of the world, exceptionally high incidence rates have been reported from some regions of China. Moderate to high incidence rates have been reported from other areas or populations, including parts of Central Asia, South Africa, South America, northern France, and among African-Americans in the United States^[3,4]. A marked change in the male to female ratio of age-adjusted incidence rates has also been observed^[5].

Squamous cell carcinoma is the predominant histologic type of EC in the world^[4]. Nevertheless, a shift in proportion of EC type from SCC to adenocarcinoma (AC) has been reported to have occurred in Western countries, notably from the 1970s in the United States and from the 1980s and early 1990s in some European countries^[6-8]. The reason for this shift is not clear, but it may be related to several factors, including transitions in lifestyle and diet, being overweight, and declining rates of *Helicobacter pylori* infection in the Western world^[7,9]. In Europe, while incidence of SCC has remained stable or declined during the past few decades, the incidence of esophageal AC has been rising. This increase has been more prominent in Northern Europe, notably in the United Kingdom and Ireland, but smaller increases have also been reported from other parts of the continent^[7,8].

Most ECs are diagnosed at an advanced stage and in patients with co-morbidity. This precludes curative treatment and makes palliative treatment a more realistic option for most of these patients^[10]. Dysphagia is the predominant symptom in more than 70% of patients with EC^[11]. Several management options have been developed in recent years to palliate malignant dysphagia. These include mechanical measures such as endoluminal stenting or surgery and antineoplastic methods such as external beam radiation, brachytherapy, chemotherapy, chemoradiotherapy, laser treatment, photodynamic therapy or ablation using injection of alcohol or chemotherapeutic agents. The optimum management of dysphagia caused by advanced primary EC is not established although continued progress has been made in recent years to achieve this goal^[10,12,13]. However, palliation remains an important aspect of treatment, with goals of relieving dysphagia, reducing the risk of aspiration, maintaining a patent orogastric pathway and nutritional status and improving the quality of life.

Esophageal intubation for the palliation of dysphagia from malignant esophageal obstruction has been practiced for over a hundred years. In 1959, Celestin^[14] described the palliation of esophageal malignancy with a plastic endoprosthesis introduced at laparotomy. In the 1970s,

Atkinson *et al*^[15] introduced an endoscopically inserted plastic prosthesis, with a more reduced complication rate. Plastic stents have been superseded by the newer range of metallic self-expanding stents that are safer and easier to place^[14-22]. The first description of the endoscopic placement of an expanding metallic spiral stent was made by Frimberger^[23] in 1983. Nevertheless, some series report little difference in the degree of palliation from dysphagia between plastic and metal stents^[14,24], although the complication rates with metallic stents are significantly lower.

However, the use of self-expanding metal stents (SEMS) is not without problems. Stent migration, incomplete expansion of the stent and tumour ingrowth and overgrowth may require further intervention for recurrent dysphagia. Insertion of stents beyond the gastro-esophageal junction has been observed to result in acid reflux^[25,26]. More recently, developments in SEMS design have resulted in the increasing use of anti-reflux SEMS^[27,28] and retrievable SEMS^[29]. Although SEMS insertion is now reported to be the most common palliative method for treating dysphagia in EC^[30], there is a paucity of evidence regarding its effectiveness in improving the quality of life and nutritional status of the patients.

The aim of this study was to review the impact on health-related quality of life (HRQoL) of SEMS implantation as palliative treatment in patients with EC. A text word literature review was performed using the PubMed and Medline databases. Although this was not a systematic review, the search terms used were as follows: esophageal AND cancer OR carcinoma AND quality of life OR HRQoL OR health-related quality of life OR patient reported outcome AND self-expandable metal stents OR esophageal stents. The reference lists of identified articles were searched for further relevant publications. Two researchers (Diamantis G and Scarpa M) independently selected the studies, limited to clinical studies published between January 1980 to July 2010 and in the English language. Unpublished data and data published in abstract form only were excluded, because these were unlikely to contain sufficient methodologic information to allow valid conclusions to be made. Whenever there was discordance regarding study inclusion the two researchers negotiated an agreement.

HRQOL AS MEASURE OF OUTCOME AFTER TREATMENT OF EC

Defining HRQoL is a complex matter and a universally accepted definition does not exist. Usually, the term quality of life (QoL) and, more specifically, HRQoL, refers to a multidimensional construct which encompasses patients' perceptions of both negative and positive aspects in at least 4 dimensions: physical, emotional and social functions, as well as disease and treatment-related symptoms^[31]. More recently, the assessment of other dimensions of QoL (e.g. spiritual well-being or sexual function) has received more attention. QoL data provide direct measures of benefit as perceived by the patients and may be

Table 1 Examples of quality of life questionnaires used to assess quality of life in patients with esophageal cancer

Category	Type of questionnaire	Advantages	Disadvantages
Generic	SF-36	Good psychometric properties	
	EQ-5D	Reliable	Limited number of domains
Cancer-specific	Spitzer QoL index	Brief and easy to complete	Limited number of domains
	EORTC QLQ C30	Fully validated The most widely used specific instrument Clinical significance assessed	Ceiling effect Site-specific modules may add to patient burden
EC-specific	HAD scale	Well validated and widely used with RSCL	Only assesses anxiety and depression
	EORTC QLQ-OES18	Fully validated	Needs to be used with QLQ-C30
	EORTC QLQ-STO22	Fully validated	Limited supporting psychometric data
	FACT-E	Provides overall summary score	

Modified from Conroy *et al*^[55]. SF-36: Medical Outcomes Study 36-Item Short Form Health Survey; HAD: Hospital Anxiety and Depression; QoL: Quality of life; EORTC QLQ: European Organization for Research and Treatment QoL Questionnaire; FACT: Functional Assessment of Cancer Therapy.

useful for helping them clarify their treatment preferences. The data are also intended to help physicians' decisions by allowing them to understand patients' experiences of treatment, and there is evidence that QoL data may have prognostic value, especially in metastatic disease.

Questionnaires are the most frequently used tools to measure QoL. Multitudes of HRQoL instruments have been described providing adequate coverage of the basic HRQoL dimensions (i.e. physical, functional, social, and emotional function). As the patients represent the most appropriate source of information for their own QoL, the patient self-report is the usual means of assessing QoL. There is no consensus as to which instruments are more appropriate. However, not all QoL instruments have been shown to have acceptable psychometric properties such as reliability, validity and responsiveness. These tools can be classified into 3 main categories: generic instruments, symptom-focused questionnaires and cancer-specific instruments (Table 1).

Esophagectomy, of all elective or emergency surgical oncology procedures performed, is currently one of the most highly rated for morbidity and mortality. Mortality rates vary between 1% and 8% and major morbidity occurs in almost 50% of the patients undergoing resection^[32,33]. The impact of surgery on QoL has been investigated in several prospective studies using validated reliable questionnaires^[34-36]. These investigations generally showed that early in the postoperative phase most aspects of QoL significantly deteriorate. Probably the only aspect of QoL that does not dramatically deteriorate after surgery is emotional function^[34-37]. Several studies have shown that scores for emotional function remain stable after surgery and this may represent patients' relief that the procedure is over, despite the slow physical recovery. Dysphagia scores are generally improved or stable after surgery and patients report fewer problems swallowing solid and soft foods than before the operation. Relief of dysphagia, however, is replaced with other symptoms such as anorexia, change in taste, nausea and diarrhea. The combined impact of multiple symptoms and general deterioration in key aspects of well-being leads to reduced overall QoL scores, but these

gradually recover within 9 mo. Although longitudinal studies show QoL recovery after surgery, one of the difficulties with interpreting these studies relates to missing data due to attrition. The 1-year survival rate after esophagectomy is about 65%; therefore, most papers reporting a recovery of QoL only include data from patients who are alive and sufficiently well to complete questionnaires.

Safieddine *et al*^[38] suggested that although combined modality therapy (chemotherapy/radiation therapy/surgical intervention) is arduous and prolonged, its effect on HRQoL in patients with operable EC is transient, since HRQoL scores return to baseline levels after induction and before surgical intervention. Similarly, surgical intervention has a significant effect on HRQoL because FACT-E scores (Functional Assessment of Cancer Therapy-Esophageal; a validated tool to measure the effect of treatment on functional, social, physical, and emotional well-being that incorporates the EC subscale and allows for a systematic evaluation of QoL specifically in the context of EC) decrease significantly 1 mo after surgical intervention but again return to baseline levels within 3 mo of surgical intervention. Significantly greater increases in FACT-E scores were observed in patients who were still alive 1 year after surgical intervention with or without disease but were observed to decrease in those who died within 1 year of surgical intervention. These researchers had similar findings in patients after esophagectomy who had not been treated with induction chemoradiotherapy.

HRQOL AFTER SEMS IMPLANTATION IN PATIENTS WITH INOPERABLE EC

Since their introduction in the early 1990s^[39,40], SEMS have virtually supplanted not only conventional prostheses but also most forms of ablative therapy in the palliation of malignant dysphagia^[41,42]. The reasons for this range from perceived to real benefits. The latter include smaller delivery systems (precluding the need for excessive dilation), ability to curve around acute angulations, and the larger internal diameter of most SEMS compared with conventional prostheses. The perceived, as opposed to proved,

Table 2 Characteristics of conventional and anti-reflux mechanism self-expanding metal stents

	Material	Length	Inner diameter	Constrainability	Foreshortening	Anti-reflux mechanism
Conventional SEMS						
Ultraflex	Nitinol	10 cm (7 cm CS)	18 mm with 23 mm PF	Braided nylon wire	20% to 40%	
	Polyurethane sheath	12 cm (9 cm CS) 15 cm (12 cm CS)	23 mm with 28 mm PF	Not reconstrainable when partially deployed		
Z stent	Stainless steel	8, 10, 12, 14 cm	18 mm with 25 mm PF and DF	Polyethylene sheath	None	
	Polyurethane covering			Reconstrainable when partially deployed		
Wallstent	Elgiloy	10 cm (8 cm CS)	20 mm with 23 mm PF and DF	Polyethylene sheath	Up to 28%	
	Polyurethane sheath	15 cm (13 cm CS)		Reconstrainable when partially deployed		
SEMS with an anti-reflux mechanism						
Dua Z-stent						Polyurethane sleeve (collapses with gastric pressure)
DO stent						Tricuspid valve
Fer-X-Ella stent						Stainless steel with polyethylene covering and windsock type valve

Modified from Sreedharan *et al*^[56]. SEMS: Self-expanding metal stents; CS: Covered segment; PF: Proximal flare; DF: Distal flare.

benefits have been heavily touted by the manufacturers and include more precise placement (contradictory data because of prosthesis foreshortening in many of the SEMS), fewer long-term complications, and long-term survival advantages. In the 20 years since their introduction, both the SEMS themselves and their delivery systems have undergone multiple modifications. As such, most SEMS have evolved from an uncovered to a covered form, diameters of the prostheses have often been increased, and attempts have been made to minimize migration, gastroesophageal reflux, and tissue ingrowth (Table 2). Although it is safer and easier to place expandable stents, they are not devoid of complications. The major complications include stent migration, stent block, bleeding, and perforation, while the minor ones are foreign body sensation, regurgitation, and chest pain. The main complication of metal stents is distal migration, with an incidence rate ranging between 10% and 30%^[43]. It is more commonly (50%) seen when covered endoprosthesis are used to treat distal esophageal lesions involving the gastroesophageal junction^[44,45]. Stents can become blocked either due to tumor ingrowth through the stent mesh (17%-36%) or tumor overgrowth (10%)^[46,47].

As mentioned in the introduction, the primary aim of treatment in patients with inoperable EC is to relieve dysphagia with minimal morbidity and mortality, and thus improve their QoL. Implantation of a SEMS has become established as a treatment modality for the palliation of malignant dysphagia. SEMS relieves dysphagia rapidly and improves the nutritional status. However, in most studies, relief of dysphagia is the only aspect of HRQoL being measured, although physical, mental and social functioning and other EC-specific aspects of HRQoL are additional important outcome measures, as explained before.

A randomized clinical trial comparing SEMS with plastic endoprosthesis published in 2002 by O'Donnell *et al*^[48]

included 50 patients suffering from dysphagia due to an inoperable EC, and measured QoL using EORTC QLQ-30, a multi-dimensional cancer-specific QoL questionnaire and an EC specific questionnaire (EORTC OES-24), allowing QoL to be measured over 26 components relating to cancer in general and EC in particular. Although the authors found no statistical significance in any of the 26 components, 21 of the 26 components showed a trend towards the metal group, five were neutral and none favored plastic stents.

Shenfine *et al*^[49] in a randomized controlled trial regarding the cost-effectiveness of palliative therapies for patients with inoperable EC studied QoL in detail using four different questionnaires including Spitzer QoL index, Karnofsky performance scale, Euroqol EQ-5D and EORTC QLQ-30. They also used proxy and self-administered questionnaires. These authors reported differences in the baseline quality of life index favoring the non-SEMS group and went on to report 1 and 6 wk QoL data for the different treatment groups. Mean QoL index for the SEMS group at 6 wk was significantly lower than for the QoL index at baseline for the same group. The authors concluded that decreased QoL in the SEMS group at 6 wk, although not statistically significant, reflected the presence of pain following the intervention; the effect of pain on quality of life may have significant implications for treatment with SEMS.

Bergquist *et al*^[50], in their randomized controlled clinical trial published in 2005, compared endoluminal brachytherapy with endoscopic stent placement for newly diagnosed patients with advanced EC or gastroesophageal junction cancer, with a primary outcome being the detailed evaluation of HRQoL. Sixty-five patients eligible for the study were enrolled; 34 were randomized to stent treatment and 31 to brachytherapy. The authors assessed dysphagia improvement as a part of disease-specific HRQoL question-

Table 3 Quality of life results after self-expanding metal stents placement for malignant dysphagia

Ref.	Yr	Study type	Investigation	Type of questionnaire	Results
Dallal <i>et al</i> ^[52]	2001	Randomized trial	Endoscopic thermal ablation <i>vs</i> SEMS in patients with inoperable EC	EORTC QLQ-30 EORTC OES-24 SF-36 HAD scale	HRQoL deteriorated in the stent group but not in the group treated with thermal ablation
Siersema <i>et al</i> ^[53]	2001	Prospective, randomized study	comparison between Ultraflex stent, Flamingo Wallstent, and Gianturco-Z stent in 100 consecutive patients with dysphagia caused by EC or carcinoma of the gastric cardia	WHO performance status Dysphagia score	Mean WHO performance status before and at 4 wk after stent placement was not different among the 3 patient groups
O'Donnell <i>et al</i> ^[48]	2002	Randomized clinical trial	SEMS <i>vs</i> plastic endoprostheses	EORTC QLQ-30 EORTC OES-24	QoL in patients with SEMS was better than in plastic stents (no statistical significance)
Homs <i>et al</i> ^[51]	2004	Randomized trial	Stent placement <i>vs</i> single dose brachytherapy for the palliation of EC	EORTC OES-23 Visual analogue pain scale EORTC QLQ-C30 Euroqol EQ-5D EQ-VAS	Treatment with single dose brachytherapy gave better overall scores on HRQoL scales compared with stent placement for the palliation of EC
Shenfine <i>et al</i> ^[49]	2005	Randomized controlled trial	Cost-effectiveness of palliative therapies for patients with inoperable EC	Spitzer QoL index Karnowsky performance scale Euroqol EQ-5D EORTC QLQ-30	Mean QoL index for the SEMS group at 6 wk was significantly lower than the QoL index at baseline for the same group
Bergquist <i>et al</i> ^[50]	2005	Randomized controlled clinical trial	Endoluminal brachytherapy <i>vs</i> endoscopic stent placement in patients with advanced EC or gastroesophageal junction cancer	EORTC OES-23 EORTC QLQ-30	Insertion of SEMS offered a more instant relief of dysphagia compared to endoluminal brachytherapy, but HRQoL was more stable in brachytherapy treatment
Madhusudhan <i>et al</i> ^[54]	2009	Prospective study	QoL after palliative stenting in patients with inoperable EC	EORTC QLQ-C30 EORTC QLQ-OES 18	Palliative stenting using SEMS resulted in significant improvement in all scales of QoL

QoL: Quality of life; HRQoL: Health-related quality of life; EC: Esophageal cancer; SEMS: Self-expanding metal stents.

naire EORTC OES-23 and found a statistically significant improvement in dysphagia grade, ability to swallow saliva, choking and coughing compared to baseline scores. There was no improvement in these outcomes for patients treated with brachytherapy. In an interim inter-group analysis at 1 mo a significant improvement in dysphagia scale favored the SEMS group. At 3 mo, some of the dysphagia-related parameters continued to show clinical improvement in the SEMS group but these did not achieve statistical significance. In the brachytherapy group, clinically significant improvements were noted in some of the parameters related to dysphagia at 3 mo and these were maintained at 6 mo. However, these data did not achieve statistical significance. General health QoL was measured using the EORTC QLQ-30 scale. In the stent group all functional scales and single symptom scales deteriorated compared to mean scores at inclusion. The largest deterioration was found for social function, followed by pain, role function and insomnia. In the brachytherapy group, a clinically relevant deterioration was found for most variables on the function and single symptom scales with physical function, global QoL and pain scales reaching statistical significance.

The same type of comparison between these two types of palliative procedures, brachytherapy and stent placement, in patients with advanced EC was published from Homs *et al*^[51] who prospectively compared generic and disease-specific HRQoL between single dose brachy-

therapy and SEMS placement. Treatment with single dose brachytherapy gave better overall scores on HRQoL scales compared with stent placement for the palliation of EC. Major improvements were seen on the dysphagia and eating scales of the disease-specific EORTC OES-23, in contrast to other scales of this disease-specific measure which remained almost stable during follow-up. In addition, pain levels remained stable or slightly increased during follow-up, indicating that adequate pain management during follow-up is important.

In a randomized trial carried out at the Western General Hospital in Edinburgh (United Kingdom)^[52], patients presenting with advanced inoperable EC were randomized to palliative therapy by endoscopic thermal ablation (34 patients) or insertion of a SEMS (31 patients), assessing HRQoL. The authors evaluated cancer-specific and EC-specific questionnaires (EORTC QLQ-30 and OES 24) along with a generic questionnaire (SF-36) and a psychometric questionnaire (HAD Scale). The baseline QoL data were reported to be similar in the two groups. However, at 1 mo the SEMS group was significantly worse in parameters of physical function, physical health, pain and emotional health. Results of the cancer-specific questionnaires were reported to be significantly worse in the SEMS group for fatigue, emotional, cognitive and social function and troublesome taste. No differences were noted in dysphagia, deglutition and eating scores.

Several randomized studies comparing different types

of SEMS have been published over the last 10 years but only a few of them have assessed HRQoL as an outcome. Siersema *et al*^[53] performed a prospective, randomized study comparing the Ultraflex stent, the Flamingo Wallstent, and the Gianturco-Z stent in 100 consecutive patients with dysphagia caused by EC or carcinoma of the gastric cardia. All patients were evaluated before stent placement and at 4-wk intervals until death. The authors used the WHO performance status in addition to the dysphagia score to evaluate QoL. The mean WHO performance status before and at 4 wk after stent placement was not different among the 3 patient groups.

Madhusudhan *et al*^[54] in their prospective study assessed the QoL using EORTC QLQ-C30 (version 3) and EORTC QLQ-OES 18 questionnaires before stenting, and at 1, 4, and 8 wk following placement of the stent. The results showed significant improvement following stenting. The general health scale and function scores increased significantly. Most symptom scores, except pain, showed improvement. The pain score deteriorated at 1 wk, as initial expansion of SEMS following its placement led to an increase in pain sensation. Over a period of 2 mo, the pain scores decreased to baseline values. The financial strain scores also showed a significant improvement.

The QoL results after SEMS placement for malignant dysphagia are displayed in Table 3.

CONCLUSION

The prime objective of palliative treatment in patients with inoperable esophageal or gastro-esophageal junctional cancers is to achieve adequate improvement in dysphagia and QoL in a short span of time with a reduced need for additional interventions. In summary, the above analysis confirms that SEMS insertion provides a swift palliation of dysphagia compared to brachytherapy. However, this difference gradually diminishes over time and, in the long run, brachytherapy appears to provide better dysphagia improvement and improved disease-specific QoL scores along with better general health-related QoL scores in these gradually deteriorating patients. On the other hand, no evidence was found to suggest that stent implantation is any better than laser treatment in terms of improving dysphagia, recurrent dysphagia or in yielding a better QoL. Nevertheless, SEMS insertion has a better technical success rate and also reduces the number of repeat interventions. Finally, QoL seems to be similar between different types of conventional SEMS.

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S- Editor Sun H L- Editor Logan S E- Editor Zheng XM

Connective tissue growth factor reacts as an IL-6/STAT3-regulated hepatic negative acute phase protein

Olav A Gressner, Ieva Peredniene, Axel M Gressner

Olav A Gressner, Ieva Peredniene, Axel M Gressner, Institute of Clinical Chemistry and Pathobiochemistry, Central Laboratory, RWTH-University Hospital, 52074 Aachen, Germany
Olav A Gressner, Wisplinghoff Medical Laboratories, Classen-Kappelmann-Str. 24, 50931 Cologne, Germany
Ieva Peredniene, Synlab Lab Services, Gubener Str. 39, 86156 Augsburg, Germany

Author contributions: All authors contributed equally to this work; all authors designed the research, analyzed the data and wrote the paper; Peredniene I and Gressner OA performed research.

Correspondence to: Axel M Gressner, Professor Emeritus of the Institute of Clinical Chemistry and Pathobiochemistry, RWTH-University, Lutherweg 2, 52074 Aachen, Germany. gressner@t-online.de

Telephone: +49-2405-4996968 Fax: +49-241-7014300

Received: August 30, 2010 Revised: October 19, 2010

Accepted: October 26, 2010

Published online: January 14, 2011

Abstract

AIM: To investigate the mechanisms involved in a possible modulator role of interleukin (IL)-6 signalling on CYR61-CTGF-NOV (CCN) 2/connective tissue growth factor (CTGF) expression in hepatocytes (PC) and to look for a relation between serum concentrations of these two parameters in patients with acute inflammation.

METHODS: Expression of CCN2/CTGF, p-STAT3, p-Smad 3/1 and p-Smad2 was examined in primary freshly isolated rat or cryo-preserved human PC exposed to various stimuli by Western blotting, electrophoretic mobility shift assay (EMSA), reporter-gene-assays and reverse-transcriptase polymerase chain reaction.

RESULTS: IL-6 strongly down-regulated CCN2/CTGF protein and mRNA expression in PC, enhanceable by extracellular presence of the soluble IL-6 receptor gp80, and supported by an inverse relation between IL-6 and CCN2/CTGF concentrations in patients' sera. The inhi-

bition of TGF β 1 driven CCN2/CTGF expression by IL-6 did not involve a modulation of Smad2 (and Smad1/3) signalling. However, the STAT3 SH2 domain binding peptide, a selective inhibitor of STAT3 DNA binding activity, counteracted the inhibitory effect of IL-6 on CCN2/CTGF expression much more pronounced than pyrrolidine-dithiocarbamate, an inhibitor primarily of STAT3 phosphorylation. An EMSA confirmed STAT3 binding to the proposed proximal STAT binding site in the *CCN2/CTGF* promoter.

CONCLUSION: CCN2/CTGF is identified as a hepatocellular negative acute phase protein which is down-regulated by IL-6 *via* the STAT3 pathway through interaction on the DNA binding level.

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Key words: Hepatocytes; Interleukin-6; Connective tissue growth factor; STAT3; Liver fibrosis; Acute phase reaction

Peer reviewer: Dr. T Choli-Papadopoulou, Associate Professor, Department of Biochemistry, Aristotle University of Thessaloniki, School of Chemistry, Thessaloniki 55124, Greece

Gressner OA, Peredniene I, Gressner AM. Connective tissue growth factor reacts as an IL-6/STAT3-regulated hepatic negative acute phase protein. *World J Gastroenterol* 2011; 17(2): 151-163 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/151.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.151>

INTRODUCTION

Fibrogenic restructuring of the liver is commonly caused by chronic inflammatory processes. Upon perpetuation of the initial inflammatory attack, a rapid synthesis of several proteins, which is stimulated by cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and particularly IL-6, takes place in order to restore homeostasis.

This process is widely known as the hepatocellular acute phase reaction upon the initial tissue injury, infection or inflammation^[1].

CYR61-CTGF-NOV (CCN) 2/connective tissue growth factor (CTGF), a member of the CCN superfamily of secreted, cysteine-rich glycoproteins, has been implicated in the pathogenesis of hepatic fibrosis and is currently suggested to be an important downstream amplifier of the effects of the profibrogenic master cytokine transforming growth factor (TGF)- β ^[2,3]. Its molecular mechanism of action is still not known in detail, but it very likely strengthens the binding of TGF β 1 to its cognate receptors^[4]. Its crucial role in fibrogenesis is documented by strong up-regulation in fibrotic liver tissue^[5-7], and even more importantly by recent studies, in which knock-down of CCN2/CTGF by siRNA lead to substantial attenuation of experimental liver fibrosis^[8,9]. Recently, we were among the first to identify that hepatocytes (PC) substantially synthesize CCN2/CTGF in cell culture and in injured liver, and that CCN2/CTGF is sensitively up-regulated by TGF β 1^[10-12].

IL-6, originally identified as a B cell differentiation factor in 1981^[13] is a pleiotropic cytokine, which is in the liver mostly synthesized by hepatic macrophages (Kupffer cells) or CD4+ T-helper (Th) cells^[14-16]. IL-6 signals through a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-6R α chain (gp80, CD126), and the signal-transducing component gp130, which is the common signal transducer for several cytokines including leukemia inhibitory factor (LIF), oncostatin M, or IL-11, and which is almost ubiquitously expressed in most tissues^[17]. In contrast, the expression of gp80 is restricted to certain cells such as PC, neutrophils, monocytes/macrophages and some lymphocytes. However, naturally occurring soluble IL-6R together with IL-6 can stimulate cells lacking gp80 receptor part, a process termed trans-signalling^[18]. As IL-6 interacts with its receptor gp80, it triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through certain transcription factors, Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs), but may also lead to an activation of MAP-kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signalling cascades^[19].

In the present study, we investigated the anti-fibrogenic effect of IL-6 comparing with the effect of other selected cytokines (IL-12, IL-2) on CCN2/CTGF. The down-regulating effect of IFN- γ on CCN2/CTGF in hepatocytes and hepatic stellate cells was already shown by others^[11,20]. On the other hand, the up-regulation of IL-6 induced by CCN2/CTGF was shown in pancreatic stellate cells^[21].

Earlier reports by van Gool *et al.*^[22] gave evidence that the stereotypical rat acute phase reactant α 2-macroglobulin acts as an inhibitor of experimental hepatitis; however, the impact of this or other acute phase proteins such as IL-6 on CCN2/CTGF production in PC and the molecular basis of CCN2/CTGF involvement in the acute phase reaction are yet unknown.

We therefore investigated the mechanisms involved in

a possible modulator role of IL-6 signalling on CCN2/CTGF expression in rat and human PC and looked for a possible association between serum concentrations of these two parameters in patients with acute inflammation. Our findings propose that CCN2/CTGF serves as a hepatocellular negative acute phase protein which is down-regulated by IL-6 *via* the STAT3 pathway.

MATERIALS AND METHODS

Reagents

Cytokines or soluble cytokine receptors: rrIL-6 (506-RL), IL-2, IL-12, rhTGF β 1 (240-B) and rhsgp130 (228-GP) were all from R&D Systems (Minneapolis, MN); rhsgp80 was prepared as described by Weiergräber *et al.*^[23] and kindly provided by the Department of Biochemistry, University Hospital of RWTH Aachen, Germany. rhIL-6 (1131567) was from Roche (Mannheim, Germany). STAT3 inhibitors: PDTC was from Sigma-Aldrich (St. Louis, MO); cell permeable STAT3 inhibitor peptide (PY*³LKTK, Cat. No. 573096) was from Calbiochem (Darmstadt, Germany). Specific MAP Kinase inhibitors used in this study were: PD98059 (Cat. No. 513000), SB203580 (Cat. No. 559398), and UO126 (Cat. No. 662005) were all from Calbiochem (Darmstadt, Germany), Edelfosine (PKI-ET18) was from Biaffin (Kassel, Germany).

Antibodies for Western blotting: rabbit anti-Smad3 (ab28379) and chicken anti- α 1-AT (ab14226) (Abcam, Cambridge, UK); goat anti-CTGF/CCN2 (L-20, sc-14939) (Santa Cruz, CA); rabbit anti-p-Smad3 (Ser423/425)/p-Smad1 (Ser463/465) (#9514), rabbit anti-p-Smad2 (Ser465/467) (#3101), rabbit anti-Smad2 (#3102), rabbit anti-phospho-STAT3 (Tyr705) (#9145) and mouse anti-STAT3 (#9139) (Cell Signalling/New England Biolabs, Ipswich, MA); mouse anti- β -actin (AC-15, Cat. No. 5441, Sigma-Aldrich).

Animals

Adult male Sprague-Dawley rats (body weight 180 to 220 g, between 0.5 and 0.8 years of age) had free access to a standard laboratory chow diet and normal tap water throughout the experimental period. All animals received care and treatment in compliance with the German Animal Protection Act, which is in accordance with the German Research Council's criteria.

Isolation and culture of rat hepatocytes

Primary rat PC were isolated from male Sprague-Dawley rats by the two-step collagenase method of Seglen^[24] modified as described before^[25]. Cell culture was performed under serum-free conditions as previously described^[10]. Supplementation of the culture medium with rrIL-6, rhTGF β 1 or STAT3 inhibitors was performed as described in the respective figure legends.

Western blotting analysis

Preparations of cytoplasmic cell extracts, determination of protein concentrations, and Western blotting analysis were performed exactly as previously described^[11,26]. Den-

sitometric quantification of the blot results was done with the Lumi-Imager (Roche, Mannheim, Germany) and the LumiAnalyst 3.0 Software (Roche).

Reverse-transcriptase polymerase chain reaction for rat *CCN2/CTGF*

Total cellular RNA was extracted with the Qiagen RNeasy Mini purification kit (Qiagen, Hilden, Germany). cDNA was reverse-transcribed using the First-Strand cDNA synthesis kit III (Invitrogen, Karlsruhe, Germany). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using the Geneamp 9700 Thermocycler PCR System (Applied Biosystems, Germany) and the following primers: *rCCN2/CTGF* (forward: 5'-CGTCCATGCTGCACAGG-3'; reverse: 5'-CAAGTTTGAGCTTTCTGG-3') as previously described^[3]. The size of the amplified fragment was 144 bp.

Electrophoretic mobility shift assay

Nuclear extracts of rat PC treated with rrIL-6 (10 µg/L, 30 min), were prepared and the electrophoretic mobility shift assay (EMSA) performed slightly modified from that previously described^[27]. In brief, for EMSA, 3.4 µg of total nuclear protein were used for each lane. The oligonucleotide sequence used to assess the two proposed STAT binding sites in the *CCN2/CTGF* promoter (proximal: -418 to -415 bp, distal: -384 to -381 bp)^[28] was 5'-AGGAATTCCTGCTGTTTGCCTCTTCAGCTACCTACTTCCT-3'. The mutated proximal STAT binding site (mt, proximal) was analyzed using the following oligonucleotide sequence: 5'-AAGAATTCCTGCTGTTTGCCTCTTCAGCTACCTACTTCCT-3'. The oligonucleotide sequence of the probe reflective of the mutant distal putative STAT binding site (mt, distal) was: 5'-AGGAATTCCTGCTGTTTGCCTCTTCAGCTACCTACTTCTT-3'. All oligos were synthesized by MWG Biotech (Ebersberg, Germany). The synthetic oligonucleotides were labeled using a T4 polynucleotide kinase (DNA 5'-End Labeling Kit; Promega, Mannheim, Germany) and [γ -³²P]ATP (Perkin Elmer, Rodgau-Jügesheim, Germany). Following incubation of the radiolabeled probes with nuclear extracts, protein-DNA complexes were resolved on a NOVEX 6% retardation gel (Invitrogen) and detected using a Typhoon 9410 Phosphor-Imager (Amersham Biosciences, Freiburg, Germany).

Generation of recombinant *CCN2/CTGF* reporter adenovirus (Ad-hCTGF-Luc)

For generation of the reporter adenovirus Ad-hCTGF-Luc, the approximately 2.5-kbp *Cla* I fragment of vector pGL3-Basic-hCTGF-Luc^[12] harbouring a fusion of human *CCN2/CTGF* gene promoter and the luciferase reporter gene was cloned into the *Cla* I site of vector pΔE1sp1A^[29] resulting in the generation of pΔE1sp1A-hCTGF-Luc. The integrity of cloning boundaries was verified by restriction analysis and sequencing with the flanking primers 5'-GCGTAACCGAGTAAGAATTTG-3' and 5'-GGC-GACCATCAATGCTGGAG-3' that were obtained from MWG-Biotech AG (Ebersberg, Germany).

The integration of the reporter cassette from pΔE1-

sp1A-hCTGF-Luc into the adenoviral backbone vector pJM17^[30] was performed by *in vitro* homologous recombination in the human embryo kidney cell line 293 using a protocol described before^[31]. Successful generation of recombinant viral particles was visualized by viral foci formation. After total infection, the viral particles were released from cells by three rounds of a freeze-thaw cycle and separated from cell debris by centrifugation at 3000 r/min for 10 min. To generate high titer viral stocks, 293 cells were re-infected at a multiplicity of infection (MOI) of 1 and grown for 3-4 d. Amplified viruses were harvested, concentrated through standard CsCl gradient centrifugation and subsequently purified using the BD Adeno-X™ Purification Filter system (BD Biosciences, Clontech, Palo Alto, CA) according to the manufacturer's instructions.

Luciferase gene reporter assay

Cells were cultured in black 96 well plates and infected with 1×10^8 virions/mL of Ad-hCTGF-luc reporter virus. After specific treatment, the luciferase activity was measured as described previously^[3].

Culture of primary human hepatocytes

Primary human hepatocytes from a 15-year-old female donor of Caucasian origin with history of traumatic head injury (BD Gentest™ Cryopreserved Human Hepatocytes, Cat. No. 454551, Lot. No. 208, BD Bioscience, Franklin Lakes, NJ) were cultured according to the distributor's instructions. Primary human hepatocytes were stored in liquid nitrogen vapor until ready and were gradually thawed for further use^[32]. Cell purification was performed using Cryopreserved Hepatocytes Purification Kit (Cat. No. 454500, BD Bioscience). The viability of the final cell suspension, checked by trypan blue exclusion, was around 70% and cell recovery was $9-11 \times 10^6$ cells/vial. For the induction assay, primary human hepatocytes were seeded in ISOMs Seeding Media supplemented with 5% FCS, 2 mmol/L L-glutamine, penicillin (100 kIU/L), streptomycin (100 g/L) (all from BioWhittaker Europe) and ascorbic acid (50 mg/L, Merck Biochemicals, Darmstadt, Germany) on type I collagen coated plastic dishes (BD Bioscience) with a density of 19×10^4 cells/cm² and cultured in a humidified atmosphere (37°C, 5% CO₂). The first change of medium was performed with HepatoStim medium (Cat. No. 355056, BD Bioscience) supplemented with L-glutamine, penicillin and streptomycin as described above and rhEGF (Cat. No. 354052, BD Bioscience) 4 h after seeding. The cells were incubated for another 24 h until the medium was changed and supplemented with the indicated concentrations of rhIL-6, gp80 and gp130 for further 24 and 48 h as described in the respective figure legends.

Patients

Patients admitted to the hospital with different severity of an acute phase reaction [$n = 36$; mean age 47 years, range 1-86 years; 27 males (age 8-86 years) and 9 females (age 1-54 years)] having serum IL-6 concentrations routinely determined as part of their disease-related diag-

nostics, were included in the study. Patients were divided into two groups according to their serum IL-6 concentrations: Group 1 - IL-6 < 100 ng/L [$n = 21$; mean age 54 years, range 20-74 years; 18 males (age 18-74 years) and 3 females (age 14-37 years)]; Group 2 - IL-6 > 100 ng/L [$n = 15$; mean age 42 years, range 1-86 years; 9 males (age 8-86 years) and 6 females (age 1-54 years)]. Peripheral venous blood samples were taken in the morning (6 to 8 AM) upon admission to the hospital. Serum was separated at 4000 *g* 30 to 60 min after clot-retraction and stored at -80°C. At the time of blood sample collection, the patients did not suffer from liver fibrosis or renal insufficiency, as indicated by normal serum activities of ALT, AST, gamma-GT (GGT), and creatinine concentrations, respectively. All blood samples were used anonymously.

Determination of human serum IL-6 and CCN2/CTGF concentrations

Quantitative determination of human IL-6 was performed using a chemiluminescence assay on the Immulite 2000 autoanalyzer (Siemens Medical Healthcare, Erlangen Germany). Serum levels of the CCN2/CTGF were determined in replicates using a human CCN2/CTGF sandwich enzyme-linked immunosorbent assay provided by DRG, Mountainside, NJ, USA (Cat. No. 090731). CCN2/CTGF protein standards were obtained from BioVendor, Heidelberg, Germany (Cat. No. RD172035100).

Statistical analysis

For statistical analysis, SPSS 16.0 (SPSS, Chicago, IL) was used, applying two-tailed unpaired Student's *t* tests with a *P* value for significance set at least at 0.05. The correlations between variables were analyzed with the Pearson correlation tests. Values of *P* < 0.05 were considered statistically significant.

RESULTS

IL-6 inhibits CCN2/CTGF expression in cultured rat hepatocytes

We tested the effects of cytokines produced by liver residing immunocompetent cells on CCN2/CTGF expression in PC (Figure 1). Application of rrIL-6 (as representative of the IL-6 family of interleukins) strikingly reduced hepatocellular CCN2/CTGF protein expression in a dose dependent manner as seen in Western blotting analysis (Figure 1A) and also inhibited the transcriptional activation of the pGL3-hCTGF-luc reporter (Figure 1B). This finding is supported by the observation that rrIL-6 suppressed mRNA level of CCN2/CTGF in PC (Figure 1C). The difference in the intensity of reduction in Western blotting analysis and reporter gene assay may be explained by the fact that consistent reduction of CCN2/CTGF *de novo* synthesis, as seen by the moderate reduction of CCN2/CTGF promoter activity, eventually results in a strong reduction of overall availability of CCN2/CTGF within the cell, as seen by an even stronger decrease of CCN2/CTGF protein expression in Western blotting

analysis. As expected the stimulation with IL-6 induced synthesis of α 1-antitrypsin, an acute phase protein in cultured rat PC (Figure 1D).

In contrast, treatment with IL-2 (as representative of the common γ chain family of interleukins), and IL-12 (as representative of the IL-12 family of interleukins), showed no change in either CCN2/CTGF protein expression (Figure 1E) or transcriptional activation of the pGL3-hCTGF-luc reporter gene (unpublished data) in cultured PC.

TGF β 1 fails to induce CCN2/CTGF expression in cells pretreated with IL-6

As previously reported^[12] rhTGF β 1 induced CCN2/CTGF protein expression in PC (Figure 2). Based on this, we tested whether rrIL-6 was able to reduce not just spontaneous, but also TGF β 1-driven CCN2/CTGF protein expression. Indeed TGF β 1 largely failed to induce CCN2/CTGF expression in cells pretreated with different dosages of rrIL-6 (Figure 2A), an observation particularly prominent at earlier time points (2, 4 h) (Figure 2B). The inhibition of TGF β 1 driven CCN2/CTGF expression by rrIL-6 did not involve a modulation of TGF β 1 induced Smad signaling as rrIL-6 had no negative effect on phosphorylation of both Smad-2 and -3 proteins (Figure 2C). However, the observed immediate suppression of CCN2/CTGF synthesis, already within the first 2 h after stimulation with IL-6, suggests a direct interaction between IL-6 induced STAT3 signalling and transcriptional activation of the CCN2/CTGF promoter.

Complexation of IL-6 and sgp80 enhances the inhibitory effect of this cytokine on CCN2/CTGF protein expression in primary human hepatocytes

In order to avoid possible species specific phenomena, we therefore used primary human hepatocytes for ongoing studies in this direction. Our aim was to investigate whether the enhancing effect of sgp80 on IL-6 signaling, as previously described by Rose-John *et al.*^[18] for other cellular systems, was also transferrable to IL-6 dependent repression of hepatocellular CCN2/CTGF protein expression.

As observed in rat PC, application of rhIL-6 strikingly reduced CCN2/CTGF protein expression in primary human hepatocytes cultured for another 24 h and 48 h after stimulation (Figure 3A and B). This effect was enhanced by pre-incubation of IL-6 with sgp80 and attenuated by co-incubation with recombinant human soluble gp130 (sgp130) complexing with IL-6, and sgp80 (Figure 3B). As expected, synthesis of classical acute phase proteins such as α 1-AT and C-reactive protein (CRP) was increased in both cell fraction (Figure 3A and B) and conditioned culture medium of primary human hepatocytes following pre-incubation of IL-6 with sgp80 (Figure 3C and D).

Inhibition of STAT3 counteracts the IL-6 induced suppression of CCN2/CTGF expression in cultured rat hepatocytes

To study the mechanism of IL-6 induced suppression of

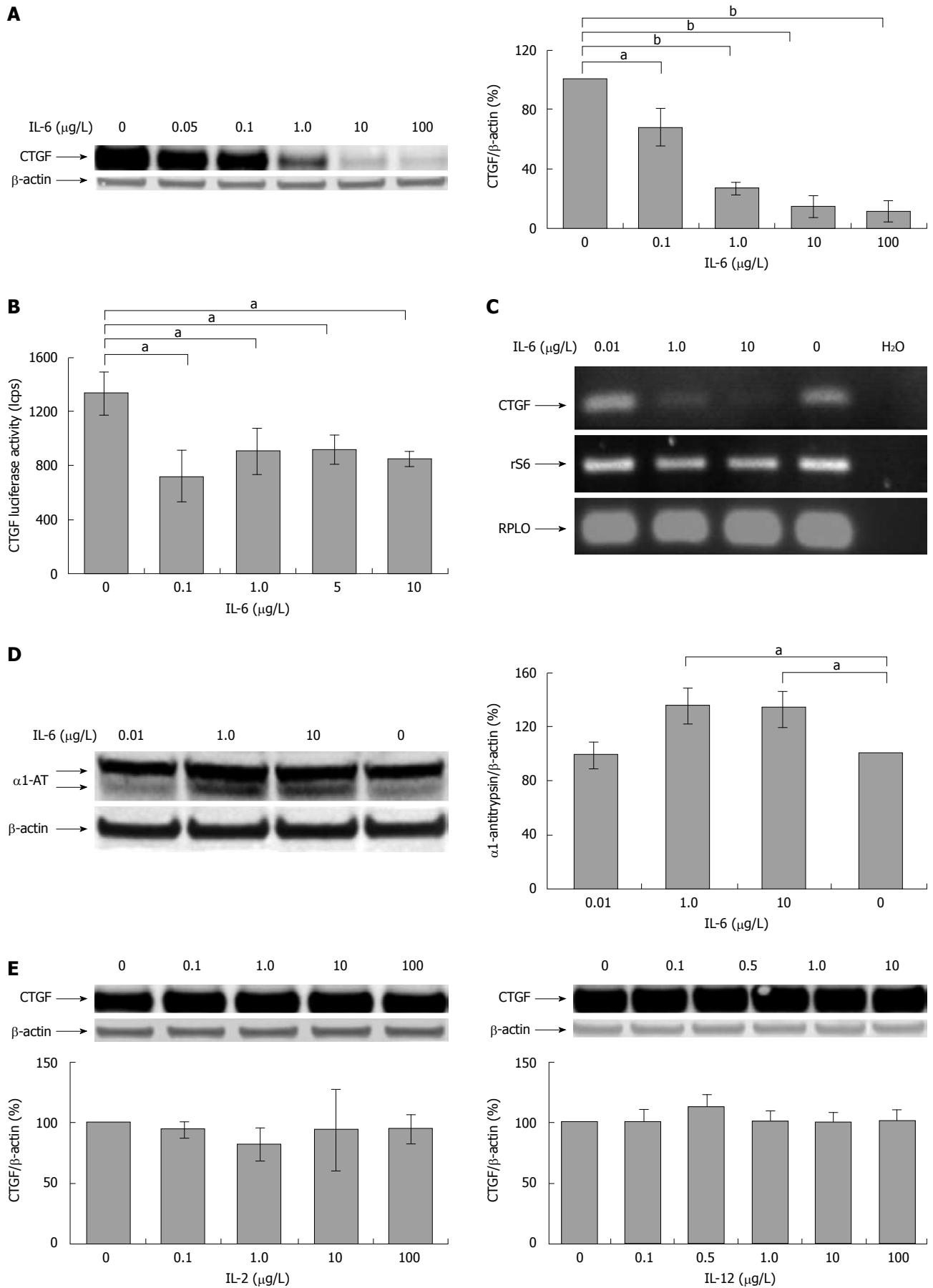


Figure 1 Interleukin-6 inhibits CYR61/CTGF/NOV 2/connective tissue growth factor expression in cultured rat hepatocytes. A: Western blotting of CYR61-CTGF-NOV (CCN) 2/connective tissue growth factor (CTGF) of rat hepatocytes (PC) cultured for 24 h under serum-free conditions with or without addition of indicated concen-

trations of rr interleukin (rrIL)-6. β -actin served as loading control. A representative blot is shown. Blots were quantified relative to β -actin using the Lumi Imager System. Quantifications represent the mean \pm SD of 3 independent cultures. $^aP < 0.05$, $^bP < 0.0001$ vs untreated control; B: CCN2/CTGF reporter gene activation. Rat PC were cultured in serum-free medium for 24 h and transfected with Ad-hCTGF-Luc then subjected to the indicated concentrations of rrIL-6 16 h after transfection and cultured for another 24 h before harvest. Mean values (\pm SD from 3 cultures) are shown. $^aP < 0.05$ vs untreated control; C: Reverse-transcriptase polymerase chain reaction (RT-PCR) of CCN2/CTGF of rat PC. Rat PC were cultured in serum-free medium and treated with rrIL-6 at indicated concentrations for 24 h. RT-PCR was performed using primers for rat CCN2/CTGF as described in Materials and Methods. rS6 and RPLO served as internal control. A representative experiment of 3 independent cultures is shown; D: Western blotting of α 1-AT of rat PC cultured for 24 h under serum-free conditions with or without addition of indicated concentrations of rrIL-6. A representative blot is shown. Blots were quantified as described in (A). $^aP < 0.05$ vs untreated control; E: Western blottings of CCN2/CTGF of PC cultured for 24 h in serum-free medium with or without addition of indicated concentrations of IL-2 or IL-12. β -actin served as loading control. Representative blots of 3 independent experiments are shown. Blots were quantified as described in (A).

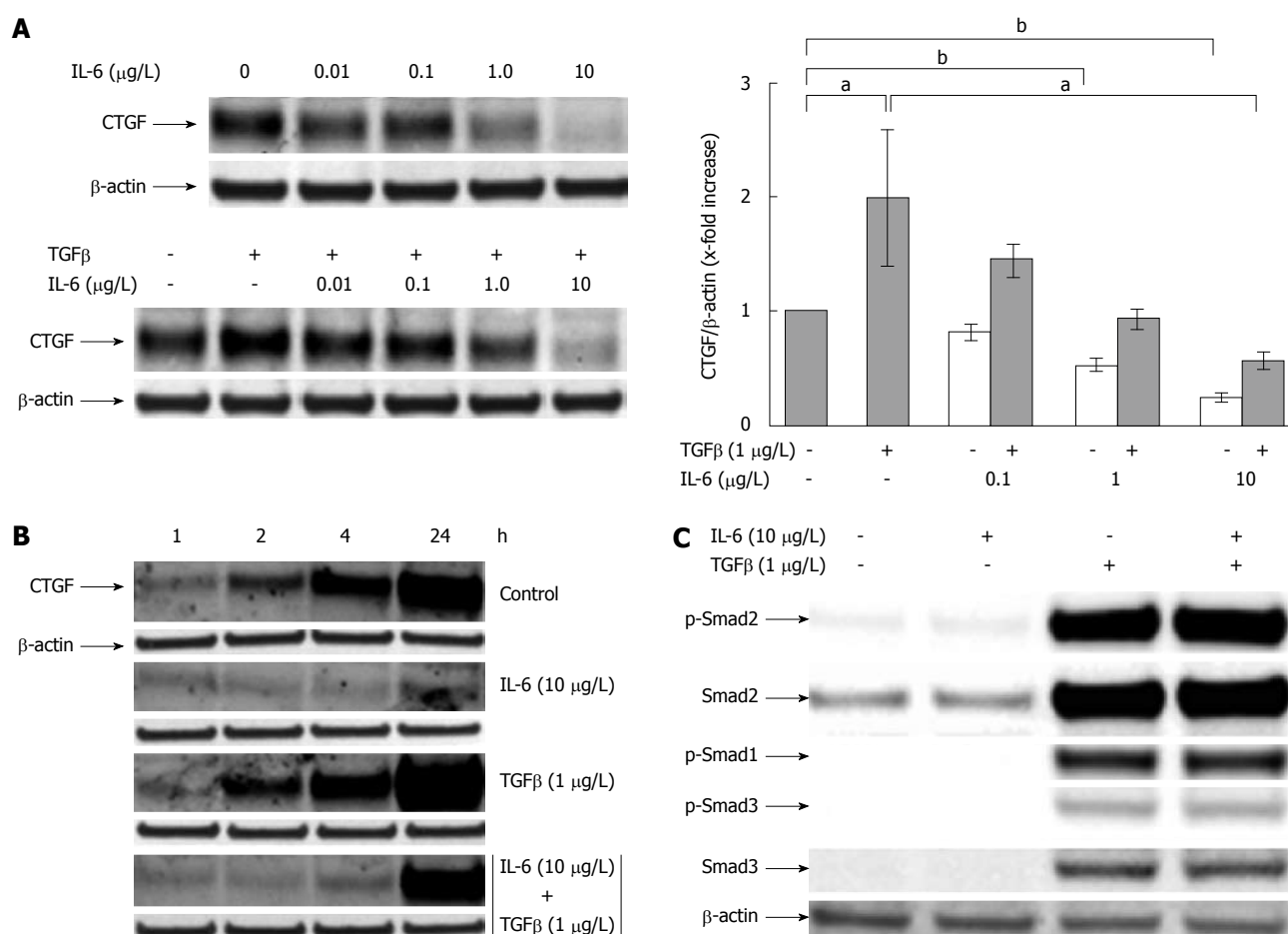


Figure 2 Interleukin-6 acts as an inhibitor of transforming growth factor β 1 induced CYR61/CTGF/NOV 2/connective tissue growth factor protein expression in cultured rat hepatocytes. A: Western blottings of CYR61/CTGF/NOV (CCN) 2/connective tissue growth factor (CTGF) of rat hepatocytes (PC) cultured under serum-free conditions with or without addition of rr interleukin (rrIL)-6 at indicated concentrations 30 min prior addition of rhTGF β 1 (1 μ g/L). The cell culture only with IL-6 at indicated concentrations served as internal control. Cells were harvested after another 24 h. β -actin served as loading control. Representative blots are shown. Blots were quantified relative to β -actin using the Lumi Imager System. Quantifications represent the mean \pm SD of 3 independent cultures. $^aP < 0.05$, $^bP < 0.0001$ vs untreated control; B: Western blottings of CCN2/CTGF of rat PC cultured as stated in (A) under serum-free conditions with or without addition of rrIL-6 (10 μ g/L) 30 min prior addition of rhTGF β 1 (1 μ g/L) where indicated. The cells were harvested after 1, 2, 4 and 24 h. β -actin served as loading control. A representative blot of 3 independent experiments is shown; C: Western blottings of phosphorylated and total Smad2 and Smad3, the latter antibody cross-reacting with Smad1. Rat PC were cultured for 24 h under serum-free conditions with or without addition of rh transforming growth factor (TGF) β 1 (1 μ g/L) and indicated concentrations of rrIL-6. Representative blots are shown.

CCN2/CTGF, the signal transduction pathway known to mediate IL-6 specific effects to the nucleus was interrupted at different levels using specific inhibitors (Figure 4). Inhibition of MAP-kinase signaling by the specific inhibitors (PD98059, SB203580, and UO126) did not abrogate the inhibitory effects of IL-6 on hepatocellular CCN2/CTGF synthesis (Figure 4A). Also, blocking of phosphatidylinositol phospholipase C signaling by administration

of edelfosine did not interfere with IL-6 signaling to the CCN2/CTGF promoter (Figure 4A).

However, exposure of cells to rrIL-6 leads to an activation of the JAK/STAT3 pathway by inducing STAT3 phosphorylation in primary rat PC^[33]. PC were pre-treated with pyrrolidine dithiocarbamate (PDTC) for 2 h and subsequently stimulated with rrIL-6. PDTC was previously proven to primarily impair STAT3 phosphorylation in

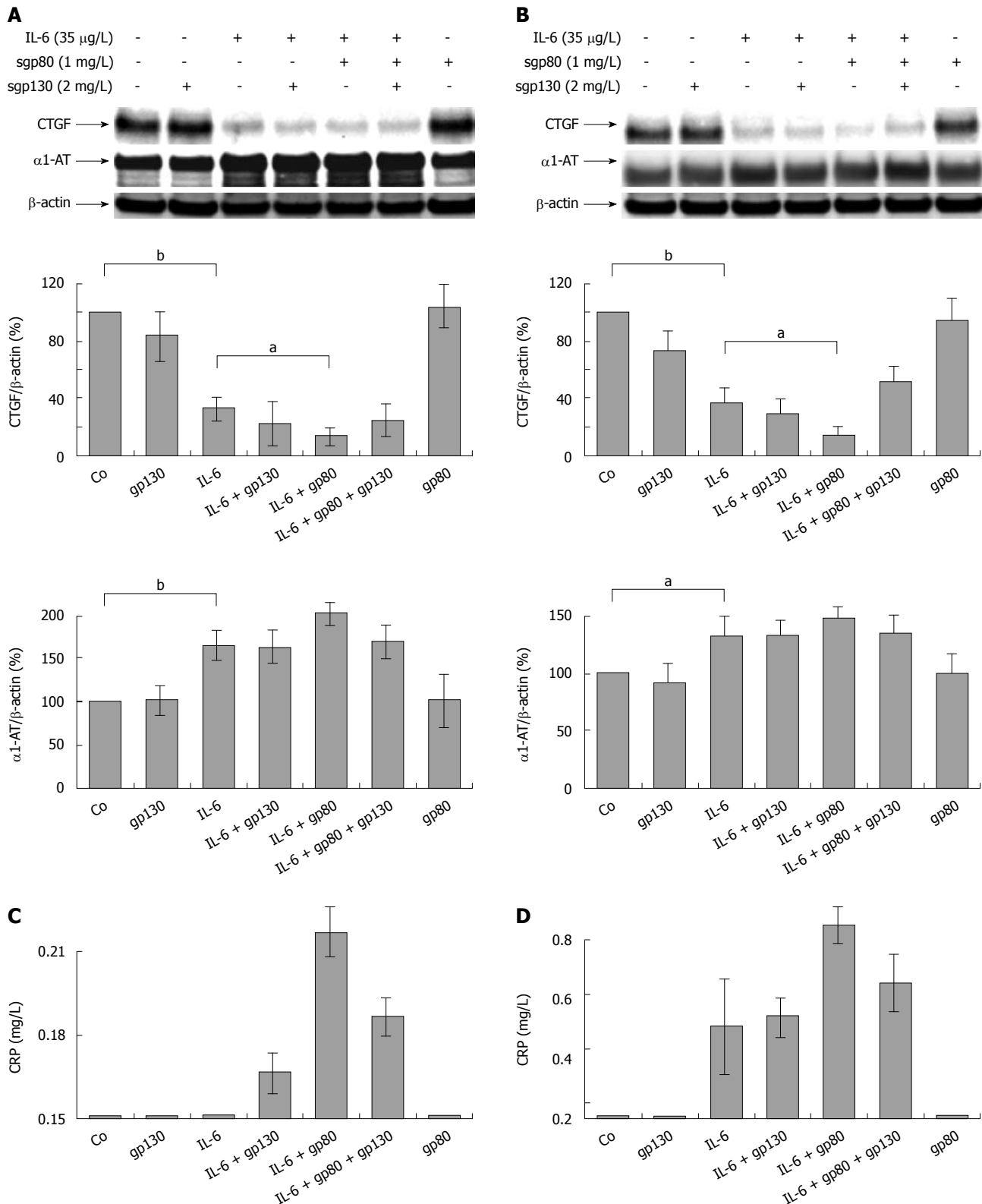
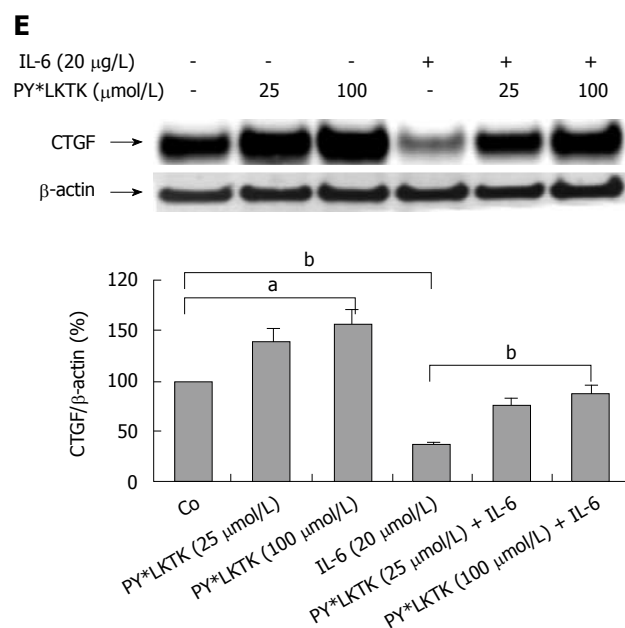
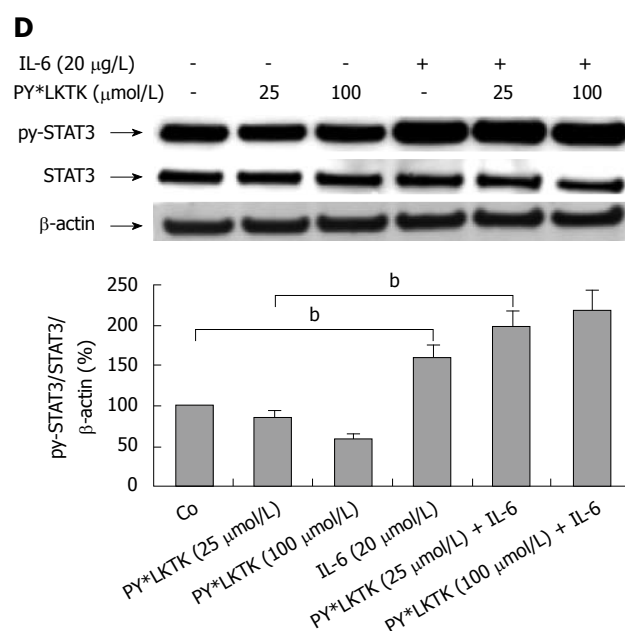
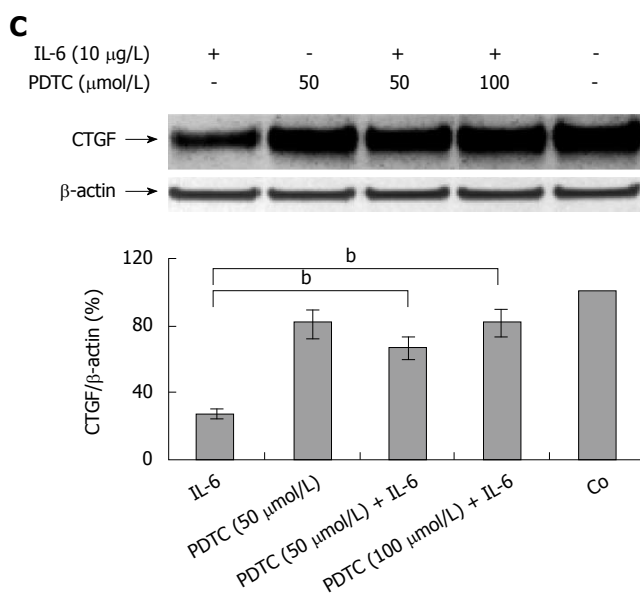
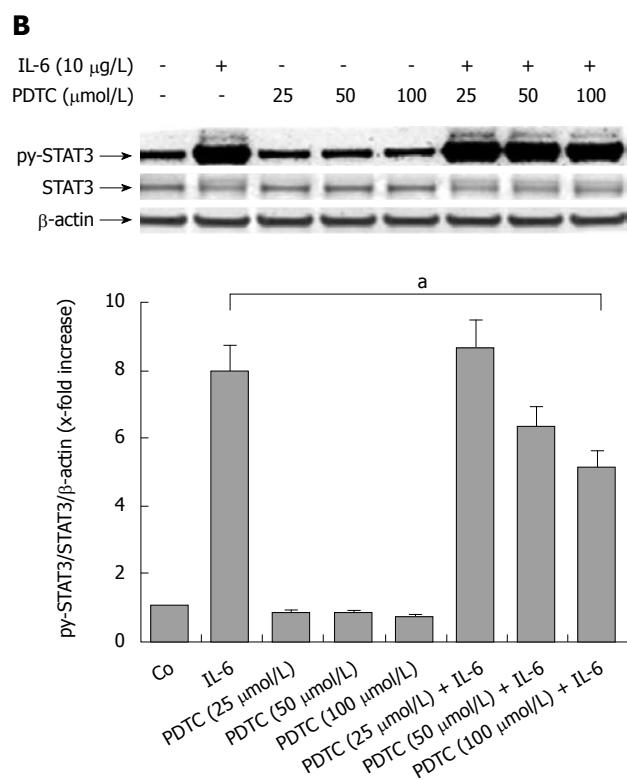
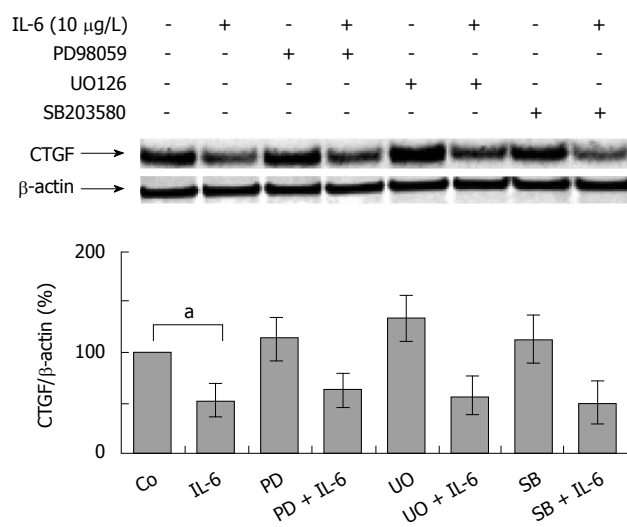
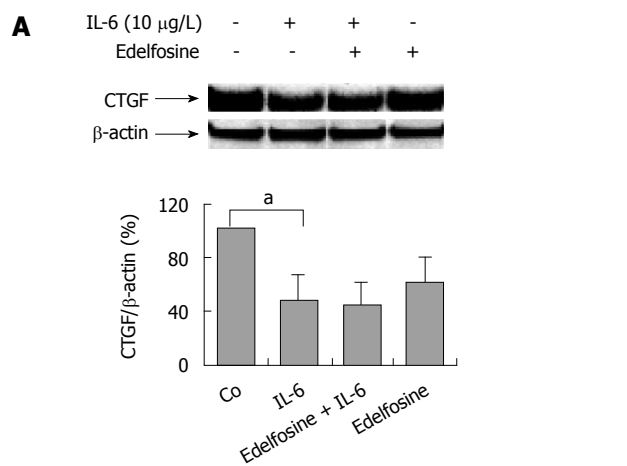


Figure 3 Soluble gp80 receptor enhances the inhibitory effect of interleukin-6 on hepatocellular CYR61/CTGF/NOV 2/connective tissue growth factor expression in primary human hepatocytes. A: Western blottings of CYR61/CTGF/NOV (CCN) 2/Connective tissue growth factor (CTGF) and α 1-AT of primary human hepatocytes cultured under serum-free conditions for 24 h and stimulated with rh interleukin (rhIL)-6 (35 µg/L), soluble gp80 receptor (sgp80, 1 mg/L) and soluble gp130 receptor (sgp130, 2 mg/L) or a complex of both. Cells were harvested after 24 h. β -actin served as loading control. Blots were quantified relative to β -actin using the Lumi Imager System. Representative blots are shown. ^a $P < 0.005$, ^b $P < 0.0001$ vs untreated or IL-6 treated control; B: Primary human hepatocytes were cultured and stimulated as described in (A). Cells were harvested after 48 h. β -actin served as loading control. Blots were quantified as described in (A). Representative blots are shown. ^a $P < 0.005$, ^b $P < 0.0001$ vs untreated or IL-6 treated control; C, D: Ultrasensitive C-reactive Protein as determined using a particle enhanced ultra sensitive assay on the Siemens BN2 nephelometer in supernatants from primary human hepatocytes cultures harvested after 24 h (C) and 48 h (D). The baseline indicates the lower detection limit of the assay at 0.15 mg/L.



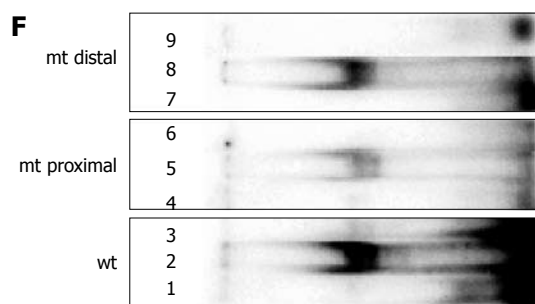


Figure 4 Interleukin-6 mediates its inhibitory effect on hepatocellular CCN2/CTGF/NOV 2/connective tissue growth factor expression through activation of the STAT3 pathway. A: Western blottings of CCN2/CTGF/NOV 2/connective tissue growth factor (CTGF) of rat hepatocytes (PC) cultured under serum-free conditions with or without addition of the phosphatidylinositol phospholipase C inhibitor edelfosine (10 μ mol/L, above) or specific MAP-Kinase inhibitors PD98059 (30 μ mol/L), UO126, (10 μ mol/L), as well as SB203580, (30 μ mol/L) (below) administered to the culture medium 30 min before the addition of rrIL-6 (10 μ g/L). Cells were harvested after 24 h. β -actin served as loading control. Blots were quantified relative to β -actin using the Lumi Imager System. A representative blot of 3 independent experiments is shown. $^aP < 0.005$. PD: PD98059; SB: SB203580; UO: UO126; B: Western blottings of phosphorylated and total STAT3 of rat PC cultured under serum-free conditions with or without addition of PDTC at indicated concentrations 2 h prior addition of rrIL-6 (10 μ g/L). Cells were harvested after 30 min. β -actin served as loading control. Representative blots of 3 independent cultures are shown. Blots were quantified relative to β -actin using the Lumi Imager System. Quantifications represent the mean \pm SD of 3 independent cultures. $^aP < 0.05$ vs IL-6 treated (PDTC untreated) control; C: Western blottings of CCN2/CTGF of rat PC cultured under serum-free conditions with or without addition of PDTC at indicated concentrations 2 h prior addition of rrIL-6 (10 μ g/L). Cells were harvested after another 2 h. β -actin served as loading control. A representative blot out of 3 is shown. Blots were quantified as described in (A). $^bP < 0.0001$ vs IL-6 treated (PDTC untreated) control; D: Western blottings of PY-STAT3 and STAT3 of rat PC cultured under serum-free conditions with or without addition of PY*LKTK at indicated concentrations 1 h prior addition of rrIL-6 (20 μ g/L). Cells were harvested after another 30 min. β -actin served as loading control. Blots were quantified relative to β -actin using the Lumi Imager System. A representative blot out of 3 is shown. $^bP < 0.0001$; E: Western blottings of CCN2/CTGF of rat PC cultured under serum-free conditions with or without addition of PY*LKTK at indicated concentrations 1 h prior addition of rrIL-6 (20 μ g/L). Cells were harvested after another 24 h. β -actin served as loading control. Blots were quantified relative to β -actin using the Lumi Imager System. A representative blot out of 3 is shown. $^aP < 0.005$, $^bP < 0.0001$; F: EMSA using nuclear lysates of PC treated with rrIL-6 (10 μ g/L; 30 min) and 32P-labeled double-stranded oligonucleotide probes containing the two proposed wild-type (wt) STAT binding sites as well as the mutated (mt) proximal and distal STAT binding sites in the CTGF promoter. Lane 1: Labeled probe containing both proposed STAT binding sites; lane 2: Nuclear extract and labeled probe containing both proposed STAT binding sites (wt); lane 3: Nuclear extract, labeled probe and 100-fold molar excess of unlabeled probe containing both proposed STAT binding sites (wt); lane 4: Labeled mutated (mt, proximal) probe; lane 5: Nuclear extract and labeled mutated (mt, proximal) probe; lane 6: Nuclear extract, labeled mutated (mt, proximal) probe and 100-fold molar excess of unlabeled mutated (mt, proximal) probe; lane 7: Labeled mutated (mt, distal) probe; lane 8: Nuclear extract and labeled mutated (mt, distal) probe; lane 9: Nuclear extract, labeled mutated (mt, distal) probe and 100-fold molar excess of unlabeled mutated (mt, distal) probe. The following specific activities were determined using scintillation counting: wt double strand oligonucleotide, 5.23×10^7 cpm/ μ g DNA; mt proximal oligonucleotide, 3.70×10^7 cpm/ μ g DNA; mt distal oligonucleotide, 3.73×10^7 cpm/ μ g DNA. The activities put on the gel were: wt double strand oligonucleotide, 33090 cpm; mt proximal oligonucleotide, 45 844 cpm; mt distal oligonucleotide, 31 556 cpm.

PC^[34]. The extent of the phosphorylation of the tyrosine residue (PY705) of STAT3, important for STAT3 dimerization and DNA binding activities, was analyzed by Western blotting analysis using a phospho-specific antibody in cells treated with rrIL-6 for up to 30 min. PDTC reduced STAT3 phosphorylation in a dose dependent manner (Figure 4B), while CCN2/CTGF protein expression, reduced by rrIL-6, could be restored up to 80%-90% of the untreated control 2 h after pre-application of PDTC (Figure 4C). One hour pre-incubation of rat hepatocytes with the cell permeable STAT3 SH2 domain binding peptide (PY*LKTK), known to be a highly selective inhibitor of STAT3 DNA binding activity^[35], in contrast to PDTC, did not impair STAT3 (PY705) phosphorylation, as expected (Figure 4D), but had a strong counteracting effect on IL-6 induced inhibition of CCN2/CTGF expression (Figure 4E). These results suggest that IL-6 mediates its repressive effect on CCN2/CTGF synthesis *via* direct interaction of activated (phosphorylated) STAT3 with the CCN2/CTGF promoter. An EMSA demonstrated that the synthetic double-stranded oligonucleotide containing the two putative STAT binding sites forms a major protein-DNA complex with nuclear extracts from rat PC treated with 10 μ g/L rrIL-6 (Figure 4F; wt, labeled). Whereas application of a mutated proximal binding site (Figure 4F; mt proximal, labeled) did affect binding of STAT3 to the CTGF promoter sequence, the mutated distal binding site (Figure 4F;

mt distal, labeled) did not. These findings suggest that the distal binding site has a lower affinity for STAT3 than the proximal site.

Inverse association between IL-6 and CCN2/CTGF serum concentrations in patients with different severity of an acute phase reaction

All the findings discussed above were based exclusively on results of *in vitro* experiments. However, results obtained from *in vitro* studies are frequently not directly transferable to the *in vivo* situation. Therefore, we investigated serum concentrations of CCN2/CTGF and IL-6 in Caucasian patients with different extent of an acute phase reaction, hypothesizing that a change in serum concentrations of IL-6 influences CCN2/CTGF serum concentrations in the respective patients.

In order to further corroborate this hypothesis, we clustered sepsis patients into two groups with defined ranges of IL-6 serum concentrations [2-99 ng/L (< 100 ng/L), 104-6100 ng/L (> 100 ng/L)], and compared CCN2/CTGF serum concentrations between both groups (Figure 5A). Results impressively demonstrate an inverse association between IL-6 and CCN2/CTGF serum concentrations with highly significant differences ($P < 0.0001$) between patients with low IL-6 concentrations and those with IL-6 concentrations > 100 ng/L. Data are shown in Tables 1-3.

Table 1 Mean and 95% CI for mean (in braces) of serum concentrations of interleukin-6 and connective tissue growth factor in two groups of patients with different severity of an acute phase reaction

	IL-6 (ng/L)	CTGF (μg/L)
Group 1: IL-6 < 100 ng/L (n = 21)	36 (1.7-54.3)	880.7 (344.0-1417.4)
Group 2: IL-6 ≥ 100 ng/L (n = 15)	1028.3 (46.6-2010.0)	391.2 (109.2-673.3)

IL-6: Interleukin-6; CTGF: Connective tissue growth factor.

Table 2 One sample statistics

	n	Mean CTGF (μg/L)	Std. deviation	Std. error mean
IL-6 < 100	21	851.18	900.50	196.51
IL-6 ≥ 100	15	391.23	509.36	131.52

IL-6: Interleukin-6; CTGF: Connective tissue growth factor.

Table 3 One-sample test

Test value = 0				
t		Mean difference CTGF (μg/L)	95% CI of the difference	
			Lower	Upper
IL-6 < 100	4.332	851.18	441.27	1261.08
IL-6 ≥ 100	2.975	391.23	109.15	673.30

IL-6: Interleukin-6; CTGF: Connective tissue growth factor.

We next observed the longitudinal development of CCN2/CTGF and IL-6 serum concentrations in one representative individual patient over a time period of 22 d. Figure 5B demonstrates an inverse relation between IL-6 and CCN2/CTGF concentrations, suggesting an indirect response of serum CCN2/CTGF concentrations to the individual's acute phase status.

DISCUSSION

It is firmly established that the fibrogenic process in the liver is prominently regulated by TGFβ1^[36-38]. However, TGFβ1 has not only multiple profibrogenic, but also immunosuppressive effects^[39-41]. *Vice-versa*, immunosuppressive agents, such as glucocorticoids, are capable of enhancing TGFβ1 induced target gene expression, in particular of CCN2/CTGF, in rat PC^[42]. We and others have previously reported that PC substantially synthesize CCN2/CTGF during culture and in injured liver, that CCN2/CTGF is sensitively up-regulated by TGFβ1^[10,11] in a Smad2 dependent mechanism and that PC are likely to be the major cellular source of CCN2/CTGF in the liver^[12]. In turn, CCN2/CTGF acts as a downstream sensitizer of TGFβ1 actions in PC^[3].

Inflammation and injury to tissue results in a variety of local and systemic events. However, although the local

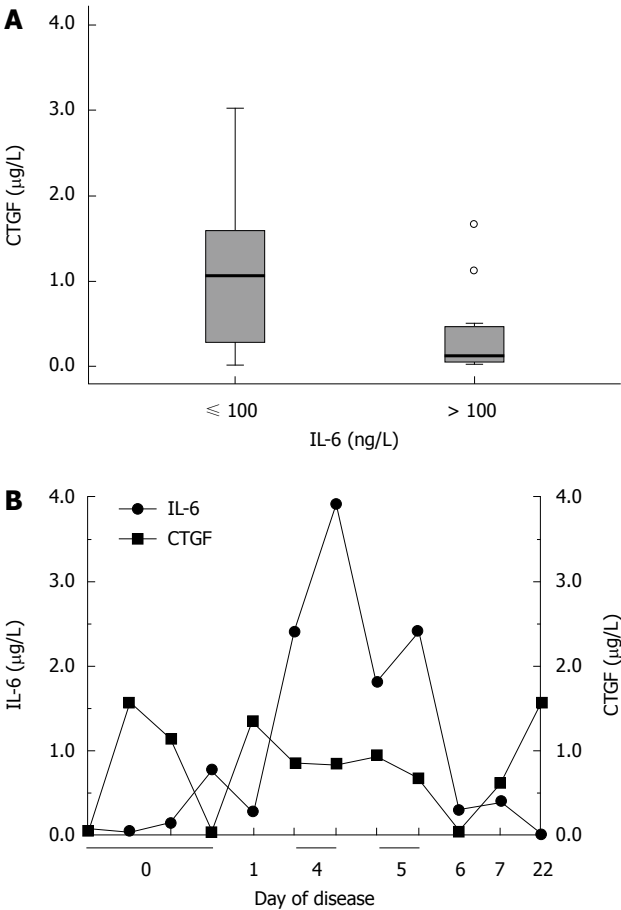


Figure 5 Inverse relation of interleukin-6 and CYR61/CTGF/NOV 2/connective tissue growth factor serum concentrations in patients with different severity of an acute phase reaction. A: Patients (n = 36) were categorized according to their interleukin (IL)-6 serum concentrations. Corresponding CYR61/CTGF/NOV (CCN) 2/connective tissue growth factor (CTGF) concentrations were significantly lower in patients with high IL-6 serum concentrations (IL-6 > 100 ng/L, n = 15) compared to those with low IL-6 serum concentrations (IL-6 ≤ 100 ng/L, n = 21). Box plot are displayed, where the dotted line indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated non-outlier values; open circles indicate outlier values; B: Longitudinal development of CCN2/CTGF and IL-6 serum concentrations in one representative individual patient (20 years old, male) over a time period of 22 d.

events of edema formation and cellular infiltration have received considerably more attention, the systemic response to inflammation is no less profound. The particular systemic event which forms the substance of this communication is the change in the circulating levels of plasma proteins which occurs after inflammatory injury, and the manner in which these changes in plasma concentration are controlled by changes in the rate of synthesis. The changes which occur are regulated at the liver by alteration of the rate of synthesis of the individual protein^[43,44].

In this study, we provide evidence that IL-6 strongly down-regulates spontaneous as well as TGFβ1-induced CCN2/CTGF protein and mRNA expression in PC, an effect enhanced by the extracellular presence of the soluble IL-6 receptor gp80. These data were confirmed by an inverse relation between IL-6 and CCN2/CTGF serum concentration in patients with different extent of

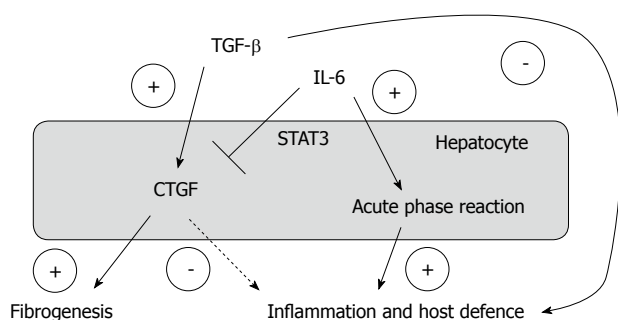


Figure 6 A simplified and schematic overview of the proposed interplay of interleukin-6 and tissue growth factor $\beta 1$ on the regulation of CTGF/NOV 2/connective tissue growth factor expression in hepatocytes and its relevance for inflammation, host defence and fibrogenesis in chronic liver disease. CTGF: Connective tissue growth factor; IL-6: Interleukin-6; TGF: Transforming growth factor.

an acute phase reaction. The inhibition of TGF $\beta 1$ driven CCN2/CTGF expression by IL-6 did not involve modulation of TGF $\beta 1$ induced Smad2 (and Smad1/3), as well as MAP-kinase or phosphoinositide 3-kinase signaling, but required activation of the STAT3 pathway. Furthermore, the difference between the potency of pyrrolidine dithiocarbamate (PDTC), an inhibitor primarily of STAT3 phosphorylation, and PY**LKTK*, a highly selective inhibitor of STAT3 DNA binding activity, as counteractors of IL-6 induced repression of CCN2/CTGF expression in PC imply that downregulation of CCN2/CTGF synthesis by IL-6 is mediated through direct interaction of activated (phosphorylated) STAT3 with the CCN2/CTGF promoter.

In the 600 bp fragment upstream of the transcription start site in the CCN2/CTGF promoter sequence putative STAT binding sites have been described (proximal: -418 to -415 bp, distal: -384 to -381 bp)^[28], but their functional relevance has not been verified yet. The immediate suppression of CCN2/CTGF synthesis within the first 30 min after stimulation with IL-6, suggest a direct interaction between IL-6 induced STAT3 signalling and transcriptional activation of the CCN2/CTGF promoter, a hypothesis herein confirmed by EMSA showing that the distal binding site seems to have a lower affinity for STAT3 than the proximal one.

Based on these results, it may be suggested that CCN2/CTGF belongs to the family of negative acute-phase-reactants, displaying a decrease of synthesis during the acute inflammatory process. Several negative acute-phase-proteins have been identified so far, e.g. albumin, transferrin, transthyretin and transcortin^[45]. But apart from transcortin, whose reduced bioavailability results in decreased glucocorticoid binding and, thus, in an enhancement of the inflammatory response, little is known of the biological function of these proteins.

Earlier studies by van Gool *et al.*^[46,47] investigated the clinical significance of the depressed acute-phase reaction in 14 patients with acute hepatitis B, and found that the acute-phase-reactant $\alpha 2$ -macroglobulin ($\alpha 2M$) was negatively correlated to the subsequent course of hepatitis, and

to the duration of the illness. This was most likely due to inflammatory inhibiting effects of $\alpha 2M$.

Therefore, it may be suggested that the observed down-regulation of negative acute-phase-proteins should have similar effects on the pathogenesis of liver fibrosis as does the upregulation of acute-phase-proteins, i.e. attenuation of the inflammatory state through an enhancement of immune stimulation, decreased tissue injury, and, thus, in an retardation of the fibrogenic process. The opposite effects may be observed in conditions of immune suppression. In other terms, CCN2/CTGF would *per se* act as an immunosuppressive and profibrogenic which is antagonized by the acute-phase-reaction as an unspecific immune response. On the contrary, recent results of Karger *et al.*^[22] show that CCN2/CTGF induces IL-6 gene expression in pancreatic stellate cells, thus, in turn, enhancing the local inflammatory reactions in the pancreas. Little is known of the immunomodulatory capacities of CCN2/CTGF, but its crucial role in fibrogenesis is documented by strong upregulation in fibrotic liver tissue^[5,6,48] and even more importantly by recent studies in which knock-down of CCN2/CTGF by siRNA leads to substantial attenuation of experimental liver fibrosis^[8,9].

In summary, our results of an inhibitory effect of IL-6 on hepatocellular CCN2/CTGF expression suggest a role of CCN2/CTGF as “negative” acute-phase-protein, whose decreased synthesis during the acute-phase-reaction results in an, at least temporary, interruption of (TGF $\beta 1$ mediated) immune suppression and fibrogenesis, amplified by CCN2/CTGF (Figure 6). However, for an appropriate assessment of this phenomenon, a detailed understanding of a possible immunomodulatory role of CCN2/CTGF is needed. Our results hopefully initiate further studies in this direction.

ACKNOWLEDGMENTS

We thank Henkel C, Department of Pathology, University Hospital Aachen, for support when using the Typhoon 9410 PhosphoImager. Ad-hCTGF-Luc was kindly provided by van de Leur E, Institute of Clinical Chemistry and Pathobiochemistry, University Hospital Aachen.

COMMENTS

Background

Hepatocytes (PC) are a major cellular source of connective tissue growth factor (CCN2/CTGF), a downstream amplifier of profibrogenic transforming growth factor (TGF)- $\beta 1$. Earlier reports identified the rat acute phase reactant $\alpha 2$ -macroglobulin ($\alpha 2M$) as an inhibitor of experimental hepatitis and fibrosis; however, the impact of acute phase reaction initiating interleukins such as interleukin (IL)-6 on CCN2/CTGF synthesis in PC is still unknown. This paper investigates the mechanisms involved in a possible modulator role of IL-6 signalling on CCN2/CTGF expression in PC and looks for a possible relation between serum concentrations of these two parameters in patients with acute inflammation.

Research frontiers

CTGF=CCN2, one of six members of cysteine-rich, secreted, heparin-binding proteins with a modular structure, is widely recognized as an important player in hepatic and non-hepatic fibrogenic pathways. Its expression is strongly increased in fibrotic tissues and TGF- β , the profibrogenic master cytokine, is a strong stimulator of CTGF synthesis in hepatocytes, biliary epithelial cells and

stellate cells. Functional activity as a mediator of fibre-fibre, fibre-matrix and matrix-matrix interactions, as an enhancer of profibrogenic TGF- β and several secondary effects owing to TGF- β enhancement, and as a down-modulator of the bioactivity of bone morphogenetic protein-7 have been shown or at least proposed. Consequently, knockdown of CTGF considerably attenuates experimental liver fibrosis. The spill-over of CTGF from the liver into the blood stream proposes this protein as a non-invasive reporter of TGF- β bioactivity in this organ. Indeed, it was shown that CTGF levels in sera correlate significantly with fibrogenic activity.

Innovations and breakthroughs

Fibrogenic restructuring of the liver is commonly caused by chronic inflammatory processes. Upon perpetuation of the initial inflammatory attack, a rapid synthesis of several proteins, which is stimulated by cytokines such as tumor necrosis factor (TNF)- α , IL-1, and particularly IL-6, takes place in order to restore homeostasis. This process is widely known as the hepatocellular acute phase reaction upon the initial tissue injury, infection or inflammation CTGF has been implicated in the pathogenesis of hepatic fibrosis and is currently suggested to be an important downstream amplifier of the effects of the profibrogenic master cytokine TGF- β which explains why experimental knockdown of CTGF considerably attenuates experimental liver fibrosis. Earlier reports gave evidence that the stereotypical rat acute phase reactant α 2M acts as an inhibitor of experimental hepatitis; however, the impact of this or other acute phase proteins such as IL-6 on CCN2/CTGF production in PC and the molecular basis of CCN2/CTGF involvement in the acute phase reaction was long unknown, thereby launching the present study, whose results identify CTGF as a hepatic negative acute phase protein.

Applications

The results of the present study, showing an inhibitory effect of IL-6 on hepatocellular CCN2/CTGF expression, suggest a role of CCN2/CTGF as "negative" acute-phase-protein, whose decreased synthesis during the acute-phase-reaction results in an, at least temporary, interruption of (TGF β 1 mediated) immune suppression and fibrogenesis, amplified by CCN2/CTGF. However, for an appropriate assessment of this phenomenon, a detailed understanding of a possible immunomodulatory role of CCN2/CTGF is needed. The results hopefully initiate further studies in this direction.

Terminology

Acute Phase Reaction: The term acute phase response summarizes the endocrine or metabolic changes observed in an organism, either locally or systemically, a short time after injuries or the onset of infections, immunological reactions, and inflammatory processes. The acute phase reaction is initiated and mediated by a number of cytokines with inflammatory activities secreted by a variety of cell types (i.e. granulocytes, monocytes, lymphocytes, etc.) in response to the inflammatory stimuli. **Acute Phase Protein:** Acute-phase proteins are a class of proteins whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) during the acute phase reaction. **CTGF=CCN2:** CTGF is a 38 kDa, cysteine-rich, secreted peptide and a classical downstream target of TGF- β . Among the many functions of the CTGF gene family are embryogenesis, wound healing and regulation of extracellular matrix production. **Liver Fibrosis:** Hepatic fibrosis is overly exuberant wound healing in which excessive connective tissue builds up in the liver. The extracellular matrix is either overproduced, degraded deficiently, or both. The trigger is chronic injury, especially if there is an inflammatory component. **TGF- β :** TGF- β is a multifunctional cytokine that regulates tissue morphogenesis and differentiation through effects on cell proliferation, differentiation, apoptosis, and extracellular matrix production. TGF- β has been implicated as a "master switch" in induction of fibrosis in many tissues including the liver.

Peer review

This work identifies CTGF as a hepatocellular negative acute phase protein which is down-regulated by IL-6 via the STAT3 pathway through interaction on the DNA binding level. The paper is well written, the biochemical documentation excellent, and the results clearly show a significant implication of CTGF in the inflammatory response of the liver.

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S- Editor Sun H L- Editor O'Neill M E- Editor Ma WH

Switching-on of serotonergic calcium signaling in activated hepatic stellate cells

Kyu-Sang Park, Pyo-Jin Sin, Dong Hyeon Lee, Seung-Kuy Cha, Min-Jeong Kim, Na-Hyun Kim, Soon-Koo Baik, Seong-Woo Jeong, In Deok Kong

Kyu-Sang Park, Pyo-Jin Sin, Seung-Kuy Cha, Min-Jeong Kim, Seong-Woo Jeong, In Deok Kong, Department of Physiology and Institute of Lifelong Health, Yonsei University Wonju College of Medicine, Wonju, Gangwon-Do, 220-701, South Korea
 Dong Hyeon Lee, Department of Physiology, College of Medicine, Pochon CHA University, Seongnam, Gyeonggi-Do, 463-712, South Korea

Na-Hyun Kim, Department of Basic Nursing Science, Keimyung University College of Nursing, Daegu, 700-712, South Korea

Soon-Koo Baik, Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju, Gangwon-Do, 220-701, South Korea

Author contributions: Park KS, Sin PJ and Kong ID designed the project; Park KS, Sin PJ, Lee DH, Cha SK, Kim MJ and Kim NH performed the experiments; Park KS, Sin PJ and Kong ID wrote the manuscript; Baik SK and Jeong SW contributed to the discussion of the data and the revision of the manuscript.

Supported by Grants from the Korean National Research Foundation (2010-0014617); the Myung Sun Kim Memorial Foundation (2009); and the Yonsei University Faculty Research Grant (2004)

Correspondence to: In Deok Kong, Professor, Department of Physiology and Institute of Lifelong Health, Yonsei University Wonju College of Medicine, 162 Ilsan-Dong, Wonju, Gangwon-Do, 220-701, South Korea. kong@yonsei.ac.kr

Telephone: +82-33-7410292 Fax: +82-33-7456461

Received: September 6, 2010 Revised: October 11, 2010

Accepted: October 18, 2010

Published online: January 14, 2011

Abstract

AIM: To investigate serotonergic Ca^{2+} signaling and the expression of 5-hydroxytryptamine (5-HT) receptors, as well as Ca^{2+} transporting proteins, in hepatic stellate cells (HSCs).

METHODS: The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of isolated rat HSCs was measured with a fluorescence microscopic imaging system. Quantitative PCR was per-

formed to determine the transcriptional levels of 5-HT receptors and endoplasmic reticulum (ER) proteins involved in Ca^{2+} storage and release in cultured rat HSCs.

RESULTS: Distinct from quiescent cells, activated HSCs exhibited $[\text{Ca}^{2+}]_i$ transients following treatment with 5-HT, which was abolished by U-73122, a phospholipase C inhibitor. Upregulation of 5-HT_{2A} and 5-HT_{2B} receptors, but not 5-HT₃, was prominent during trans-differentiation of HSCs. Pretreatment with ritanserin, a 5-HT₂ antagonist, inhibited $[\text{Ca}^{2+}]_i$ changes upon application of 5-HT. Expression of type 1 inositol-5'-triphosphate receptor and type 2 sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase were also increased during activation of HSCs and serve as the major isoforms for ER Ca^{2+} storage and release in activated HSCs. Ca^{2+} binding chaperone proteins of the ER, including calreticulin, calnexin and calsequestrin, were up-regulated following activation of HSCs.

CONCLUSION: The appearance of 5-HT-induced $[\text{Ca}^{2+}]_i$ response accompanied by upregulation of metabotropic 5-HT₂ receptors and Ca^{2+} transporting/chaperone ER proteins may participate in the activating process of HSCs.

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Key words: Hepatic stellate cells; 5-hydroxytryptamine; Intracellular Ca^{2+} transient; Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; Inositol-5'-triphosphate receptor; Endoplasmic reticulum chaperone

Peer reviewer: Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Park KS, Sin PJ, Lee DH, Cha SK, Kim MJ, Kim NH, Baik SK, Jeong SW, Kong ID. Switching-on of serotonergic calcium signaling in activated hepatic stellate cells. *World J Gastroenterol*

2011; 17(2): 164-173 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i2/164.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.164>

INTRODUCTION

Hepatic stellate cells (HSCs), also known as “Ito cells” or “fat-storing cells”, localize between hepatocytes and sinusoids (space of Disse) in mammalian livers. In their healthy state, HSCs control retinoid homeostasis, sinusoidal blood flow, macromolecule transport, and potentially act as antigen-presenting cells in the liver^[1,2]. However, in response to hepatic injury, HSCs undergo gross morphological and functional changes, transforming to a myofibroblast-like phenotype in a process called “activation” or “trans-differentiation”^[3,4]. Manifestations of activated HSCs include: (1) the expression of contractile cytoskeletal proteins such as α -smooth muscle actin (α -SMA)^[5,6]; (2) enhanced extracellular matrix synthesis^[7,8]; (3) increased cell size and proliferation^[9]; (4) decreased size of lipid droplets^[8,10]; and (5) well developed endoplasmic reticulum (ER), Golgi bodies, and compacted microfilaments^[11,12]. In particular, the deposition of cross-linked collagen during the activation process may result in cirrhotic changes accompanied by life-threatening hepatic dysfunction.

Serotonin [5-hydroxytryptamine (5-HT)] is a neurotransmitter that also acts as a multifunctional hormone in various tissues^[13], where it modulates proliferation and differentiation of muscle, neurons, and mammary glands^[14-16]. Serotonin released from platelets at sites of injury plays an important role in liver regeneration and fibrosis^[17]. It has been reported that patients with cirrhosis of the liver and portal hypertension have increased plasma serotonin levels^[18]. The expression levels of 5-HT_{2A} and 5-HT_{2B} are increased in the liver after hepatectomy as well as in activated HSCs^[2,17]. Moreover, 5-HT₂ receptor antagonists suppress cell proliferation and expression of key fibrogenic factors in activated HSCs^[2,19]. Among the mammalian 5-HT receptors (5-HT₁ to 5-HT₇), the 5-HT₂ receptor family is coupled to the G_{q/11} protein and increases intracellular Ca²⁺ concentration ([Ca²⁺]_i) mobilized from ER reservoirs^[20].

As the major intracellular calcium storage site, the ER possesses various kinds of calcium regulatory proteins that participate in: (1) pumping Ca²⁺ into the ER lumen, such as the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA); (2) releasing Ca²⁺ into the cytosol, such as IP₃ or ryanodine receptors; and (3) buffering Ca²⁺, such as calreticulin and calnexin, which are also known as chaperones. ER Ca²⁺ homeostasis is maintained by a balance between Ca²⁺ release and replenishment^[21]. The free Ca²⁺ concentration in the ER ([Ca²⁺]_{ER}) ranges from 60-400 μ mol/L, and disturbances in [Ca²⁺]_{ER} homeostasis can affect many of the functions of the ER including protein synthesis, secretion^[22], protein folding^[23], and sensitivity of cells to apoptosis^[24]. Further, [Ca²⁺]_{ER} homeostasis might be critically required for the activation process of HSCs in order to

keep up with accelerated protein synthesis. However, until now, the compensatory changes in ER protein expression involved in Ca²⁺ homeostasis and chaperone function have not been clearly elucidated.

[Ca²⁺]_i may be important for the activation of HSCs, primarily because [Ca²⁺]_i regulates the transcription of genes critical for cell function^[25], and secondly because contractile elements such as α -SMA respond sensitively to [Ca²⁺]_i^[26]. We hypothesized that serotonin, acting as an autocrine or paracrine mediator, can elicit a Ca²⁺ signal, and this signal might be involved in the activation of HSCs. Moreover, there may be an alteration in the ER function of HSCs such as Ca²⁺ release and protein folding. In this study, we isolated and cultured rat HSCs on plastic dishes *in vitro*, which has been widely accepted as an appropriate model for the study of activated HSCs^[8,27]. Appearance of [Ca²⁺]_i transients induced by 5-HT and the upregulation of 5-HT₂ receptors and ER proteins were observed during HSC activation. These observed changes may participate in an activation signal as well as adaptive changes during the trans-differentiation of HSCs.

MATERIALS AND METHODS

Isolation of rat HSCs

HSCs were isolated from male Sprague-Dawley rats (150-250 g) by means of a collagenase/pronase perfusion and Nycodenz-gradient centrifugation, as previously described^[28,29]. HSCs were cultured with DMEM containing fetal bovine serum (10%) and antibiotics-antimycotics (Invitrogen, Carlsbad, CA, USA) in a humidified incubator (5% CO₂, 37°C). The purity of HSCs was > 95% as assessed by their typical microscopic morphology and positive immunocytochemical staining for desmin at 24 to 48 h after seeding.

Quantitative reverse transcription-polymerase chain reaction analysis

Total cellular RNA was isolated and purified from HSCs at different culture periods, and reverse transcription (RT) was performed with random hexamers. Quantitative real time PCR using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Sequence specific oligonucleotide primers for the genes of interest were designed based on rat sequences deposited in the GenBank database (Tables 1 and 2), and the amplification program included the activation of AmpliTaq Gold at 95°C for 10 min, followed by 45 cycles of a two-step PCR reaction with denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The constitutively expressed housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was selected as an endogenous control to correct for potential variation in RNA loading and efficiency of amplification reactions.

Fluorescent [Ca²⁺]_i measurement

HSCs at 3 d or 2 wk after isolation were seeded on glass

Table 1 Primers for reverse transcription-polymerase chain reaction

Name	Sequence	Accession code	Position	Product (bp)
5-HT _{1A}				
(+)	5'-TCAGCTACCAAGTGATCACC-3'	NM_012585.1	98-117	211
(-)	5'-GTCCACTTGTGAGCACCTG-3'		308-289	
5-HT _{1B}				
(+)	5'-TACACGGTCTACTCCACGGT-3'	NM_022225.1	610-629	258
(-)	5'-TCGCACTTTGACTTGGTTCAC-3'		867-847	
5-HT _{2A}				
(+)	5'-GTGTCCATGTTAACCATCCT-3'	NM_017254	446-465	376
(-)	5'-GTAGGTGATCACCATGATGG-3'		821-802	
5-HT _{2B}				
(+)	5'-CATGCATCTCTGTGCCATTTC-3'	NM_017250	652-672	352
(-)	5'-TGTTAGGCGTIGAGGTGGC-3'		1003-985	
5-HT _{3A}				
(+)	5'-TCCTCAACGTGGATGAGAAG-3'	NM_024394.1	553-572	352
(-)	5'-ATGTTGATGTCCTGGATGGT-3'		904-885	
5-HT _{3B}				
(+)	5'-AAGCCCATCCAGGTGGTCTC-3'	NM_022189.1	459-478	428
(-)	5'-GACATGTTGACCCCTGAAGAC-3'		886-867	
5-HT ₄				
(+)	5'-TCATGGTGCTGGCCATTAC-3'	NM_012853.1	640-659	377
(-)	5'-CTCATCATCACAGCAGAGGA-3'		1016-997	
5-HT _{5A}				
(+)	5'-GAACAGGAGGAAGGAAGAGA-3'	NM_013148	1535-1554	109
(-)	5'-TAAGTCTCCTTGGTGTGAGG-3'		1643-1624	
5-HT _{5B}				
(+)	5'-TTCACCGTACTCGTGGTAAC-3'	L10073.1	453-472	132
(-)	5'-GGTCGAGGCTACCAAGTTAT-3'		584-565	
5-HT ₆				
(+)	5'-CCTGAGAGTGTGCTGAATTG-3'	NM_024365.1	1716-1735	129
(-)	5'-AGCCACACTACACAAGCAAC-3'		1844-1825	
5-HT ₇				
(+)	5'-GTGTGTCCACTGTCAAATCC-3'	NM_022938	2072-2091	148
(-)	5'-TCACTCATCTCCAGTTACCG-3'		2219-2200	

5-HT: 5-hydroxytryptamine.

coverslips and loaded with fura-2/AM (5 μ mol/L) in a dark room for 30 to 60 min at room temperature. Dye-loaded cells were then washed and transferred to a perfusion chamber on a fluorescence microscope (IX-70, Olympus, Tokyo, Japan). The HSCs were alternately excited at 340 and 380 nm by a monochromatic light source (LAMDA DG-4; Sutter, Novato, CA, USA), and fluorescence images were captured at 510 nm with an intensified CCD camera (Cascade; Roper, Duluth, GA, USA). Images were analyzed using the Metafluor 6.1 software package (Universal Imaging Corporation, Downingtown, PA, USA).

Immunocytochemistry

HSCs cultured on coverslips were fixed in 4% paraformaldehyde and immunocytochemical staining was performed using an antibody for α -SMA (Sigma Chemical Co., St Louis, MO, USA). After incubating with a biotinylated secondary antibody, an avidin-conjugated peroxidase complex was added to the slides and 3-amino-9-ethylcarbazole (AEC) was used as the chromogen.

Electrophysiology

Whole-cell membrane currents were recorded using the gramicidin-perforated patch-clamp technique as described

previously^[28]. All experiments were performed at room temperature (20–24°C). The internal solution for the perforated patch clamp contained (in mmol/L): 140 KCl, 5 EGTA, 10 HEPES, 0.5 CaCl₂, 5 NaCl, and gramicidin (50 μ g/mL) (pH 7.2). The external solution contained (in mmol/L): 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, and 10 glucose (pH 7.4).

Statistical analysis

Quantitative data are expressed as the mean \pm SE. Statistical comparisons were made by the two-tailed Student's *t*-test and ANOVA. Differences with *P* < 0.05 were considered to be significant. PCR from each cDNA sample was done in triplicate and *n* indicates the number of experiments. For quantitative comparisons, the expression level of each gene was normalized to that of GAPDH and presented as relative expression ratio (target/GAPDH) by applying the formula $2^{-\Delta\Delta Ct[30]}$.

RESULTS

Serotonergic signaling and receptor expression during HSC activation

We isolated HSCs using density gradient-based separation with Nycodenz. Most of the harvested cells (> 95%)

Table 2 Primers for quantitative reverse transcription-polymerase chain reaction

Name	Sequence	Accession code	Position	Product (bp)
5-HT _{2A}				
(+)	5'-GGGTACCTCCCACCGACAT-3'	NM_17254	234-252	101
(-)	5'-TTCCAGCAATGGTGAGAATAATC-3'		334-311	
5-HT _{2B}				
(+)	5'-CGCCATCCCAGTCCCTATTA-3'	NM_17250	781-800	101
(-)	5'-AGAGCATGAAACTGCCAAAGC-3'		881-861	
IP ₃ R 1				
(+)	5'-GCAGAAGCAGATTGGCTATG-3'	NM_1007235	2072-2091	261
(-)	5'-GTCTCAATCAGGATGTCAGC-3'		2332-2313	
IP ₃ R 2				
(+)	5'-CAAGAAGTTCAGAGACTGCC-3'	NM_31046.3	396-415	295
(-)	5'-ACGCATGGCATTCTTCTCCA-3'		690-671	
IP ₃ R 3				
(+)	5'-GATGTGGTGTGCTGCAGAA-3'	NM_13138.1	390-409	137
(-)	5'-TGTGTGCTCTCATGTGCAG-3'		526-507	
RyR 1				
(+)	5'-CTGAATGTCTGCTCTCCAAG-3'	AC_165142.3	35577-35596	112
(-)	5'-GAAGGGCAGAGAGACAAGAT-3'		35688-35669	
RyR 2				
(+)	5'-ATGTAGGCTTCTCCAGAGC-3'	XR_8338.1	11405-11414	136
(-)	5'-TGCAGTACCTTCTCTCTGA-3'		11540-11521	
RyR 3				
(+)	5'-TACCTTGCCGTGACACAAC-3'	XM_001080527.1	13957-13976	123
(-)	5'-AGTCACAGATGACAGGATCG-3'		14079-14060	
SERCA 1				
(+)	5'-CCAAGGAGCCTCTTATCAGT-3'	NM_017254	2516-2535	111
(-)	5'-CCTCTGCATACAAGAACCAC-3'		2626-2607	
SERCA 2				
(+)	5'-AGTTCATCCGCTACCTCATC-3'	M_23114	2297-2316	119
(-)	5'-CACCAGATTGACCCAGAGTA-3'		2415-2396	
SERCA 3				
(+)	5'-CTCATGCAGAAGGAGTTCAC-3'	NM_172812	1563-1582	140
(-)	5'-CGCTCAATTACACTCTCAGG-3'		1702-1683	
Calreticulin				
(+)	5'-AGAAGACTGGGATGAACGAG-3'	NM_22399.1	683-701	109
(-)	5'-GTCCTCAGGCTTCTTAGCAT-3'		791-772	
Calsequestrin-2				
(+)	5'-CAGATGGCTATGAGTTCCTG-3'	NM_17131.2	988-1007	118
(-)	5'-CAGTAAGCAACAAGCAGAGG-3'		1105-1086	
Calnexin				
(+)	5'-GTGTTTGCTACTGGTCCTTG-3'	NM_172008.1	21-40	146
(-)	5'-ATGGAGGAGTGCTGGTATCT-3'		166-147	
TGF- β type 1 R				
(+)	5'-ACCAGCTATTGCCCATAGAG-3'	L_26110	1011-1030	106
(-)	5'-GGCAGAATCATGTCTCACAG-3'		1116-1097	
α -SMA				
(+)	5'-GCAGAGCAAGAGGGATCCT-3'	X_06801	222-242	73
(-)	5'-CATGTCGTCCCAGTGGTGAT-3'		294-274	
Cav1.2 (α 1c)				
(+)	5'-GACCCGTAGGAGCACGTTTG-3'	NM_012517	2327-2346	71
(-)	5'-CCTCCCGGTCAGGATCT-3'		2397-2380	

5-HT: 5-hydroxytryptamine; SERCA: Sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase; α -SMA: α -smooth muscle actin; RyR: Ryanodine receptor; TGF: Transforming growth factor.

exhibited positive intra-cytoplasmic staining for desmin and glial fibrillary acidic proteins (GFAP). Expression of HSC trans-differentiation markers was tested at 1 d, 1 wk and 2 wk after isolation. In activated HSCs (2 wk after isolation), bundles of α -SMA were clearly observed as cytoskeletal fibers in immunocytochemical staining (Figure 1A), which was not evident in quiescent cells. In a voltage-clamp mode, nimodipine (10 μ mol/L)-sensitive L-type Ca²⁺ currents were recorded only for activated

HSCs (Figure 1C). The expression level of α -SMA and the L-type Ca²⁺ channel (Cav1.2) were proportional to the activation period elicited by culturing cells on plastic dishes (Figure 1B and D). Transforming growth factor- β 1 (TGF- β 1), an abundant isoform of TGF in both normal and cirrhotic liver, is known as the main profibrogenic cytokine^[31]. We observed that the type I receptor for TGF- β 1 (T β -RI) was also upregulated during activation (Figure 1E), while the expression of 28S RNA as well as

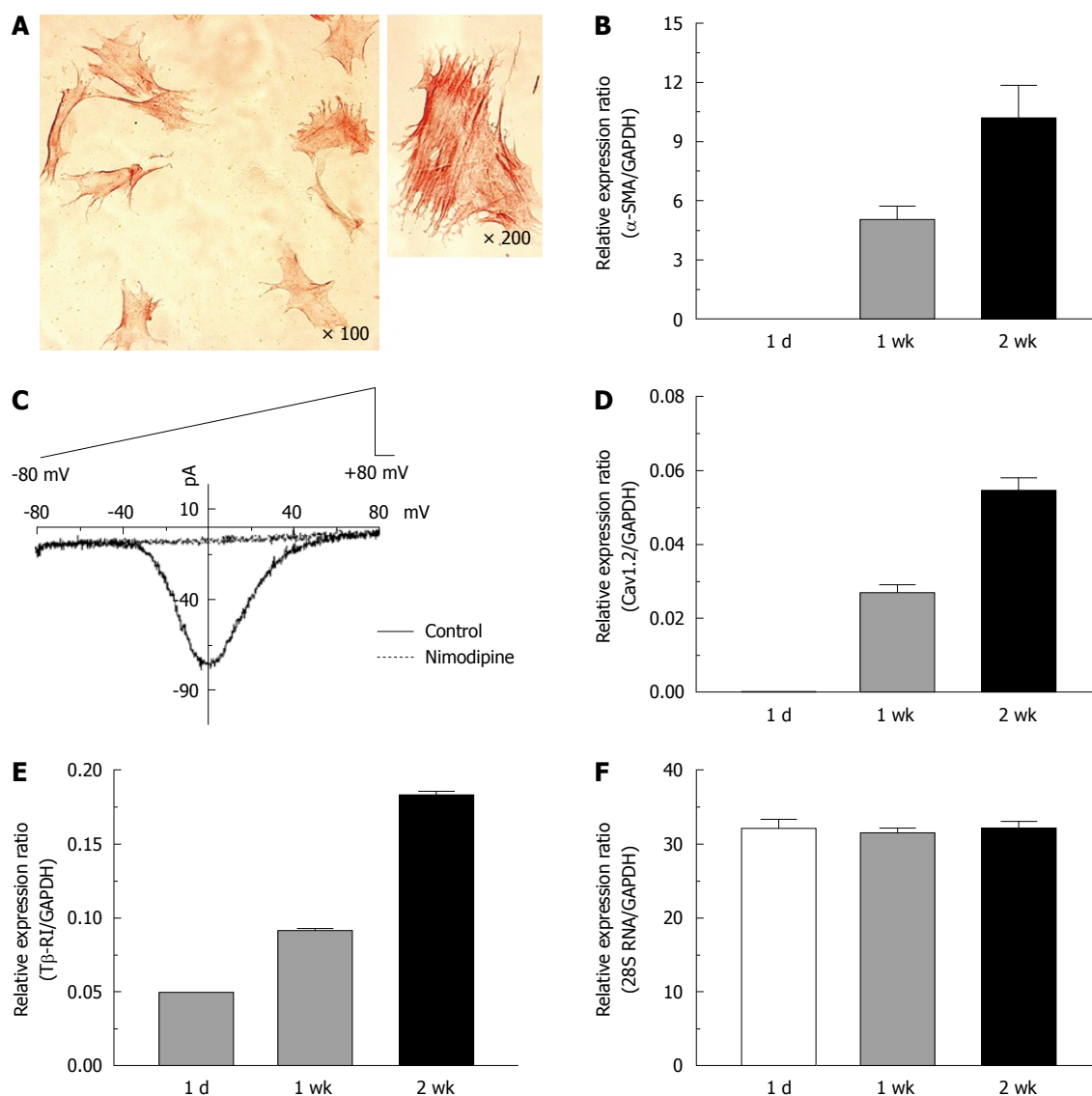


Figure 1 Expression of α -smooth muscle actin, L-type calcium channels and type 1 transforming growth factor- β receptors in activated rat hepatic stellate cells. A: Immunocytochemical staining for α -smooth muscle actin (α -SMA) was performed on hepatic stellate cells (HSCs) cultured for 1 wk; C: Whole cell Ca^{2+} currents in a voltage-clamp mode were recorded from 2 wk-cultured HSCs, and were completely blocked by nimodipine (10 $\mu\text{mol/L}$); Changes in the transcript levels of α -SMA (B), the $\alpha_1\text{c}$ subunit of the L-type Ca^{2+} channel (Cav1.2) (D), the type 1 receptor of transforming growth factor- β (T β -RI) (E), and 28S RNA (F) during HSC culturing (1 d, 1 wk and 2 wk) were measured by quantitative real-time reverse transcription-polymerase chain reaction analysis. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as a relative expression ratio (target/GAPDH). Data are presented as the mean \pm SE ($n = 3$).

GAPDH was not changed during the activation process of HSCs (Figure 1F).

Serotonergic signaling has been suggested as a candidate for triggering activation of HSCs^[2,17]. We focused on $[\text{Ca}^{2+}]_i$ signaling in HSCs, which has been emphasized by previous work as having an important role in the activation process^[26,32]. As shown in Figure 2A and B, strong $[\text{Ca}^{2+}]_i$ transients followed by a slow plateau increase were recorded in response to 5-HT (10 $\mu\text{mol/L}$) application only from most of the activated HSCs (2 wk after isolation; 81 cells out of 92 cells), but not from quiescent cells (3 d after isolation; 0 out of 11 cells). The 5-HT-induced $[\text{Ca}^{2+}]_i$ increase was dose-dependent in activated HSCs (Figure 2C). Consistent with a previous report^[33], ATP also evoked $[\text{Ca}^{2+}]_i$ transients in activated HSCs while acetylcholine did not (Figure 3).

Among the 5-HT receptors, 5-HT₂ is known to release Ca^{2+} from the ER while 5-HT₃ acts as a ligand-gated cation channel^[20]. We estimated the steady-state mRNA levels of 5-HT receptor isotypes (5-HT₁ to 5-HT₇) using reverse transcription-polymerase chain reaction (RT-PCR) and found that the 5-HT_{2A} and 5-HT_{2B} receptors, but not 5-HT₃, were abundantly transcribed (Figure 2D). Consistent with the observed changes in $[\text{Ca}^{2+}]_i$, the expression of 5-HT_{2A} was increased by about 17-fold after 2 wk of isolation (5-HT_{2A}/GAPDH; from 0.004 at 1 d to 0.067 at 2 wk). 5-HT_{2B} was also found to be upregulated in activated HSCs (from 0.003 to 0.008) using quantitative RT-PCR (Figure 2E).

It has been recognized that 5-HT₂ receptors are coupled with the G_{q/11}-phospholipase C pathway. Figure 4A and B show that the 5-HT-induced $[\text{Ca}^{2+}]_i$ changes were abolished

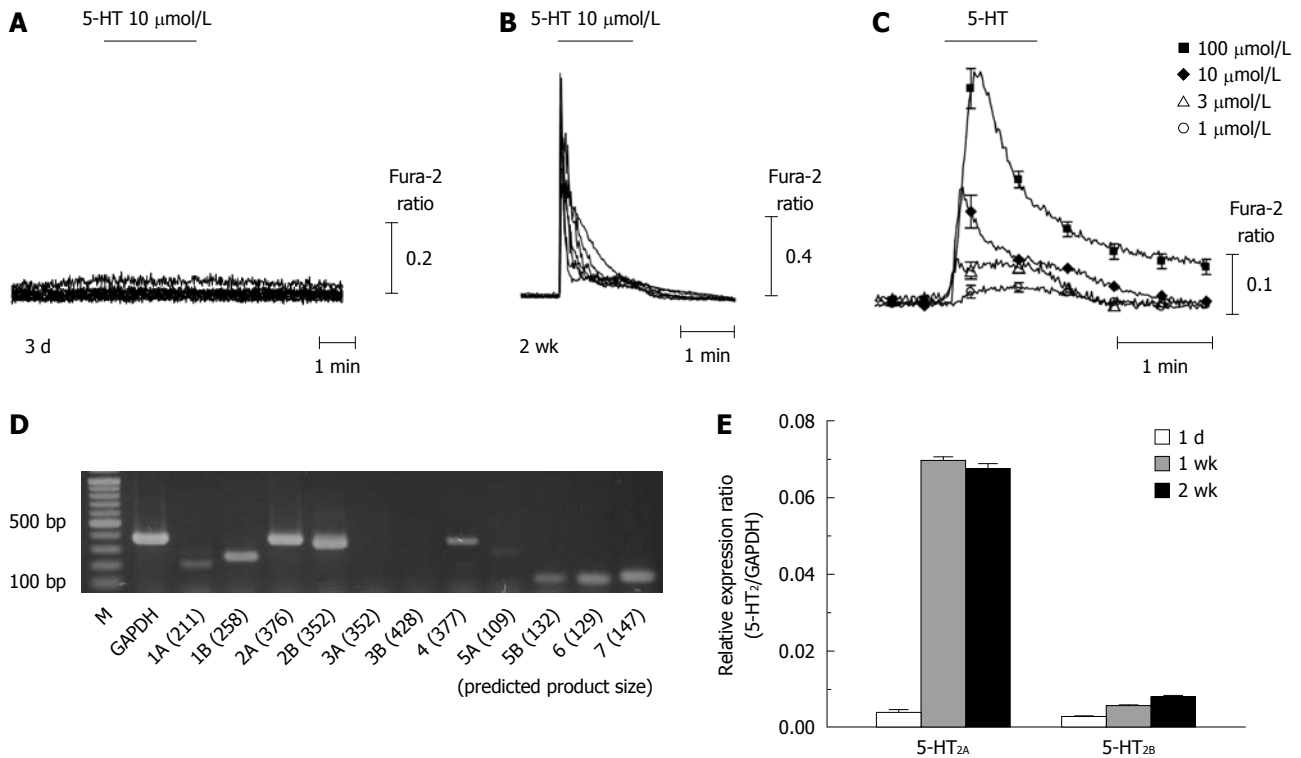


Figure 2 5-hydroxytryptamine-induced intracellular Ca^{2+} concentration changes and the expression of 5-hydroxytryptamine₂ receptors in quiescent and activated hepatic stellate cells. A, B: 5-hydroxytryptamine (5-HT)-induced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) transients were recorded from hepatic stellate cells (HSCs) at 3 d (A) and 2 wk (B) after isolation; C: Averages of $[\text{Ca}^{2+}]_i$ changes (from 13-40 cells/each trace) in response to 5-HT (1-100 $\mu\text{mol/L}$) application to 2 wk-cultured HSCs are shown; D: Steady-state mRNA levels of the 5-HT receptor isotypes in 2 wk-cultured HSCs were compared using reverse transcription-polymerase chain reaction (RT-PCR); E: Using quantitative RT-PCR, the transcriptional changes in 5-HT₂ receptors among 1 d-, 1 wk- and 2 wk-cultured HSCs were compared. Expression levels were normalized to GAPDH and expressed as a relative expression ratio (target/GAPDH, $n = 3$). Data are presented as the mean \pm SE.

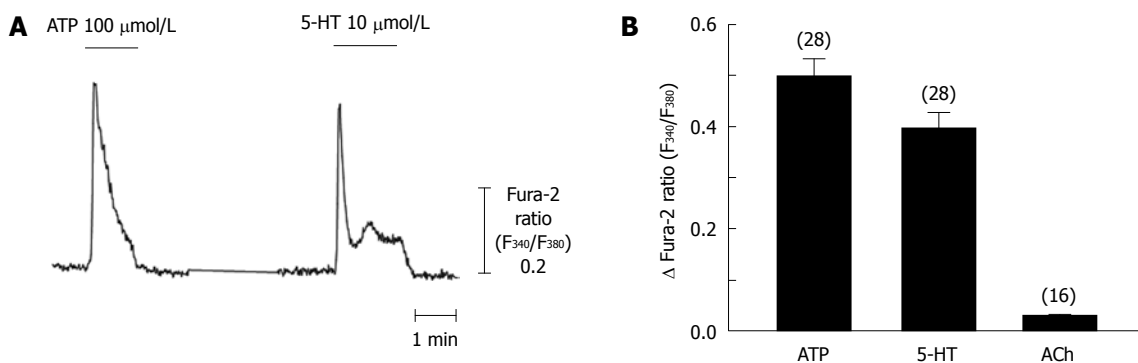


Figure 3 Comparison of intracellular Ca^{2+} concentration responses to various metabotropic receptor agonists in activated hepatic stellate cells. Intracellular Ca^{2+} concentration changes following application of ATP (100 $\mu\text{mol/L}$), 5-hydroxytryptamine (5-HT) (10 $\mu\text{mol/L}$), or acetylcholine (ACh, 10 $\mu\text{mol/L}$) were measured in 2 wk-cultured hepatic stellate cells ($n = 3-6$, 16-28 cells). Data are presented as the mean \pm SE.

by pretreatment with 1 $\mu\text{mol/L}$ U73122, a phospholipase C inhibitor (0.05 ± 0.05 peak changes of Fura-2 ratio from 0.66 ± 0.12 , $n = 13$). We also observed that $[\text{Ca}^{2+}]_i$ transients induced by 5-HT were not altered in extracellular Ca^{2+} -free conditions (data not shown). These results suggest that 5-HT activates phospholipase C to produce IP_3 , which induces Ca^{2+} release from ER in activated HSCs. To confirm the receptor subtype, we tested blocking effects of a universal 5-HT₂ antagonist, ritanserin, which does not discriminate among 5HT₂ isotypes. 5-HT-induced $[\text{Ca}^{2+}]_i$ responses were attenuated by pretreatment

with 10 $\mu\text{mol/L}$ ritanserin by 46.3% (0.34 ± 0.08 from 0.89 ± 0.10 , $n = 11$).

Upregulation of calcium transporting and binding proteins in the ER

In mammalian cells, there are three major subtypes of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA1, 2, and 3) which pump Ca^{2+} into the ER lumen. We observed SERCA2 to be the dominant subtype in HSCs. SERCA2, especially SERCA2b, is considered to be a house-keeping protein expressed constitutively

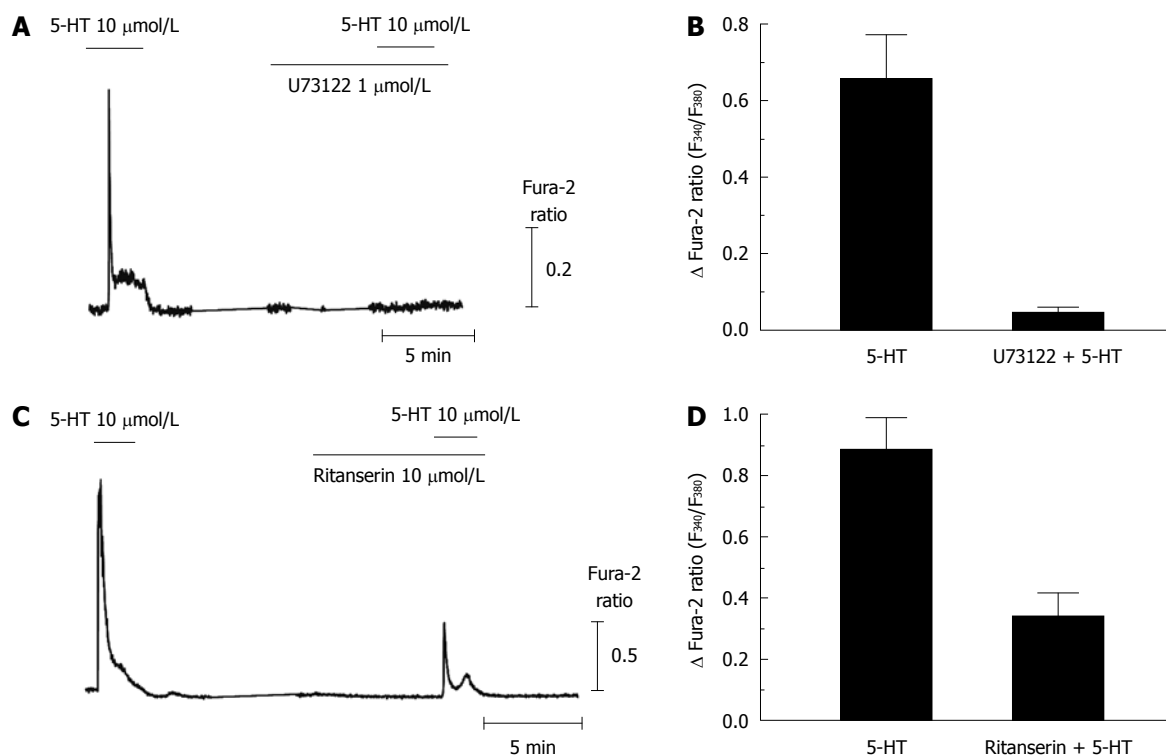


Figure 4 5-hydroxytryptamine-induced intracellular Ca^{2+} concentration transients via metabotropic 5-hydroxytryptamine₂ receptor in activated hepatic stellate cells. A, B: 5-hydroxytryptamine (5-HT)-induced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) transients were completely abolished by pretreatment with U73122 (1 $\mu\text{mol/L}$), a phospholipase C blocker ($n = 3$, 11 cells); C, D: Ritanserin (10 $\mu\text{mol/L}$), a 5-HT₂ antagonist, inhibited the $[\text{Ca}^{2+}]_i$ responses to 5-HT in activated hepatic stellate cells (2 wk-cultured cells; $n = 3$, 13 cells). Data are presented as the mean \pm SE.

in most kinds of cells; however, in HSCs, the expression of SERCA2 tends to increase during activation. Specifically, the relative expression ratio of SERCA2 (SERCA2/GAPDH) at 1 d after isolation was 0.058, and increased to 0.106 after 1 wk in culture and 0.164 after 2 wk in culture *in vitro* (Figure 5A). The expression of SERCA3 was also increased during culture (SERCA3/GAPDH; 0.4×10^{-3} at 1 d and 6.9×10^{-3} at 2 wk).

Among the three isoforms (types 1 through 3) of the IP_3 receptor, the type 1 IP_3 receptor was the main subtype expressed in activated HSCs. We observed that the expression of the type 1 IP_3 receptor increased by about 7-fold ($\text{IP}_3\text{R 1/GAPDH} = 0.037$) after 1 wk of culture, and 20-fold (0.100) after 2 wk of culture compared to (0.005) levels 1 d after isolation (Figure 5B). In contrast, the expression level of ryanodine receptors, which are a family of Ca^{2+} -releasing channel proteins expressed in the ER, either did not change or was decreased during the activation of HSCs (Figure 5C).

We investigated whether Ca^{2+} binding chaperones of the ER could be up-regulated following the activation process of HSCs. There were similar increases in the expression levels of calreticulin (calreticulin/GAPDH; from 0.204 at 1 d to 0.655 at 2 wk), calnexin (calnexin/GAPDH; from 0.240 at 1 d to 0.750 at 2 wk), and calsequestrin in HSCs. In the case of calsequestrin, the expression level in HSCs at 1 d after isolation was undetectable, but was markedly increased (calsequestrin/GAPDH; 0.217) after 2 wk of culturing (Figure 5D).

DISCUSSION

Trans-differentiation of HSCs is accompanied by marked increases in protein synthesis, including collagen, elastin, and glycoproteins^[7]. It is well known that Ca^{2+} homeostasis in the ER is critical for the synthesis, folding, and secretion of protein^[22,23]. In HSCs, the depletion of ER Ca^{2+} stores inhibits protein synthesis and increases intracellular degradation of collagen^[34]. Maintaining a high Ca^{2+} gradient across the ER membrane (around 1000-fold) is accomplished by active Ca^{2+} transport by SERCAs. Among the three different isoforms of SERCAs, SERCA2 is considered to be a house-keeping protein expressed in the ER of most cell types, including HSCs^[34]. We observed that SERCA2 was the main isotype in quiescent and activated HSCs (Figure 5A). During activation, the expression of SERCA2 (and also SERCA3) was increased, which likely helped to maintain appropriate ER Ca^{2+} concentrations.

Chaperone proteins in the ER facilitate the folding of newly synthesized proteins and glycoproteins. In particular, calreticulin and calnexin are important chaperones involved in a “quality control” system for protein synthesis^[35]. In addition, these chaperones act as Ca^{2+} binding proteins in the ER. Overexpression of calreticulin increases the total amount of Ca^{2+} in intracellular stores, whereas calreticulin-deficient cells have reduced ER Ca^{2+} storage capacity^[36]. Impaired collagen synthesis has been observed in cells derived from mice possessing genetic defects in ER chaperone proteins^[37]. In this study, we observed for

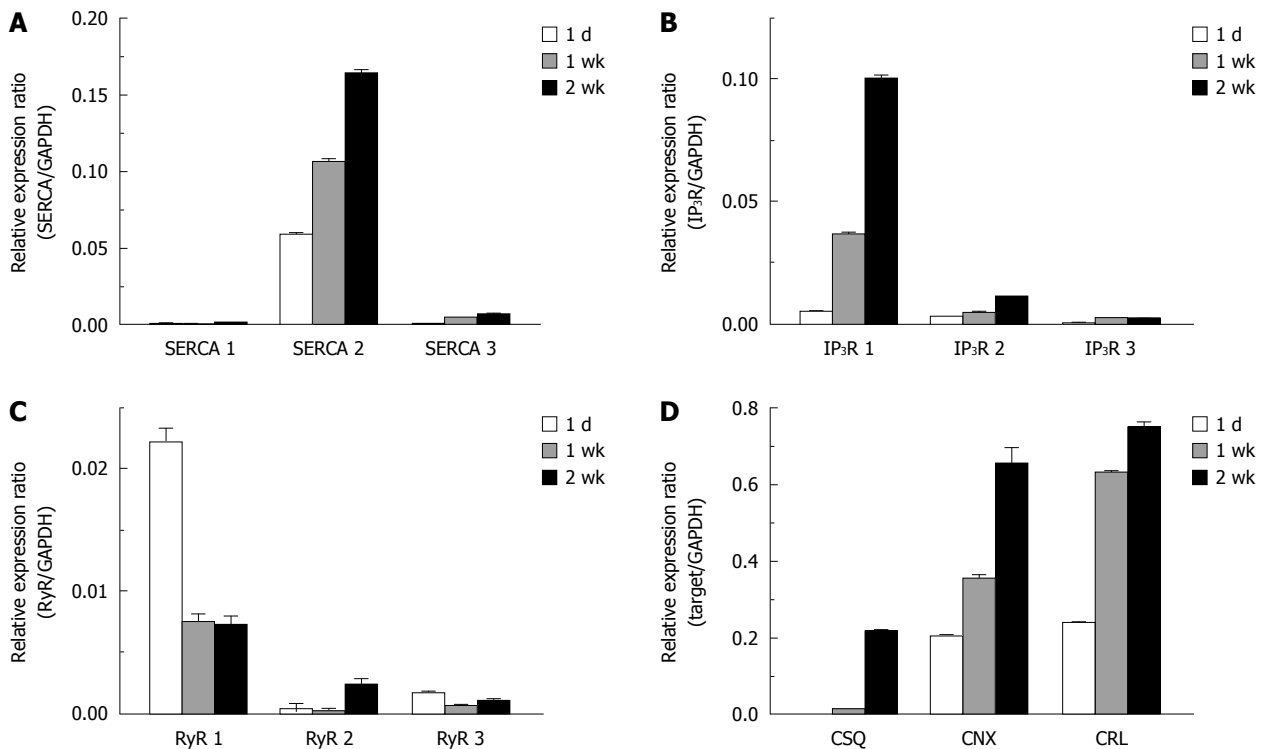


Figure 5 Up-regulation of endoplasmic reticulum Ca²⁺ transporting and binding proteins in activated hepatic stellate cells. Changes in the expression level of 3 isoforms of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA) (A), inositol triphosphate receptor (IP₃R) (B), ryanodine receptor (RyR) (C) and Ca²⁺ binding chaperones (D) during the culture periods (1 d, 1 wk and 2 wk) were measured by quantitative real-time reverse transcription-polymerase chain reaction analysis. Expression levels were normalized to GAPDH and expressed as a relative expression ratio (target/GAPDH). Data are presented as the mean \pm SE ($n = 3$). CSQ: Calsequestrin, CNX: Calnexin, and CRL: Calreticulin.

the first time that the expression of ER Ca²⁺ binding proteins was markedly increased during the activation process of HSCs, which might be an important adaptive change for trans-differentiation.

Upon stimulation from the extracellular space, ER Ca²⁺ is the main source for releasing Ca²⁺ and is responsible for enabling biologic signaling mediated by Ca²⁺. In addition, Ca²⁺ release from the ER stimulates store-operated Ca²⁺ entry into the cytosol, which eventually increases the refilling of the ER Ca²⁺ reservoir. It has been shown that cytosolic Ca²⁺ signaling is important for proliferation and differentiation of HSCs^[25]. Similar to myofibroblast-like cells, activated HSCs can have a contractile response to [Ca²⁺]_i changes, which may increase vascular resistance leading to portal hypertension *in vivo*^[32]. During trans-differentiation, the expression of L-type calcium channels increases, which may contribute to cytosolic Ca²⁺ signaling in HSCs^[26,38]. In the present study, we observed that 5-HT increased [Ca²⁺]_i only in activated HSCs *via* a serotonergic receptor. Until now, 5-HT-induced [Ca²⁺]_i changes have not been reported in HSCs. Physiologic concentrations of 5-HT in plasma are known to be less than 100 nmol/L, but those in cirrhotic patients are significantly elevated (3-4 fold) compared to controls^[39]. Moreover, intrahepatic neighboring cells secrete 5-HT to act as an autocrine/paracrine regulator^[40]. Thus, we hypothesize that local 5-HT concentration close to the releasing cells might be higher than the plasma level and repetitive exposure may

have additive effects on [Ca²⁺]_i-mediated changes in the process of HSC activation.

We observed that 5-HT elicited a [Ca²⁺]_i response *via* the metabotropic 5-HT₂ receptor in activated HSCs. This was demonstrated by the findings that 5-HT-induced [Ca²⁺]_i transients were (1) completely blocked by a PLC inhibitor; (2) not altered by nominally Ca²⁺ free conditions; and (3) reduced by a 5-HT₂ blocker. 5-HT_{2A} is known to mediate mitogenic effects in fibroblasts^[41], while 5-HT_{2B} is involved in the development of the heart and enteric nervous system^[42]. However, we did not discriminate whether the 5-HT_{2A} and/or 5-HT_{2B} receptor mediated the serotonergic Ca²⁺ signaling in activated HSCs. We also observed that the type I IP₃ receptor (IP₃R 1) is the main isoform expressed in activated HSCs, which is consistent with a recent report by Kruglov *et al.*^[32]. The expression level of IP₃R 1 was increased during the activation process (Figure 5B).

Various ligands for G_{q/11}-coupled metabotropic receptors could be important extracellular stimuli, as they generate IP₃ by activating phospholipase-C. Interestingly, it has been reported that the expression of the P2Y metabotropic purinoceptor (P2Y6) is rapidly upregulated following activation of HSCs, with a similar increase in ATP-induced [Ca²⁺]_i transients^[33]. The same study also reported that extracellular UDP increases the transcription of procollagen in activated HSCs *via* activation of the P2Y receptor, and this effect is partially inhibited by a P2Y receptor blocker. These results add further support to the

hypothesis that Ca^{2+} signaling released from ER stores is associated with HSCs undergoing the process of activation. We also observed that ATP increased $[\text{Ca}^{2+}]_i$, which might be mediated by the metabotropic P2Y receptor (Figure 3). However, acetylcholine did not induce calcium changes, indicating that muscarinic acetylcholine receptors do not functionally exist in activated HSCs, even in the presence of machinery for ER Ca^{2+} release.

In this study, we observed the pronounced increase in serotonergic $[\text{Ca}^{2+}]_i$ response related to the upregulation of metabotropic 5-HT₂ receptors, type 1 inositol-5'-triphosphate receptor, type 2 sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, and Ca^{2+} binding ER chaperone proteins following trans-differentiation of HSCs. These changes may be involved in the pathophysiologic (pro-fibrotic) process of rat HSCs as well as being a compensatory mechanism for maintaining ER Ca^{2+} homeostasis and protein synthesis/maturation. Switching on and off of the serotonergic signaling pathway might be implicated in potential treatment for portal hypertension. Yet, the biological relevance of a 5-HT-induced $[\text{Ca}^{2+}]_i$ transient in HSCs remains to be clarified. Moreover, it is not obvious whether simply switching-off this serotonergic signaling is an ideal target for developing treatments for liver cirrhosis. While there is evidence to suggest that 5-HT₂ antagonists reduce proliferation and increase cell death of isolated HSCs^[2,19], a recent study found that fibrotic changes induced by CCl₄ are not ameliorated by a 5-HT₂ antagonist^[29,43]. Further studies to elucidate the detailed role of serotonergic signaling in HSCs are needed in order to develop therapeutic approaches to hepatic fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSCs) are known to initiate hepatic fibrosis by trans-differentiating into myofibroblast-like cells. Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) have been suggested as a stimulus for the activation of HSCs.

Research frontiers

Recent data showed that activated HSCs responded to 5-hydroxytryptamine (5-HT) in a profibrogenic manner, which can be suppressed by 5-HT₂ antagonists. In this study, the authors demonstrated that 5-HT generated $[\text{Ca}^{2+}]_i$ transients released from endoplasmic reticulum (ER) in trans-differentiated HSCs, which was consistent with the upregulation of 5-HT₂ receptors.

Innovations and breakthroughs

Serotonergic $[\text{Ca}^{2+}]_i$ signaling has not been reported in HSCs, until now. It is also a novel finding that the expression of ER Ca^{2+} binding proteins was markedly increased during the activation process of HSCs.

Applications

The identification of $[\text{Ca}^{2+}]_i$ signaling and the expressional changes of Ca^{2+} handling proteins in the process of HSC activation could help us to understand the pathophysiology and develop therapeutic approaches to hepatic fibrosis.

Terminology

IP₃ receptor and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase are ER proteins involved in Ca^{2+} release from, and refilling into, ER. Calsequestrin, calnexin, and calreticulin are ER Ca^{2+} binding chaperone proteins. Upregulation of all these proteins is important not only for $[\text{Ca}^{2+}]_i$ signaling but also for maintaining ER Ca^{2+} levels needed for protein synthesis/maturation.

Peer review

The manuscript by Park *et al* reports the results of investigations on the serotonergic Ca^{2+} signaling, and the expression of 5-HT receptors and Ca^{2+} transporting proteins in rat HSCs. By employing reverse transcription-polymerase chain reac-

tion, and fluorescent (fura-2) and electrophysiological techniques, as well as immunocytochemistry, the authors conclude that the increase in serotonergic $[\text{Ca}^{2+}]_i$ responses accompanied by the upregulation in 5-HT₂ receptors and Ca-transport proteins attests to their role in HSC activation. It is worthy of publication.

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S- Editor Sun H L- Editor Logan S E- Editor Zheng XM

Systematic review on the surgical treatment for T1 gallbladder cancer

Seung Eun Lee, Jin-Young Jang, Chang-Sup Lim, Mee Joo Kang, Sun-Whe Kim

Seung Eun Lee, Department of Surgery, Chung-Ang University College of Medicine, Seoul, 140-757, South Korea
Jin-Young Jang, Chang-Sup Lim, Mee Joo Kang, Sun-Whe Kim, Department of Surgery, Seoul National University College of Medicine, Seoul, 110-744, South Korea

Author contributions: Lee SE, Jang JY, Lim CS, Kang MJ and Kim SW designed research; Lee SE, Kang MJ and Lim CS performed research; Lee SE and Jang JY analyzed the data; Lee SE wrote the paper.

Correspondence to: Jin-Young Jang, MD, PhD, Department of Surgery, Seoul National University College of Medicine, 28 Yeongeong-dong, Jongno-gu, Seoul, 110-744, South Korea. jangjy4@snu.ac.kr

Telephone: +82-2-20722194 Fax: +82-2-7412194

Received: August 12, 2010 Revised: September 9, 2010

Accepted: September 16, 2010

Published online: January 14, 2011

Eight patients (1.1%) with T1a GB cancer and 52 patients (9.3%) with T1b GB cancer died of recurrent GB cancer ($P < 0.01$).

CONCLUSION: Simple cholecystectomy represents the adequate treatment of T1a GB cancer. There is no definite evidence that extended cholecystectomy is advantageous over simple cholecystectomy for T1b GB cancer.

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Key words: Gallbladder; Cancer; Cholecystectomy; Simple; Extended

Peer reviewer: Jutta Elisabeth Lüttges, Professor, Doctor of Medicine and Professor of Pathology, Head of Department of Pathology, General Hospital Saarbrücken, Herchenbacher str.60, Riegelsberg, 66292, Germany

Abstract

AIM: To evaluate the efficacy of simple and extended cholecystectomy for mucosa (T1a) or muscularis (T1b) gallbladder (GB) cancer.

METHODS: Original studies on simple and extended cholecystectomy for T1a or T1b GB cancer were searched from MEDLINE (PubMed), Cochrane Library, EMBase, and CancerLit using the search terms of GB, cancer/carcinoma/tumor/neoplasm.

RESULTS: Twenty-nine out of the 2312 potentially relevant publications met the eligibility criteria. Of the 1266 patients with GB cancer included in the publications, 706 (55.8%) and 560 (44.2%) had T1a and T1b GB cancer, respectively. Simple cholecystectomy for T1a and T1b GB cancer was performed in 590 (83.6%) and 375 (67.0%) patients, respectively ($P < 0.01$). In most series, the treatment of choice was simple cholecystectomy for T1a GB cancer patients with a 5-year survival rate of 100%. Lymph node metastasis was detected in 10.9% of the T1b GB cancer patients and in 1.8% of the T1a GB cancer patients, respectively ($P < 0.01$).

Lee SE, Jang JY, Lim CS, Kang MJ, Kim SW. Systematic review on the surgical treatment for T1 gallbladder cancer. *World J Gastroenterol* 2011; 17(2): 174-180 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/174.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.174>

INTRODUCTION

Gallbladder (GB) cancer confined to mucosa (T1a) or muscularis (T1b) is classified as an early cancer. Because of the high prevalence of advanced stage GB cancer at presentation, early GB cancer has been less studied among all GB cancers. However, on the basis of studies performed on GB cancer, less than 10% are early GB cancers and the proportion is growing because they tend to be diagnosed after laparoscopic cholecystectomy.

Most authors would agree that T1a GB cancer is a local disease and simple cholecystectomy represents its adequate treatment, provided that the resection margin is not involved. It was reported that the incidence of lymph node metastasis of GB cancer is less than 5%^[1-9].

Recurrent GB cancer has been reported only in the form of case reports^[2-4,10-12] and the 5-year survival rate of most GB cancer patients is approaching 100%^[1,3,13-21]. Particularly, laparoscopic cholecystectomy is believed to be the adequate treatment of T1a GB cancer^[4,12,14,16-20,22,23]. Although port site recurrence^[24] and possible tumor seeding caused by pneumoperitoneum^[25] are concerned, few cases have been reported^[10,24].

It has been argued that T1b GB cancer may have spread regionally or systematically at presentation. Therefore, whether T1b GB cancer should be treated with simple cholecystectomy or extended cholecystectomy still remains debatable. Some authors believe that T1b GB cancer should be considered simply as a local disease with the following reasons. First, lymphovascular and perineural invasion and lymph node metastasis are rarely found in patients with pT1b GB cancer^[26]. Second, the outcome of patients with pT1b GB cancer in terms of survival after simple cholecystectomy is excellent and similar to that of patients with pT1a GB cancer when the resection margins are not invaded^[10,17]. Third, extended cholecystectomy does not increase the long-term survival rate of patients with pT1b GB cancer^[15,17]. Fourth, no difference has been observed in survival rates between patients who underwent subsequent laparotomy with additional resection and those who did not^[7,15,17]. On the other hand, others believe that lymphovascular and perineural invasion and lymph node metastasis are more frequently found in patients with pT1b GB cancer^[11,13,14], and pT1b GB cancer recurs more frequently than pT1a GB cancer^[7,11,18]. Extended cholecystectomy increases the long-term survival rate of patients with pT1b GB cancer^[5,11,27].

Few early GB cancer cases are available and randomized trials are difficult to conduct for the assessment of appropriate surgical procedures. Therefore, a pooled systematic analysis of the efficacy of each surgical procedure for early GB cancer is essential to establish the appropriate management of T1a and T1b GB cancer. This study was to evaluate the efficacy of simple and extended cholecystectomy for T1a or T1b GB cancer.

MATERIALS AND METHODS

Search strategy

Original studies on surgery for T1 GB cancer were searched from MEDLINE (PubMed, 1966-2008), Cochrane Library (1996-2008), EMBase (1970-2008), and CancerLit (1970-2008) using the search terms of GB, cancer/carcinoma/tumor/neoplasm.

Selection criteria

Of the identified studies, only publications in the English language were included. Selection was confined to peer-reviewed articles. Unpublished data, abstracts, case reports and case series containing less than 5 patients were excluded. Studies in which the T stage was inadequately assessed and diagnosis was other than adenocarcinoma were excluded. All surgical studies on histologically proven

T1a and T1b GB cancer were included. T1a and T1b GB cancer was defined as a cancer confined to the mucosa to muscularis, respectively. Only studies describing surgical intervention (simple cholecystectomy or extended cholecystectomy, *etc.*) for T1a and T1b GB cancer were included. Only the most recent publications were included when the selected articles included the same or overlapping data in multiple publications.

Data extraction

Two authors (Lee SE and Kang MJ) reviewed each article and performed data extraction independently according to the predefined inclusion criteria. General information pertaining to the study design, patient number and follow-up length were recorded. Types of intervention were classified into simple cholecystectomy and extended cholecystectomy, respectively. Because the extent of extended cholecystectomy varied in each article, we defined it as cholecystectomy, regional lymph node dissection, liver resection beyond wedge resection and/or resection of other organs, respectively. The overall 5-year survival rate of patients with GB cancer was defined as the primary outcome. Secondary outcomes included mortality, morbidity, and recurrence of GB cancer. The extracted data were then cross-checked by the two authors to eliminate the discrepancy.

Statistical analysis

Data are presented as median (range) unless otherwise stated. Interpretative analyses were performed based on pooled as opposed to individuals. Since patient data could not account for censored, missing or incomplete follow-up, survival analyses could not be conducted based on these data.

RESULTS

Search results

Overall, the search identified 2312 potentially relevant publications. After the titles and abstracts were reviewed, 252 publications were potentially eligible and full text reviews were conducted. Finally, 29 studies^[1,3-22,24,26-32] that specifically assessed the surgical outcomes of patients with T1a and T1b GB cancer met the eligible criteria (Figure 1). There were no randomized studies and all studies were retrospective in nature. Furthermore, the majority of studies involved a small number of patients (median 15, range 5-89) except for nation-wide surveys^[1,5,7,16] (Table 1).

Characteristics of the study population

Twenty-nine publications included 1266 patients with T1 GB cancer. Of these 1266 patients, 706 (55.8%) and 560 patients (44.2%) had T1a and T1b GB cancer, respectively.

Types of surgical intervention

The operative procedures performed in the 1266 patients are summarized in Table 2. Simple cholecystectomy for T1a and T1b GB cancer was performed in 590 (83.6%) and 375 (67.0%) patients, respectively ($P < 0.01$). Ex-

Table 1 Characteristics of included studies on surgical treatment of patients with T1 gallbladder cancer

Authors	Yr	No. of patients	Primary outcome		Secondary outcome		
			3 YSR	5 YSR	Morbidity	Mortality	Recurrence
Ogura <i>et al</i> ^[13]	1991	366 ¹					NS
Shirai <i>et al</i> ^[26]	1992	89	NS	NS	NS		
Ouchi <i>et al</i> ^[11]	1994	15	NS		NS		
Chijiwa <i>et al</i> ^[28]	1994	5	NS		NS		
de Aretxabala <i>et al</i> ^[14]	1997	24	NS		NS		NS
Mori <i>et al</i> ^[22]	1997	9	NS	NS	NS		
Benoist <i>et al</i> ^[1]	1998	36 ²					
Z'graggen <i>et al</i> ^[29]	1998	9		NS	NS		
Shimada <i>et al</i> ^[30]	2000	17			NS		
Suzuki <i>et al</i> ^[30]	2000	25	NS		NS		
Wakai <i>et al</i> ^[6]	2001	25	NS		NS		
Puhalla <i>et al</i> ^[24]	2002	9		NS			
Waghlikar <i>et al</i> ^[7]	2002	14	NS		NS		
Kim <i>et al</i> ^[17]	2002	19	NS		NS		
Ouchi <i>et al</i> ^[18]	2002	234 ³	NS		NS		
Wakai <i>et al</i> ^[10]	2002	15	NS		NS		
Cucinotta <i>et al</i> ^[4]	2005	12	NS	NS	NS		
Yildirim <i>et al</i> ^[15]	2005	13					
Eguchi <i>et al</i> ^[31]	2005	12	NS	NS	NS		
Sun <i>et al</i> ^[19]	2005	15	NS		NS		
Otero <i>et al</i> ^[32]	2006	51	NS		NS		
Yagi <i>et al</i> ^[8]	2006	13	NS		NS		
Chan <i>et al</i> ^[12]	2006	33	NS	NS	NS		
Cangemi <i>et al</i> ^[27]	2006	15	NS				
Kang <i>et al</i> ^[16]	2007	11	NS				
You <i>et al</i> ^[3]	2008	52	NS				
Kwon <i>et al</i> ^[20]	2008	20	NS		NS		
Kohya <i>et al</i> ^[21]	2008	15	NS		NS		NS
Goetze <i>et al</i> ^[5]	2008	93 ⁴	NS		NS		

¹Japan survey from 172 major hospitals; ²French cooperative group AURC (Association Universitaire de Recherche en Chirurgie);

³Japan nationwide survey on laparoscopic cholecystectomy from 253 hospitals; ⁴German Registry of Incidental Gallbladder Carcinoma of the German Society of Surgery. NS: Not stated.

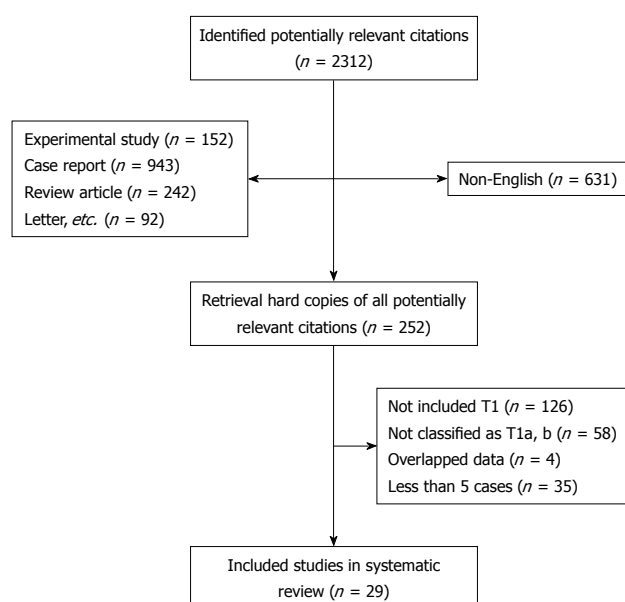


Figure 1 Flowchart of included articles.

tended cholecystectomy for T1a and T1b GB cancer was performed in 110 (15.6%) and 168 (30.0%) patients, respectively ($P < 0.01$).

Postoperative morbidity and mortality

Five articles (17.2%)^[1,3,15,16,24] discussed the postoperative morbidity related to the operative procedure. Complications occurred in 11 patients (11/52, 21.2%) following simple cholecystectomy, and in 21 patients (21/75, 28%) after extended cholecystectomy. Five postoperative deaths (1.0%)^[1,24] occurred after simple cholecystectomy and the causes of death included acute myocardial infarction and pulmonary embolism. Seven postoperative deaths (1.5%)^[1,10,11,24] occurred after extended cholecystectomy. Three patients died of co-morbidities of the disease, two patients died of hemorrhage, one patient died of peritonitis and one died of liver failure.

Lymph node metastasis

Of the 29 reviewed articles, information on lymph node metastasis was available from 17 publications (58.6%)^[3,5-7,10,11,13-18,24,26-28,31] (Table 3). Lymph node metastasis was found in 1.8% and 10.9% of T1a and T1b GB cancer patients, respectively ($P < 0.01$). Of the 17 publications, only 5 included lymph node metastasis cases^[3,5,13,15,17] (Table 4).

Recurrence

Information pertaining GB cancer recurrence was available from 26 out of 29 articles (89.76%)^[1,3,5-8,10-13,15-22,24,26-30,32].

Table 2 Surgical procedures for mucosa and T1b gallbladder cancer *n* (%)

	T1a (<i>n</i> = 706)	T1b (<i>n</i> = 560)	<i>P</i> value	Total (<i>n</i> = 1266)
Simple cholecystectomy	590 (83.6)	375 (67.0)	< 0.01	965 (76.2)
Open cholecystectomy	321 (54.4)	286 (76.3)	< 0.01	607 (62.9)
Laparoscopic cholecystectomy	269 (45.6)	89 (23.7)		358 (37.1)
Extended cholecystectomy	110 (15.6)	168 (30.0)	< 0.01	278 (22.0)
2nd operation	16 (14.5)	43 (25.6)		59 (21.2)
Major hepatectomy	6 (0.8)	16 (2.9)	NS	22 (1.7)
Pancreatoduodenectomy	0	1 (0.2)	NS	1

NS: Not stated.

Table 3 Lymph node metastasis of T1 gallbladder cancer *n* (%)

	T1a (<i>n</i> = 280)	T1b (<i>n</i> = 276)	<i>P</i> value	Total (<i>n</i> = 556)
Lymphovascular invasion	7 (2.5)	33 (12.0)	< 0.01	40 (7.2)
Perineural invasion	1 (0.4)	7 (2.5)	NS	8 (1.4)
Lymph node metastasis	5 (1.8)	30 (10.9)	< 0.01	35 (6.3)

NS: Not stated.

Table 4 Studies reporting lymph node metastasis of T1 gallbladder cancer *n* (%)

	T1a	T1b	Odds ratio	95% CI
Goetze <i>et al</i> ^[5]	0/21	1/72 (1.4)	1.296	1.159-1.448
Yildirim <i>et al</i> ^[13]	0/5	1/8 (12.5)	1.714	1.063-2.765
You <i>et al</i> ^[3]	0/27	2/25 (8.0)	2.174	1.610-2.935
Kim <i>et al</i> ^[17]	0/10	1/9 (11.1)	2.250	1.342-3.771
Ogura <i>et al</i> ^[13]	5/201 (2.5)	25/165 (15.2)	7.000	2.616-18.733

Eight patients (1.1%) with T1a GB cancer^[3,4,10-12,18,26] and 52 patients (9.3%) with T1b GB cancer^[4-7,11,12,17-19,27,32] died of recurrent GB cancer ($P < 0.01$). The studies reporting recurrent GB cancer are listed in Table 5. Cases of T1a GB cancer recurrence and the recurrence pattern of T1b GB cancer are summarized in Tables 6 and 7, respectively.

Survival rate

Twenty-one publications^[1,3,5-11,13-21,27,30,32] described the 5-year survival rate of GB cancer patients. The 5-year survival rate of patients with T1a and T1b GB cancer is 45%-100% and 37.5%-100%, respectively (Table 8).

Only 6 publications^[5,6,11,15,17,27] compared the survival rates of patients after simple cholecystectomy or extended cholecystectomy. No significant difference was observed in the survival rate of patients with T1a GB cancer after different operative procedures. However, 3 publications^[5,11,27] showed a significantly longer survival time of patients with T1b GB cancer after extended cholecystectomy (Table 9).

Outcome of patients after laparoscopic cholecystectomy and open cholecystectomy

Only two studies^[4,12] compared the survival rates of patients with T1 GB cancer after laparoscopic cholecystectomy and open cholecystectomy. No significant difference

Table 5 Studies reporting recurrence cases of T1 gallbladder cancer *n* (%)

	T1a	T1b	Odds ratio	95% CI
You <i>et al</i> ^[3]	1/27 (3.7)	0/25	0.510	0.39-0.67
Shirai <i>et al</i> ^[26]	2/78 (2.6)	0/11	0.874	0.81-0.95
Wakai <i>et al</i> ^[10]	1/13 (7.7)	0/2	0.923	0.79-1.08
Z'graggen <i>et al</i> ^[29]	0/3	1/6 (16.7)	1.200	0.84-1.72
Wagholikar <i>et al</i> ^[7]	0/2	5/12 (41.7)	1.286	0.91-1.82
Goetze <i>et al</i> ^[5]	0/21	14/72 (19.4)	1.362	1.19-1.56
Cangemi <i>et al</i> ^[27]	0/4	5/11 (45.4)	1.667	1.01-2.77
Kim <i>et al</i> ^[17]	0/10	1/9 (11.1)	2.250	1.34-3.77
Chan <i>et al</i> ^[12]	1/14 (7.1)	3/19 (15.8)	2.438	0.23-26.29
Otero <i>et al</i> ^[32]	0/25	9/26 (34.6)	2.471	1.71-3.57
Ouchi <i>et al</i> ^[11]	1/8 (12.5)	3/7 (42.9)	5.250	0.40-68.94
Ouchi <i>et al</i> ^[18]	1/167 (0.6)	3/67 (4.5)	7.781	0.79-76.19
Cucinotta <i>et al</i> ^[4]	1/5 (20)	6/7 (85.7)	24.000	1.14-505.2
Wakai <i>et al</i> ^[6]	0	2/25 (8.0)		

was observed in the survival rate of patients after the two operative procedures. However, it must be emphasized that the total number of patients included was small. In addition, 6 studies^[17-20,22,30] evaluated the safety of laparoscopic cholecystectomy on the basis of recurrent GB cancer and survival rate of GB cancer patients, showing a low recurrence rate and a high survival rate (Table 10). Meta-analysis could not be performed because no studies reported the hazard ratio for overall survival rate according to the surgical procedure.

DISCUSSION

This review evaluated the surgical outcomes of patients with T1 GB cancer. The evidence was of low quality, because it was obtained from the reviewed articles, and most studies were case series. No study was controlled, and all reviewed articles were retrospective in nature. Furthermore, such studies usually involved a small number

Table 6 Recurrence cases of T1a gallbladder cancer after surgical treatment (*n* = 8)

	Operation	Time of recurrence (mo)/site	Survival after op (mo)
Shirai <i>et al</i> ^[26]	NA ¹	NA/CBD	76
	NA ¹	NA/CBD	66
Ouchi <i>et al</i> ^[11]	Simple cholecystectomy	NA/CBD	45
Ouchi <i>et al</i> ^[18]	Laparoscopic cholecystectomy ²	NA/NA	NA
Wakai <i>et al</i> ^[10]	Laparoscopic cholecystectomy	NA/P. seeding	52
Cucinotta <i>et al</i> ^[4]	Laparoscopic cholecystectomy	7/Port site	20
Chan <i>et al</i> ^[12]	Laparoscopic cholecystectomy	48.3/liver, lung	51
You <i>et al</i> ^[3]	Laparoscopic cholecystectomy	3/CBD	19

¹Cystic duct margin (+) patients; ²Gallbladder perforation during laparoscopic cholecystectomy. CBD: Common bile duct; NA: Not available.

Table 7 Recurrence pattern of T1b gallbladder cancer after surgical treatment *n* (%)

	Simple cholecystectomy (<i>n</i> = 375)	Extended cholecystectomy (<i>n</i> = 185)
Loco-regional recurrence	12 (50.0)	
CBD	3 (6.4)	
Lymph node	5 (10.6)	
Port site	4 (8.5)	
Systemic recurrence	12 (50.0)	3 (60)
Liver	7 (14.9)	2 (40)
Peritoneal seeding	4 (8.5)	
Lung	1 (2.0)	1 (20)
Unknown	23 (48.9)	2 (40)
Total ^b	47 (12.5)	5 (2.7)

^b*P* < 0.01. CBD: Common bile duct.

Table 8 Five-year survival rates of patients with T1a and T1b gallbladder cancer after surgical treatment

	N	5 YSR (%)			
	T1 (T1a/T1b)	T1a (SC/EC)	T1b (SC/EC)	T1 (SC/EC)	
Benoist <i>et al</i> ^[1]	36 ¹ (13/23)	45	44	44	
You <i>et al</i> ^[3]	52 (27/25)	96.3	96	96.2	
de Aretxabala <i>et al</i> ^[14]	24 (11/13)	100	75	75	
Kang <i>et al</i> ^[16]	11 (3/8)	100	100	100	
Sun <i>et al</i> ^[19]	15 (10/5)	100	100	100	
Kwon <i>et al</i> ^[20]	20 (18/2)	100	100	100	
Kohya <i>et al</i> ^[21]	15 (8/7)	100	100	100	
Yildirim <i>et al</i> ^[15]	13 (5/8)	100	80	90	
		(100/100)	(50/100)		
Kim <i>et al</i> ^[17]	19 (10/9)	100	100	100	
		(100/100)	(100/100)		
Ouchi <i>et al</i> ^[11]	15 (8/7)	(71/100)	(42/100)		
Cangemi <i>et al</i> ^[27]	15 (4/11)	100	(37.5/100)		
Ogura <i>et al</i> ^[13]	366 (201/165)	82.6	72.5		
Ouchi <i>et al</i> ^[18]	234 (167/67)	99	95		
Otero <i>et al</i> ^[32]	51 (25/26)	70			
Goetze <i>et al</i> ^[5]	93 (21/72)		(42/79)		
Wakai <i>et al</i> ^[6]	25 (0/25)		87 (100/75)		
Wagholikar <i>et al</i> ^[7]	14 (2/12)			68	
Shimada <i>et al</i> ^[9]	17 (10/7)			86.7	
Eguchi <i>et al</i> ^[31]	25 (19/6)			92	
Wakai <i>et al</i> ^[10]	15 (13/2)			(90/100)	
Yagi <i>et al</i> ^[8]	13 (12/1)			100	

¹All patients underwent simple cholecystectomy. SC: Simple cholecystectomy; EC: Extended cholecystectomy.

Table 9 Five-year survival rates of patients with T1b gallbladder cancer after different surgical procedures

	N (SC/EC)	5 YSR (%)		P value
		SC	EC	
Wakai <i>et al</i> ^[6]	25 (17/8)	100	75	NS
Kim <i>et al</i> ^[17]	9 (6/3)	100	100	NS
Yildirim <i>et al</i> ^[15]	8 (5/3)	50	100	NS
Ouchi <i>et al</i> ^[11]	7 (5/2)	42	100	< 0.05
Cangemi <i>et al</i> ^[27]	11 (8/3)	37.5	100	< 0.01
Goetze <i>et al</i> ^[5]	72 (49/23)	42	79	0.03

NS: Not significant; SC: Simple cholecystectomy; EC: Extended cholecystectomy.

Table 10 Clinical outcome of laparoscopic cholecystectomy for T1a and T1b gallbladder cancer

	N		Recurrence		5 YSR (%)	
	T1a	T1b	T1a	T1b	T1a	T1b
Mori <i>et al</i> ^[22]	7	2	0	0		
Kim <i>et al</i> ^[17]	9	6	0	1 (port site)	100	100
Sun <i>et al</i> ^[19]	10	5	0	0	100	100
Eguchi <i>et al</i> ^[31]	13	5	0	0	100	100
Kwon <i>et al</i> ^[20]	18	2	0	0	100	100
Ouchi <i>et al</i> ^[18]	167	67	1 ¹	3 ¹	99	95

¹Site was not described.

of patients, making it difficult to draw a statistically sound conclusion. The inclusion of heterogeneous groups of patients who underwent surgery at different centers by different specialists also made interpretation of results challenging. However, the relatively low incidence of this condition makes it impractical to conduct adequately powered randomized controlled trials to compare different surgical and/or nonsurgical interventions. Therefore, a systematic review of evidence despite a lower scientific level was needed.

Simple cholecystectomy for T1a GB cancer was performed in 84% of patients. Of these patients, 46% underwent laparoscopic cholecystectomy. Lymph node metastasis was found only in 1.8% GB cancer patients with a recurrence rate of 1.1%. Because 50% of the recurrence occurred in the common bile duct, pathological confirmation of cystic duct margin would be important. If cystic duct margin was proved to be positive for GB cancer,

resection of the common bile duct should be performed. Although the 5-year survival rate of patients with T1a GB cancer was 45%-100%, the publications^[1,11,32] showing a low survival rate did clarify that most deaths were not related to GB cancer. The remaining publications reported a 5-year survival rate of over 90%, indicating that simple cholecystectomy is the adequate surgical treatment of T1a GB cancer. In particular, recent reports^[17-20,30] showed a 100% 5-year survival rate of patients with GB cancer following laparoscopic cholecystectomy, indicating that laparoscopic cholecystectomy is a safe procedure for GB cancer. However, because a report^[10] on peritoneal seeding due to GB perforation during laparoscopic cholecystectomy and a report^[4] on port site recurrence following laparoscopic cholecystectomy, careful dissection to avoid perforation of GB should be performed and GB should be retrieved using a plastic bag.

Simple cholecystectomy and extended cholecystectomy for T1b GB cancer were performed in 67% and 30% patients, respectively. More patients with T1b GB cancer underwent extended cholecystectomy ($P < 0.01$). Despite a lack of evidence, many authors agreed that aggressive approach would be needed for T1b GB cancer. This review validated the rationale behind this approach to a certain extent. Lymph node metastasis of T1b GB cancer was 11%, which was significantly higher than that (2%) of T1a GB cancer ($P < 0.01$). The publications^[3,5,13,15,17] reporting lymph node metastasis of T1a and T1b GB cancer showed that the incidence of lymph node metastasis of T1b GB cancer is 1.3-7 times higher than that of T1a GB cancer (Table 4). The recurrence rate of T1b GB cancer was 9%, which was significantly higher than that (1%) of T1a GB cancer ($P < 0.01$). The publications^[3-7,10-12,17,18,26,27,29,32] reporting the recurrence rate of T1a and T1b GB cancer showed that the recurrence rate of T1b GB cancer is 1.3-24 times higher than that of T1a GB cancer (Table 7). In addition, the recurrence rate of GB cancer was higher after simple cholecystectomy than after extended cholecystectomy (12.5% *vs* 2.7%, $P < 0.01$). Although the recurrence sites were not available in 50% of the cases reviewed, no patient showed loco-regional recurrence after extended cholecystectomy in the remaining cases. The 5-year survival rate of patients with T1b GB cancer was 37.5%-100%. Studies^[1,5,11,15,27] showing a 5-year survival rate of less than 50% discussed simple cholecystectomy cases. Of these publications, 3^[5,11,27] showed a significantly higher survival rate after extended cholecystectomy than after simple cholecystectomy. However, the total number of patients included was too small to comment on statistical significance. Except for these studies, the rest showed a 5-year survival rate of over 80% for patients with GB cancer irrespective of surgical procedure. Although there was no definite evidence that extended cholecystectomy was advantageous over simple cholecystectomy in this review, regional lymph node dissection should be included in any surgical procedure for T1b GB cancer considering that the lymph node metastasis rate was relatively high. Although the recurrence rate of GB cancer was high and the survival rate of GB cancer

patients was low after simple cholecystectomy, extended cholecystectomy was recommended for T1b GB cancer in several guidelines^[33,34]. In view of the inconclusive results obtained from this review, multicenter prospective studies should be performed to clarify the surgical strategy for T1b GB cancer.

In conclusion, T1 GB cancer should be treated based on the outcomes of this review. Simple cholecystectomy for T1a GB cancer is the adequate treatment and laparoscopic cholecystectomy is a safe procedure for T1b GB cancer, provided the port site recurrence is considered. Careful dissection should be conducted considering that the peritoneal seeding is associated with bile spillage. Resection margin of cystic duct should be confirmed histopathologically with possible common bile duct resection in mind. There is no definite evidence that extended cholecystectomy is advantageous over simple cholecystectomy for T1b GB cancer. Because lymph node metastasis is considerable, regional lymphadenectomy should be performed for the treatment and staging of GB cancer.

COMMENTS

Background

Most authors agree that mucosa (T1a) gallbladder (GB) cancer is a local disease and simple cholecystectomy represents its adequate treatment provided that the resection margin is not involved. It has been argued that muscularis (T1b) GB cancers may have spread regionally or systematically at presentation. Therefore, whether T1b cancers should be treated with simple cholecystectomy or extended cholecystectomy still remains debatable.

Research frontiers

Few early GB cancer cases are available and randomized trials are difficult to conduct for the assessment of appropriate surgical procedures. Therefore, a pooled systematic analysis of the efficacy of each surgical procedure for early GB cancer is essential to establish the appropriate management of T1a and T1b GB cancer.

Innovations and breakthroughs

Original published studies on surgery for T1 GB cancer were searched from MEDLINE (PubMed, 1966-2008), Cochrane Library (1996-2008), EMBase (1970-2008), and CancerLit (1970-2008).

Applications

Simple cholecystectomy for T1a GB cancer is its adequate treatment and laparoscopic cholecystectomy is safe procedure for T1b GB cancer provided the port site recurrence is considered. There is no definite evidence that extended cholecystectomy is advantageous over simple cholecystectomy for T1b GB cancer.

Terminology

GB cancer confined to T1a or T1b is as an early cancer.

Peer review

This review evaluated the surgical outcomes of T1 GB cancer. The evidence is of low quality, because it was obtained from the reviewed articles, and most studies were case series. No study was controlled and all reviewed articles were retrospective in nature. Furthermore, such studies usually involved a small number of patients, making it difficult to draw a statistically sound conclusion. The inclusion of heterogeneous groups of patients who underwent surgery at different centers by different specialists also made interpretation of results challenging. However, the relatively low incidence of GB cancer makes it impractical to conduct adequately powered randomized controlled trials comparing different surgical and/or nonsurgical interventions. Therefore, systematic review of evidence despite a lower scientific level is needed.

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S- Editor Sun H L- Editor Wang XL E- Editor Zheng XM

LY294002 potentiates the anti-cancer effect of oxaliplatin for gastric cancer *via* death receptor pathway

Jie Liu, Xue-Qiong Fu, Wei Zhou, Hong-Gang Yu, Jie-Ping Yu, He-Sheng Luo

Jie Liu, Xue-Qiong Fu, Wei Zhou, Institute of Gastroenterology and Hepatology, Wuhan University Medical School, Wuhan 430060, Hubei Province, China

Hong-Gang Yu, Jie-Ping Yu, He-Sheng Luo, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Author contributions: Liu J, Zhou W and Fu XQ performed the majority of experiments; Yu HG provided vital reagents and analytical tools and was also involved in editing the manuscript; Luo HS and Yu JP designed the study and provided financial support for this work; Liu J wrote the manuscript.

Supported by The National Natural Science Foundation of China, No. 30470782

Correspondence to: He-Sheng Luo, Professor, Department of Gastroenterology, Renmin Hospital of Wuhan University, 238 Jiefang Street, Wuhan 430060, Hubei Province, China. luotangwh@gmail.com

Telephone: +86-27-88042134 Fax: +86-27-88042292

Received: September 14, 2010 Revised: December 14, 2010

Accepted: December 21, 2010

Published online: January 14, 2011

tion and cell apoptosis *in vitro*, and increased tumor growth inhibition and cell death in the tumor mass *in vivo*. In MKN45 and AGS cells, oxaliplatin treatment promoted both protein kinase B (Akt) and NFκB activation, while pretreatment with LY294002 significantly attenuated oxaliplatin-induced Akt activity and NFκB binding. LY294002 promoted oxaliplatin-induced Fas ligand (FasL) expression, Fas-associated death domain protein recruitment, caspase-8, Bid, and caspase-3 activation, and the short form of cellular caspase-8/FLICE-inhibitory protein (c-FLIPs) inhibition. *In vivo*, LY294002 inhibited oxaliplatin-induced activation of Akt and NFκB, and increased oxaliplatin-induced expression of FasL, inhibition of c-FLIPs, and activation of caspase-8, Bid, and caspase-3.

CONCLUSION: Combination of oxaliplatin and LY294002 was therapeutically promising for gastric cancer treatment. The enhanced sensitivity of the combined treatment was associated with the activation of the death receptor pathway.

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Abstract

AIM: To examine the effects of combined treatment of oxaliplatin and phosphatidylinositol 3'-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) for gastric cancer.

METHODS: Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptotic cells were detected by flow cytometric analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Western blotting and immuno-precipitation were used to examine protein expression and recruitment, respectively. Nuclear factor κB (NFκB) binding activities were investigated using electrophoretic mobility shift assay. Nude mice were used to investigate tumor growth.

RESULTS: Treatment with combined oxaliplatin and LY294002 resulted in increased cell growth inhibi-

Key words: Gastric cancer; Oxaliplatin; Phosphatidylinositol 3'-kinase/Akt pathway; Death receptor pathway; Apoptosis; LY294002

Peer reviewers: Jun-Hyeog Jang, Professor, Chief, Department of Biochemistry, Inha University School of Medicine, Jung-Gu, Incheon 400-712, South Korea; Long-Bin Jeng, MD, Organ Transplantation Center, China Medical University Hospital, 2 Yuh-Der Road, Taichung 40447, Taiwan, China; Tatsuo Kanda, MD, PhD, Division of Digestive and General Surgery, Graduate School of Medical and Dental Sciences, Niigata University, Niigata City 951-8510, Japan

Liu J, Fu XQ, Zhou W, Yu HG, Yu JP, Luo HS. LY294002 potentiates the anti-cancer effect of oxaliplatin for gastric cancer *via* death receptor pathway. *World J Gastroenterol* 2011; 17(2): 181-190 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/181.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.181>

INTRODUCTION

Gastric cancer is the second leading cause of cancer death in the world and the top lethal cancer in Asia^[1]. The management of gastric cancer is usually a multi-approach involving surgery, chemotherapy, and radiotherapy. Approximately half of gastric cancer patients present with non-operable tumors^[2]. As such, besides surgical resection, chemotherapy is the main adjuvant treatment for postoperative and advanced gastric cancer^[2]. Combined chemotherapy regimens are currently accepted as the first-line treatment for this disease^[3].

Oxaliplatin, a third-generation platinum coordination complex of the 1,2-diaminocyclohexane families, generates covalent adducts between platinum and two adjacent guanines or guanine and adenine in cell DNA, which leads to disruption of DNA replication and transcription^[4,5]. Oxaliplatin was shown to be effective in the treatment of advanced gastric cancer when combined with 5-fluorouracil and leucovorin, and has also been used in adjuvant chemotherapy for gastric cancer. Despite the improvement in the efficacy of chemotherapeutic drugs used in the treatment of metastatic gastric cancer, the response rates in the advanced diseases are approximately 47.9% for the most effective drug combinations, and the vast majority of patients relapse, with a median survival of only 11.2 mo^[6]. Recently, the combination of chemotherapy and a targeted therapeutic agent was shown to be promising for the treatment of advanced gastric cancer.

Several studies have reported that protein kinase B (Akt) is a key molecule for protecting cells from apoptosis, likely due to phosphorylation and inactivation of a variety of key pro-apoptotic targets. The Akt-mediated survival-signaling pathway is an attractive target for cancer chemotherapy^[7-10]. In gastric cancer, over expression and activation of Akt have also been detected, and anomalous expression of Akt induces cell survival^[7,11]. In addition, inhibition of Akt activity stimulates apoptosis and enhances the sensitivity of gastric cancer to chemotherapy in a variety of mammalian cells^[12-14].

In the present study, we examined the role of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) in augmenting the anti-cancer effects of oxaliplatin in gastric cancer. We found that LY294002 sensitizes gastric cancer cells to oxaliplatin in both *in vitro* and *in vivo* studies. Furthermore, the death receptor pathway was involved in regulating Akt-mediated apoptosis in response to chemotherapy in gastric cancer.

MATERIALS AND METHODS

Cell culture

Human gastric carcinoma cell lines MKN45 and AGS were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All the cell lines were cultured in RPMI 1640 medium (GIBCO, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 10 U/mL penicillin,

and 10 µg/mL streptomycin in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Antibodies and reagents

Phosphatidylinositol 3'-kinase (PI3K) inhibitor (LY294002) and oxaliplatin were purchased from Alexis Biochemicals (San Diego, CA, USA). The primary antibodies against human Akt1, phosphorylated Akt at Ser⁴⁷³ (phospho-AktSer⁴⁷³), phospho-AktThr³⁰⁸ (Cell Signaling Technology, Beverly, MA, USA), short form of cellular caspase-8/FLICE-inhibitory protein (c-FLIPs), long form of c-FLIP (c-FLIP_L), Fas ligand (FasL), Fas, Fas-associated death domain protein (FADD), caspase-8, caspase-3, Bid, nuclear factor κB (NFκB)-p65 and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in Western blotting. The primary antibodies against human phospho-AktSer⁴⁷³, NFκB-p65, FasL, active caspase-8, t-Bid, c-FLIPs, and active caspase-3 (Cell Signaling Technology, Beverly, MA, USA) were used in immunohistochemistry.

Cell transfection

FasL siRNA was purchased from Santa Cruz Biotechnology. MKN45 and AGS cells were transiently transfected with FasL siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. FasL expression was detected by Western blotting.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells (4×10^3 cells/well) were plated in 96-well plates in 100 µL of RPMI 1640 without FBS, and incubated for 24 h. Various concentrations (0-4 µmol/L) of the anticancer drugs were added to the culture medium. The viability of cells was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturers' specifications (Roche Applied Science, Indianapolis, IN, USA). Briefly, MTT was added at a concentration of 500 mg/L, and cells were incubated for 4 h at 37°C. The absorbance readings of each well were determined using a computer-controlled microtiter plate reader at 570 nm wavelength. The percentage cell survival was defined as the relative absorbance of untreated vs treated cells.

Apoptosis analysis

Cells were treated with various concentrations (0-20 µmol/L) of anticancer drugs and suspended at chosen time points (24 h). Next, 2×10^6 cells were centrifuged and washed twice with ice-cold phosphate-buffered saline. Apoptotic cells were detected by flow cytometry using Annexin V-Fluorescein and propidium iodide (Molecular Probes, Invitrogen, Eugene, OR, USA).

Western blotting and immunoprecipitation

Cells were lysed in ice-cold lysis buffer (25 mmol/L Tris/HCl, pH 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L Na₃VO₄, 50 mmol/L β-glycerophosphate, 10 mmol/L

NaF, 1% Triton X-100, and 0.5 mmol/L phenylmethyl sulfonylfluoride) containing a protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). Protein concentration was determined by Protein Assay (Bio-Rad laboratories, Hercules, California, USA). Western blotting was performed and subjected to the standard protocol. Total cellular proteins (40 µg protein) were separated on SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad laboratories). Anti-actin antibody was used to ascertain equal loading of protein. Specific antibodies diluted in TBS-T containing 5% nonfat milk were used to detect indicated proteins. The appropriate horseradish peroxidase (HRP) conjugated secondary antibodies were used at 1:3000 for all antibodies. Positive antibody reactions were detected with the enhanced chemoluminescence system and Hyperfilm X-ray film.

For immunoprecipitation of the Fas death-inducing signaling complex (DISC), cells were lysed and the lysate (300 mg protein/sample) was incubated with 0.4 mg anti-Fas antibody overnight at 4°C. Immunoprecipitates were separated by 10% SDS-PAGE and immunoblotted with anti-FADD.

Electrophoretic mobility shift assay

NFκB binding assays were performed using nuclear extracts and biotin-labeled NFκB oligonucleotides (Panomics, Fremont, CA, USA). Electrophoretic mobility shift assay (EMSA) was performed using an EMSA Gel-Shift Kit. For EMSA, an equal amount of nuclear extracts was incubated for 30 min with an NFκB-specific 32P-labeled oligonucleotide and binding mix as described previously^[15]. Samples were electrophoresed at 100 V and 4°C, transferred to Biotodyne nylon membranes (Pierce Biotechnology, Rockford, IL, USA), and then cross-linked in an ultraviolet cross-linker (Stratagene Inc., La Jolla, CA, USA). Protein gels were visualized using streptavidin-HRP followed by chemiluminescence detection. The nucleotide sequence of biotin-labeled NFκB was 5'-AGCTATGTGGGTTTTCCTCATGAGC-3'.

In vivo assay for tumor growth

MKN45 (5×10^6) was implanted subcutaneously into the flank of nude mice (6 in each group, male BALB/c nu/nu, 4-6 wk of age) (Institute of Materia Medica, CAS, Shanghai, China). When the tumors were 100-150 mm³ in size, oxaliplatin (1.3 mg/kg) and/or LY294002 (25 mg/kg) were injected into the intraperitoneal space every four days. Tumor growth was monitored by measuring tumor volume, which was calculated by the formula: $V \text{ (mm}^3\text{)} = \text{width}^2 \text{ (mm}^2\text{)} \times \text{length (mm)}/2$. The mice were sacrificed 6 wk later, and tumors were harvested and evaluated with hematoxylin and eosin and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The expression of phospho-AktSer⁴⁷³, p65 subunit of NFκB (NFκB-p65), and several proteins in the death receptor pathway was examined by immunohistochemistry as described previously^[16].

TUNEL assay

To detect apoptotic cells in tumor tissue sections, an *in situ* apoptosis detection kit (Roche Diagnostics) was used. Tumor sections were incubated with proteinase K, rinsed with ddH₂O, dewaxed with dimethylbenzene, and rehydrated with gradient ethanol. A 3% H₂O₂ solution was used to block endogenous peroxidase. After incubation with equilibration buffer and terminal deoxynucleotidyl transferase enzyme, sections were incubated with antidigoxigenin-peroxidase conjugate. Peroxidase activity in each section was shown by diaminobenzidine. Finally, sections were counterstained with hematoxylin. Positive cells were identified and counted (three random fields per slide) under light microscope (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis

All data were expressed as mean ± SD. Comparisons of the difference of mean values were assessed using Student's two-tailed *t* test. Differences were considered statistically significant for $P < 0.05$ and $P < 0.01$. All means were calculated from at least three independent experiments.

RESULTS

LY294002 increased oxaliplatin-induced cell proliferation inhibition and apoptosis in gastric cancer cells

MKN45 and AGS cells were treated with various doses of oxaliplatin (0, 0.25, 1, 4 µmol/L for cell growth inhibition, and 0, 5, 10, 20 µmol/L for cell apoptosis) for 24 h with or without the pretreatment of LY294002 (25 µmol/L). Cell growth inhibition was evaluated by MTT assay. Apoptotic cells were investigated by flow cytometry. LY294002 significantly increased oxaliplatin-induced growth inhibition (In MKN45, oxaliplatin *vs* oxaliplatin + LY294002: $3.2\% \pm 0.1\%$ *vs* $4.1\% \pm 0.1\%$, $P > 0.05$, $6.7\% \pm 1.1\%$ *vs* $11.5\% \pm 1.3\%$, $P < 0.05$, $12.5\% \pm 1.3\%$ *vs* $29.7\% \pm 1.7\%$, $P < 0.01$, and $13.7\% \pm 3.1\%$ *vs* $29.8\% \pm 3.3\%$, $P < 0.01$; in AGS, $6.6\% \pm 0.1\%$ *vs* $7.1\% \pm 0.2\%$, $P > 0.05$, $8.4\% \pm 1.4\%$ *vs* $14.3\% \pm 1.2\%$, $P < 0.05$, $16.5\% \pm 2.5\%$ *vs* $41.1\% \pm 3.8\%$, $P < 0.01$, and $18.4\% \pm 2.1\%$ *vs* $35.3\% \pm 4.3\%$, $P < 0.01$) and apoptosis (in MKN45, oxaliplatin *vs* oxaliplatin + LY294002: $1.7\% \pm 0.1\%$ *vs* $2.6\% \pm 0.3\%$, $P > 0.05$, $14.3\% \pm 3.4\%$ *vs* $26.3\% \pm 4.3\%$, $P < 0.05$, $28.0\% \pm 4.7\%$ *vs* $44.2\% \pm 5.12\%$, $P < 0.01$, and $41.4\% \pm 4.7\%$ *vs* $63.1\% \pm 9.3\%$, $P < 0.01$; in AGS, $3.2\% \pm 0.1\%$ *vs* $4.1\% \pm 1.2\%$, $P > 0.05$, $13.4\% \pm 3.8\%$ *vs* $22.7\% \pm 3.5\%$, $P < 0.05$, $26.6\% \pm 4.1\%$ *vs* $42.5\% \pm 4.8\%$, $P < 0.01$, and $40.9\% \pm 5.9\%$ *vs* $69.8\% \pm 6.5\%$, $P < 0.01$) (Figure 1).

LY294002 inhibited basal and oxaliplatin-induced phosphorylation of Akt and NFκB/DNA binding activities

MKN45 and AGS cells were treated with oxaliplatin (20 µmol/L) and LY294002 (25 µmol/L) used singly or in combination for 24 h. For combined treatment, pretreatment of LY294002 was followed by oxaliplatin. Oxaliplatin induced an increase in the phosphorylation of Akt (Ser⁴⁷³) in MK45 and AGS cells. LY294002 significantly

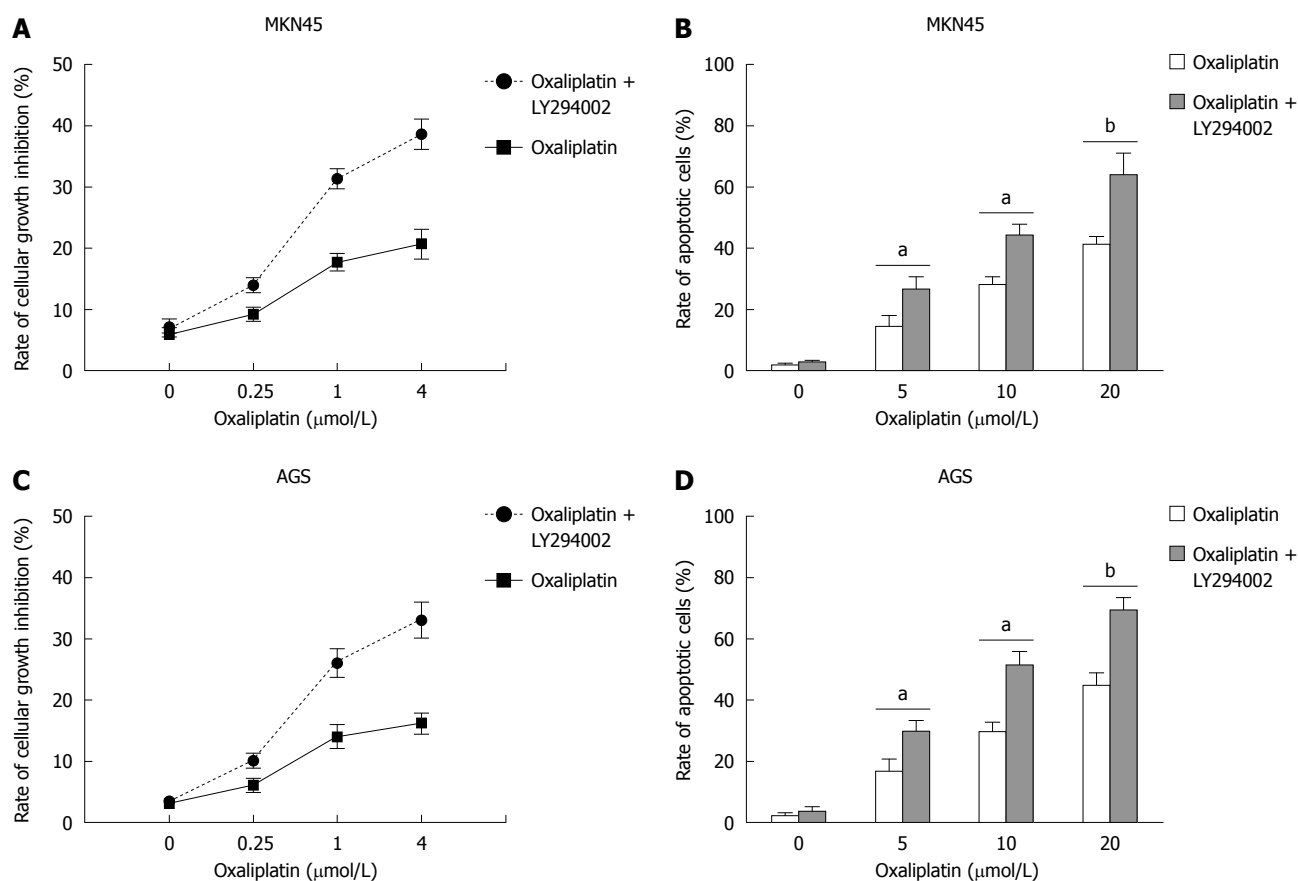


Figure 1 LY294002 increased oxaliplatin-induced cell proliferation and apoptosis in gastric cancer cells. MKN45 and AGS cells were treated with various doses of oxaliplatin (0-20 μmol/L) for 24 h with or without LY294002 pretreatment (25 μmol/L). A, C: Cell growth inhibitory rates were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; B, D: Apoptosis of cells was investigated by flow cytometry. LY294002 significantly increased oxaliplatin-induced growth inhibition and apoptosis. ^a $P < 0.05$, ^b $P < 0.01$ vs oxaliplatin alone.

reduced oxaliplatin-induced phosphorylation of Akt (Ser⁴⁷³) (Figure 2A and B). Oxaliplatin and LY294002 did not modulate the phosphorylation of Akt at Thr308 (data not shown). NFκB activity in MKN45 and AGS cells was examined using EMSA. Oxaliplatin stimulated NFκB/DNA binding activity in MKN45 and AGS cells (Figure 2C and D). When oxaliplatin was combined with LY294002, NFκB/DNA binding activity was decreased.

Effects of oxaliplatin, LY294002, or combination in recruitment of FADD, expression of FasL and c-FLIPs, and activation of caspase-8, Bid and caspase-3

Several molecules of the death receptor pathway were investigated using Western blotting. In MKN45 and AGS cells, oxaliplatin increased FasL expression, recruited FADD, and activated caspase-8, caspase-3, and Bid cleavage (t-Bid formation) (Figure 3). LY294002 significantly promoted the oxaliplatin-induced changes. Oxaliplatin reduced the c-FLIPs, while LY294002 enhanced this effect of oxaliplatin. Oxaliplatin and LY294002 did not modulate the expression of the c-FLIP_L (data not shown).

FasL siRNA attenuated oxaliplatin-, LY294002-, or combination-induced cell apoptosis

To further investigate whether LY294002 promoted oxali-

platin-induced apoptosis through the death receptor pathway, MKN45 and AGS cells transfected with FasL siRNA were treated with oxaliplatin, LY294002, or a combination of both. FasL expression was inhibited by FasL siRNA in MKN45 and AGS cells (Figure 4A). FasL silencing decreased LY294002- (in MKN45, LY294002 *vs* LY294002 + FasL siRNA: $10.5\% \pm 1.3\%$ *vs* $4.1\% \pm 0.6\%$, $P < 0.05$; in AGS, $14.6\% \pm 0.7\%$ *vs* $4.0\% \pm 0.7\%$, $P < 0.05$), oxaliplatin- (in MKN45, oxaliplatin *vs* oxaliplatin + FasL siRNA: $39.4\% \pm 3.6\%$ *vs* $10.7\% \pm 3.9\%$, $P < 0.01$; in AGS, $45.1\% \pm 4.1\%$ *vs* $11.8\% \pm 2.8\%$, $P < 0.01$), or combination- (in MKN45, combination *vs* combination + FasL siRNA: $55.7\% \pm 7.6\%$ *vs* $15.4\% \pm 2.4\%$, $P < 0.01$; in AGS, $63.4\% \pm 5.8\%$ *vs* $18.6\% \pm 4.5\%$, $P < 0.01$) induced cell apoptosis (Figure 4B).

Effects of oxaliplatin, LY294002, or combination on tumor growth and apoptosis in vivo

Four experimental groups were examined: (1) control group; (2) LY294002 group; (3) oxaliplatin group; and (4) combined oxaliplatin and LY294002 therapy group. Tumor growth curves were plotted to compare differences in anti-tumor efficiency in the course of the experiments (Figure 5A). TUNEL assay was performed to detect apoptotic cells in tumor tissue sections (Figure 5B).

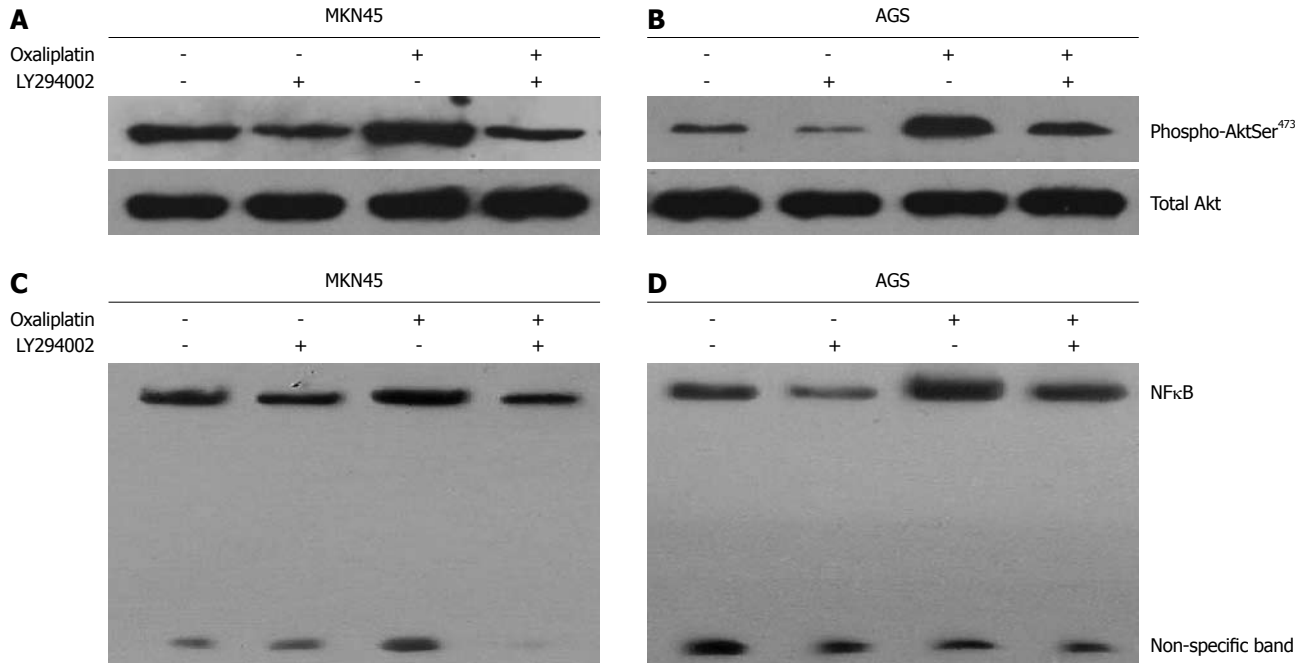


Figure 2 LY294002 inhibited basal and oxaliplatin-induced phosphorylation of Akt and nuclear factor κ B/DNA binding activities. MKN45 and AGS cells were incubated with oxaliplatin (20 μ mol/L) or LY294002 (25 μ mol/L) used singly or in combination for 24 h. A, B: Oxaliplatin enhanced the phosphorylation of Akt (Ser⁴⁷³, but not Thr308), while LY294002 inhibited the induction of Akt activity in MKN45 and AGS cells; C, D: Oxaliplatin increased nuclear factor κ B (NF κ B)/DNA binding activity, while LY294002 inhibited the induction of NF κ B/DNA binding activity.

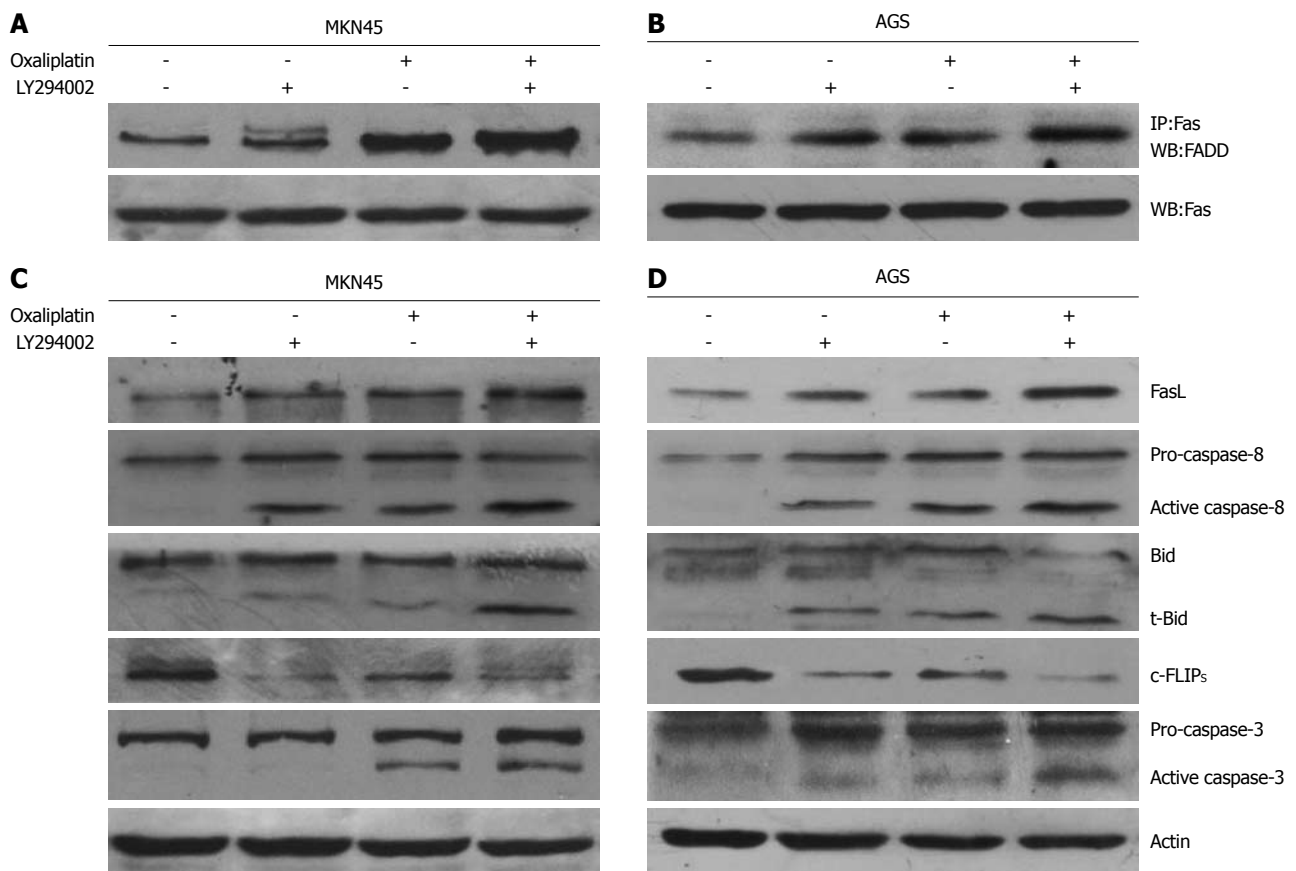


Figure 3 Effects of oxaliplatin, LY294002, or combination on recruitment of Fas-associated death domain protein, expression of Fas ligand and short form of cellular caspase-8/FLICE-inhibitory protein, and activation of caspase-8, Bid, and caspase-3. A-D: In MKN45 and AGS cells, oxaliplatin led to increased Fas ligand (FasL) expression, Fas-associated death domain protein (FADD) recruitment, caspase-8 and caspase-3 activation, and Bid cleavage (t-Bid formation). LY294002 significantly promoted these oxaliplatin-induced changes. Oxaliplatin reduced short form of cellular caspase-8/FLICE-inhibitory protein (c-FLIP_s) expression, while LY294002 enhanced this effect of oxaliplatin. Oxaliplatin and LY294002 did not modulate long form of cellular caspase-8/FLICE-inhibitory protein expression (data not shown). IP: Immunoprecipitation; WB: Western blotting.

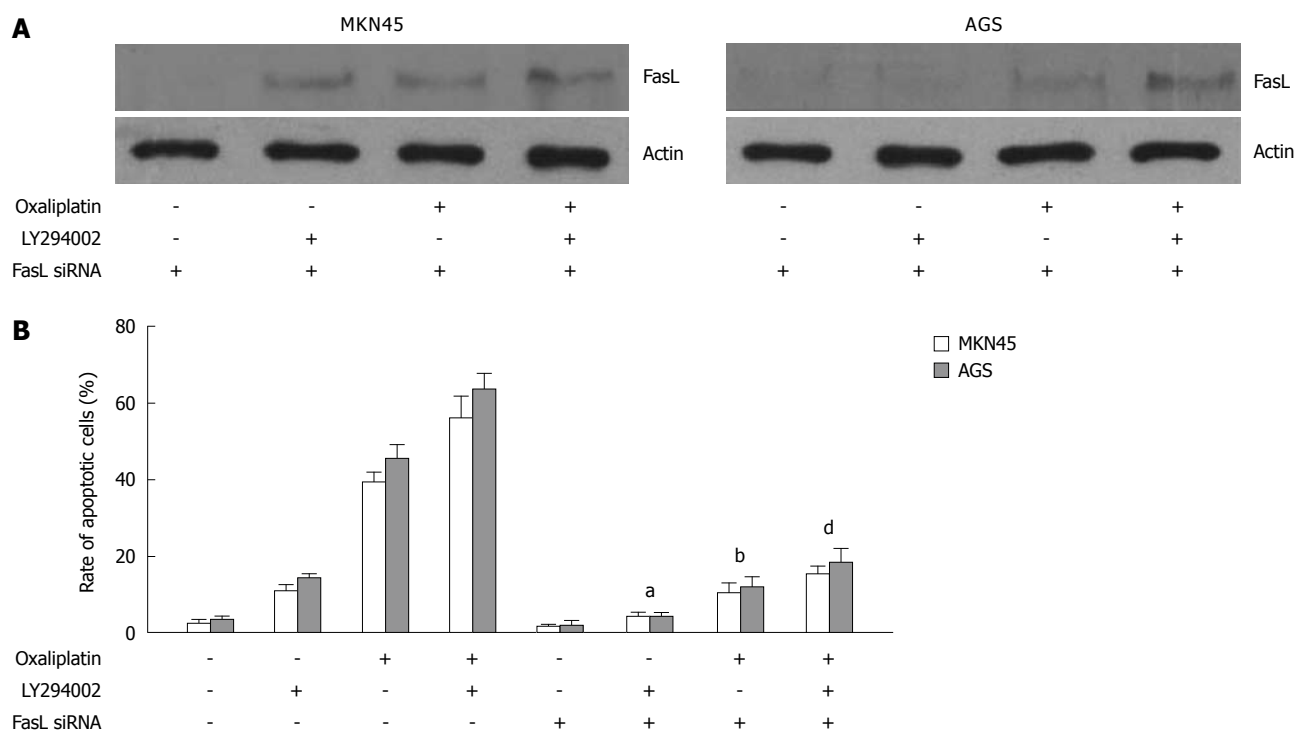


Figure 4 Fas ligand siRNA attenuated oxaliplatin-, LY294002-, or combination-induced cell apoptosis. A: Fas ligand (FasL) expression was inhibited by FasL siRNA in MKN45 and AGS cells; B: FasL silencing decreased oxaliplatin-, LY294002-, or combination-induced cell apoptosis. ^a $P < 0.05$ vs LY294002 treatment; ^b $P < 0.01$ vs oxaliplatin treatment; ^d $P < 0.01$ vs combination of oxaliplatin and LY294002.

At the end of 6 wk, tumor volume in combined oxaliplatin and LY294002 therapy group was greatly reduced compared with oxaliplatin group ($763 \pm 155 \text{ mm}^3$ vs $1789 \pm 233 \text{ mm}^3$, $P < 0.01$). Oxaliplatin combined with LY294002 significantly enhanced cell death in the tumor mass *via* apoptosis when compared with oxaliplatin treatment alone.

Immunohistochemical analysis was performed to evaluate the expression of death receptor pathway molecules (Figure 5C). LY294002 inhibited oxaliplatin-induced activation of Akt and NF κ B, and increased oxaliplatin-induced expression of FasL, inhibition of c-FLIPs, and activation of caspase-8, Bid and caspase-3.

DISCUSSION

Oxaliplatin is a diaminocyclohexane platinum anti-cancer agent. Although oxaliplatin produces DNA crosslinking similar to those of cisplatin^[17], cisplatin-resistant cells generally remain sensitive to oxaliplatin^[18]. Furthermore, oxaliplatin induces fewer complications compared with other platinum derivatives such as cisplatin and carboplatin that induce nephrotoxicity^[19] and myelosuppression^[20], respectively. Recently, oxaliplatin was shown to be effective in the treatment of advanced gastric cancer when combined with 5-fluorouracil and leucovorin, and has also been used in adjuvant chemotherapy for gastric cancer. However, despite the improvement in the efficacy of chemotherapeutic drugs used in the treatment of metastatic gastric cancer, the response rate and relative 5-year survival rate in the advanced disease remain low^[21].

The PI3K/Akt signaling pathway plays a critical role in cell cycling, cell growth, protein translation, and suppression of apoptosis by Akt-mediated phosphorylation^[22-24], and also promotes tumor growth, survival, and aggressiveness^[25,26]. In gastric cancer, several studies have reported that the majority of patients exhibit increased expression and activation of Akt^[11,27]. Over expression of phosphorylated Akt was associated with poor overall survival, disease-free survival, and high tumor recurrence in gastric cancer patients^[28]. In gastric carcinoma cell lines, phosphorylation of Akt is required for cell growth and survival^[28]. Thus, blocking the constitutively active PI3K/Akt signaling pathway may provide a novel strategy for targeted cancer therapy.

In this study, the specific PI3K inhibitor LY294002 promoted oxaliplatin-induced growth inhibition and cell apoptosis in MKN45 and AGS cells, suggesting that LY294002 enhanced the chemotherapeutic sensitivity to oxaliplatin in gastric cancer cells. Previous *in vitro* and *in vivo* studies demonstrated that activation of the PI3K pathway was associated with the therapeutic efficacy of several chemotherapeutic agents including 5-FU, paclitaxel, cisplatin, irinotecan, and doxorubicin^[29-32], while activation of the PI3K pathway induced chemoresistance in cancer cells. To explore the possible mechanisms of LY294002 in sensitizing gastric cancer cells to oxaliplatin, we examined the phosphorylation levels of Akt in oxaliplatin treated MKN45 and AGS cells. We found increased expression of phosphorylated Akt at Ser⁴⁷³ after treatment with oxaliplatin in MKN45 and AGS cells, which is in agreement with a previous study in cholangiocarcinoma cells^[33]. LY294002

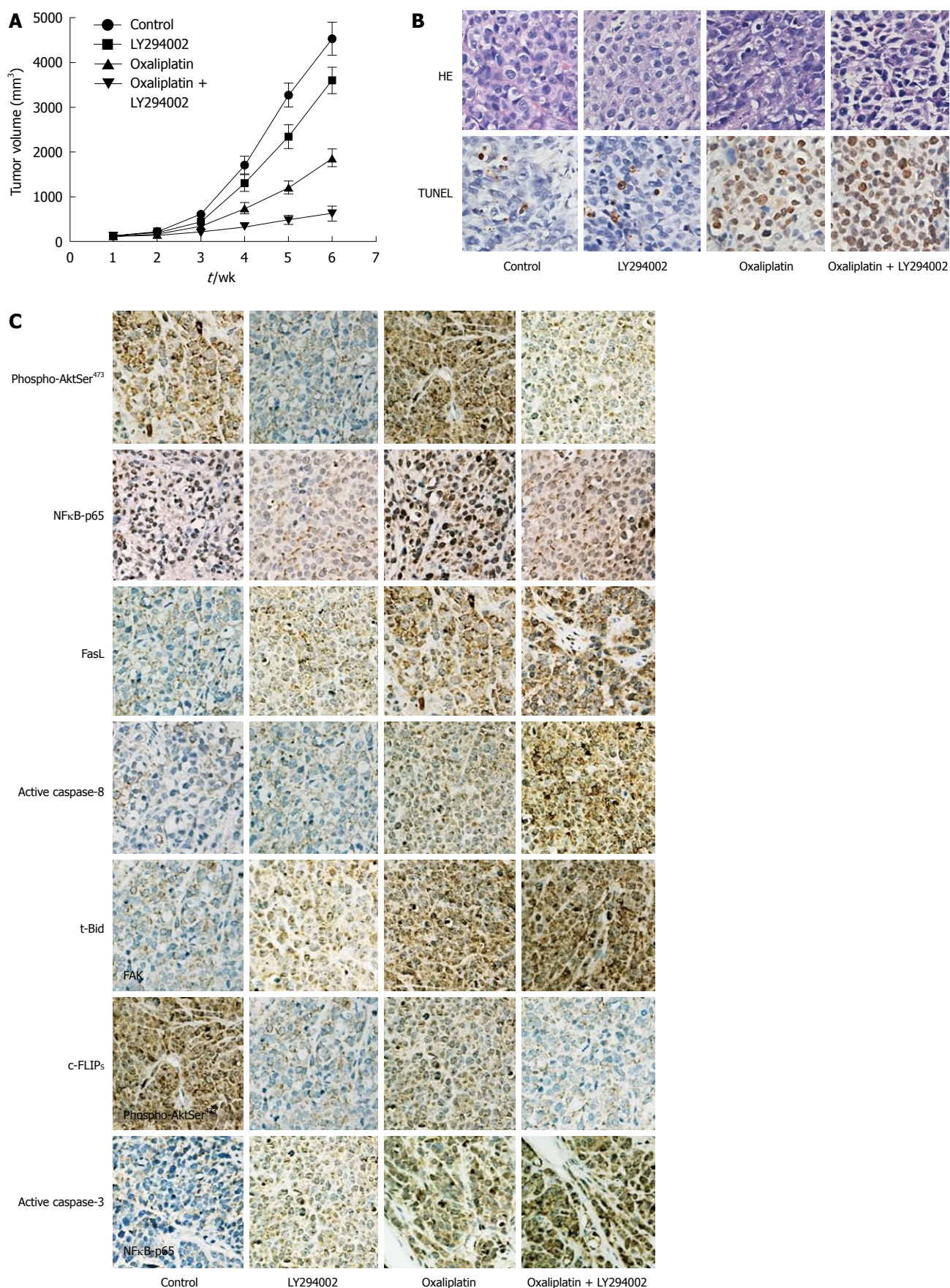


Figure 5 Effects of oxaliplatin, LY294002, or combination on *in vivo* tumor growth and apoptosis. A: Tumor volumes of nude mice in each group are presented. Each time point represents the mean tumor volume for each group; B: Detection of apoptotic cells in tumor tissue was performed by transferase-mediated dUTP nick end labeling (TUNEL) assay; C: The expression of phospho-AktSer⁴⁷³, nuclear factor κ B (NF κ B)-p65, Fas ligand (FasL), short form of cellular caspase-8/FLICE-inhibitory protein (c-FLIPs), Bid, caspase-8, and caspase-3 was investigated by immunohistochemical analysis.

blocked basal and oxaliplatin-induced phosphorylation of Akt, and resulted in an increased apoptotic rate compared with oxaliplatin alone, suggesting that Akt phosphorylation might regulate oxaliplatin resistance in gastric cancer cells. The significant increase in oxaliplatin-induced cytotoxicity in gastric cancer pretreated with LY294002 indicates that the resistance of gastric cancer cells to chemotherapeutic agents can be modulated.

NF κ B plays an important role in suppression of apoptosis. Akt phosphorylates I κ B (NF κ B inhibitor) kinases, leading to degradation of I κ B, as well as NF κ B activation^[34]. Although many studies strongly support the anti-apoptotic role of NF κ B, there are some evidences that NF κ B can induce apoptosis^[35-37]. In the present study, oxaliplatin enhanced NF κ B/DNA binding activity, while LY294002 blocked anticancer drug-induced activation of NF κ B. These data indicate that activation of Akt/NF κ B in gastric cancer cells may be a key mechanism in inhibiting oxaliplatin-induced apoptosis. It is possible that additional components of the PI3K/Akt pathway may be involved in the chemoresistance of gastric cancer cells.

To further define the role of LY294002 in the regulation of oxaliplatin-induced apoptosis, we examined expression of molecular markers of the death receptor-signaling pathway. LY294002 dramatically increased oxaliplatin-induced FasL expression, FADD redistribution into membrane lipid rafts, caspase-8 and caspase-3 activation, and Bid cleavage in MKN45 and AGS cells. Next, we down-regulated FasL using FasL siRNA in LY294002-, oxaliplatin-, or combination-treated MKN45 and AGS cells. Oxaliplatin, LY294002, or combination treatment-induced apoptosis was attenuated by FasL silencing, suggesting that the death receptor pathway might be involved in the cell apoptosis induced by oxaliplatin or LY294002 in gastric cancer cells. However, the precise mechanism whereby oxaliplatin or LY294002 induces FasL expression remains unknown.

Apoptosis mediated by Fas is regulated by c-FLIP expression^[38]. There are two isoforms of c-FLIP: the full-length c-FLIP_L and c-FLIP_S^[39,40]. c-FLIP_S is considered solely anti-apoptotic and confers resistance to receptor-mediated apoptosis by blocking proteolytic activation of caspase-8 at the Fas DISC, while c-FLIP_L exhibits dual roles^[41,42]. Additionally, c-FLIP_S and c-FLIP_L are differently regulated^[43-45]. The PI3K pathway is an important regulator of c-FLIP_S, but not c-FLIP_L, expression in human gastric cancer cells^[45]. In this study, oxaliplatin-induced apoptotic death was accompanied by suppression of c-FLIP_S in MKN45 and AGS cells. Compared with oxaliplatin alone, combination of oxaliplatin and LY294002 produced enhanced down-regulation of c-FLIP_S. c-FLIP_L expression was not significantly changed by treatment with LY294002 or oxaliplatin. These findings indicate that the anti-apoptotic function of c-FLIP_S may be more potent than that of c-FLIP_L in oxaliplatin-induced apoptosis, and that Akt is involved in regulation of c-FLIP_S in human gastric cancer cells.

We also examined the effects of the combined treatment of oxaliplatin and LY294002 in an *in vivo* xeno-

graft model. LY294002 significantly increased oxaliplatin-induced tumor growth and cell death in the tumor mass *via* apoptosis. Moreover, altered expression levels of FasL, Bid, caspase-8, caspase-3, and c-FLIP_S were found in the tumor xenograft. These data suggest that combination of oxaliplatin and LY294002 elicited a strong antitumor effect in gastric cancer *in vivo*, and that the death receptor pathway might mediate the additive cytotoxicity of oxaliplatin and LY294002.

In summary, we present a novel therapeutic approach for treatment of gastric cancer using the combined oxaliplatin and the PI3K/Akt inhibitor LY294002, that may be mediated, at least in part, by modification of the death receptor pathway.

ACKNOWLEDGMENTS

The authors thank Hong Xia (Institute for Gastroenterology and Hepatology, Wuhan University Medical School) for his valuable discussions and suggestions.

COMMENTS

Background

Gastric cancer remains a leading cause of cancer death worldwide. Besides surgical resection, chemotherapy is important treatment for gastric cancers. Despite the improvement in the efficacy of chemotherapeutic drugs, the response rates and the median survival remain low.

Research frontiers

Traditional cancer therapy predominantly utilizes cytotoxic chemotherapeutic agents. The cytotoxic events are affected mainly through disruption of various aspects of DNA synthesis and repair or disturbance of mitosis, processes which are common to all dividing cells. For this reason, most chemotherapeutic agents are often accompanied with substantial adverse effects. Target-protein-based cancer therapy has become available in clinical practice. Phosphatidylinositol 3-kinase (PI3K) inhibitors have potential to target specific pathways involved in tumor cell growth.

Innovations and breakthroughs

The PI3K/Akt pathway has been shown to be involved in the chemoresistance of gastric cancer. In both *in vitro* and *in vivo* studies, the targeted inhibition of PI3K/Akt results in increased oxaliplatin-induced apoptosis and inhibition of cellular proliferation of gastric cancer. Furthermore, the activation of the death receptor pathway may be an important mechanism by which PI3K/Akt inhibition is involved in oxaliplatin-induced apoptosis.

Applications

By understanding how LY294002 enhances the therapeutic effect of oxaliplatin in gastric cancer cells, this study provides information about the potential therapeutic intervention in patients with gastric adenocarcinoma.

Terminology

Oxaliplatin: A third-generation platinum coordination complex of the 1,2-diaminocyclohexane families, generates covalent adducts between platinum and two adjacent guanines or guanine and adenine in cell DNA. LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, a specific inhibitor of PI3K.

Peer review

This is a well-written report on the synergistic anti-tumor effects of the combined treatment with oxaliplatin and LY294002 in gastric cancer cells. The data and results are straight-forward and clearly support the conclusion that targeting PI3K/Akt results in increased oxaliplatin-induced apoptosis and inhibition of cellular proliferation of gastric cancer.

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S- Editor Sun H L- Editor Ma JY E- Editor Zheng XM

Percutaneous endoscopic gastrostomy and gastro-oesophageal reflux in neurologically impaired children

Mike Thomson, Prithviraj Rao, David Rawat, Tobias G Wenzl

Mike Thomson, Prithviraj Rao, David Rawat, Centre for Paediatric Gastroenterology, Royal Free and University College Medical School, Pond Street, London NW3 2QG, United Kingdom
Mike Thomson, Centre for Paediatric Gastroenterology, Sheffield Childrens NHS Trust, Western Bank, Sheffield, S10 2TH, United Kingdom

Tobias G Wenzl, Kinderklinik, Universitätsklinikum Aachen, Pauwelsst. 30, D 52074, Aachen, Germany

Author contributions: Thomson M, Rawat D and Wenzl TG made the majority of contributions to conception and design and acquisition of data; Rao P was involved in editing the manuscript as well as in analysis and interpretation of data; Thomson M and Rao P drafted the article; all authors revised the manuscript critically for important intellectual content and for the final approval of the version to be published.

Correspondence to: Dr. Mike Thomson, Centre for Paediatric Gastroenterology, Sheffield Childrens NHS Trust, Western Bank, Sheffield, S10 2TH, United Kingdom. mike.thomson@sch.nhs.uk
Telephone: +44-114-2717673 Fax: +44-114-2267956

Received: September 16, 2009 Revised: December 31, 2009

Accepted: January 7, 2010

Published online: January 14, 2011

Abstract

AIM: To investigate the effects of percutaneous endoscopic gastrostomy (PEG) feeding on gastro-oesophageal reflux (GOR) in a group of these children using combined intraluminal pH and multiple intraluminal impedance (pH/MII).

METHODS: Ten neurologically impaired children underwent 12 h combined pH/MII procedures at least 1 d before and at least 12 d after PEG placement.

METHODS: Prior to PEG placement (pre-PEG) a total of 183 GOR episodes were detected, 156 (85.2%) were non-acidic. After PEG placement (post-PEG) a total of 355 episodes were detected, 182 (51.3%) were non-acidic. The total number of distal acid reflux events statistically significantly increased post-PEG placement (pre-PEG total 27, post-PEG total 173, $P = 0.028$) and the

mean distal pH decreased by 1.1 units. The distal reflux index therefore also significantly increased post-PEG [pre-PEG 0.25 (0-2), post-PEG 2.95 (0-40)]. Average proximal pH was lower post-PEG but the within subject difference was not statistically significant ($P = 0.058$). Median number of non-acid GOR, average reflux height, total acid clearance time and total bolus clearance time were all lower pre-PEG, but not statistically significant.

CONCLUSION: PEG placement increases GOR episodes in neurologically impaired children.

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Key words: Percutaneous endoscopic gastrostomy; Gastro-oesophageal reflux; Multiple intraluminal impedance

Peer reviewers: Jon C Gould, MD, FACS, Associate Professor of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States; Dr. Seyed Mohsen Dehghani, MD, Associate Professor, Department of Pediatrics, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran; David Ian Watson, Professor, Head, Flinders University Department of Surgery, Room 3D211, Flinders Medical Center, Bedford Park, South Australia 5042, Australia

Thomson M, Rao P, Rawat D, Wenzl TG. Percutaneous endoscopic gastrostomy and gastro-oesophageal reflux in neurologically impaired children. *World J Gastroenterol* 2011; 17(2): 191-196 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/191.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.191>

INTRODUCTION

The enteric nervous system contains more neurones than the spinal cord^[1]. Insults to the central nervous system therefore may result in dysfunction of the gastro-intes-

tinal tract such as oro-motor dysfunction, rumination, gastro-oesophageal reflux (GOR), delayed gastric emptying and constipation. All these problems may contribute to feeding difficulties and ultimately sub-optimal nutrition in neurologically impaired children. In children with severe oro-motor dysfunction calorie supplementation of oral feeds is often not successful and adjunctive methods are required in order to achieve adequate nutrient intake. The long-term use of a fine bore nasogastric tube (NGT), though most widely used, has several limitations. These include nasal discomfort, laryngeal irritation and penetration, recurrent pulmonary aspiration, tube blockage, tube displacement, oral aversion and possibly impedance of the progressive maturation of the oral feeding pattern from sucking to chewing^[2]. Percutaneous endoscopic gastrostomy (PEG) technique has become increasingly popular for the provision of nutrition in disabled children^[3] and there are a variety of commercially available devices of variable lengths and calibres that are suitable even in young children^[4].

Several studies have demonstrated the clinical advantages to patients fed *via* PEG when compared with other feeding techniques^[5,6]. Randomised comparisons of feeding in patients with dysphagia secondary to neurological impairment, demonstrated that PEG-fed children achieved better weight gain than those fed *via* NGT^[7,8]. Post-operative follow up however, is essential to monitor weight gain and the development of GOR^[9]. Indeed nutritional rehabilitation using a feeding gastrostomy of disabled children is associated with increased mortality and morbidity secondary to GOR^[10].

Oesophageal pH monitoring is regarded as the investigation of first choice in children with unusual presentations of GOR disease (GORD), such as apnoea and recurrent respiratory disease^[11-14]. However pH measurements cannot detect GOR in the pH range 4.0-7.0 due to the proximity to the physiological oesophageal pH^[15-17] and thus misses many episodes of postprandial reflux in young infants and intragastrically fed children due to neutralisation of gastric contents by milk formula for 1-2 h after a meal. Therefore the term acid (pH < 4) and/or non-acid (pH ≥ 4) GOR should be preferred over the term GOR. Because GOR-associated symptoms are not necessarily confined to acid GOR, a pH-independent technique, known as multiple intraluminal impedance (MII) has been established^[18-21], which detects a typical decrease of electrical impedance (resistance) during the passage of a bolus through a measuring segment. The use of multiple segments along a catheter allows the analysis of movement, direction and height attained by the bolus, making it possible to distinguish antegrade and retrograde bolus movement. Simultaneous use of integrated pH sensors can help determine the pH of the reflux episodes as well.

The aim of this study was to measure GOR in neurologically impaired children before and after insertion of a PEG using the combined pH/MI procedure.

MATERIALS AND METHODS

The study included 10 neurologically impaired patients (5 male, 5 female), nine being diagnosed with cerebral palsy and one with Down's syndrome. All had severe feeding difficulties requiring long-term nutritional support and were admitted to the Centre for Paediatric Gastroenterology, Royal Free Hospital, London, UK for insertion of a PEG. Patients underwent a daytime 12-h impedance procedure for detection of acid and non-acid GOR before (Pre-PEG) and after (Post-PEG) PEG placement.

Pre- and Post-PEG study

Patients were of median age 4.9 years (range 0.5-16.8 years). Impedance procedure took place 1-79 d (median 1.5 d) prior to PEG placement. All patients were bolus fed of which four patients were fed orally and six were fed *via* NGT during the study.

Patients were of median age 5.3 years (range 0.8-17 years). The impedance procedure took place 12-384 d (median 55 d) after PEG placement, and this represented a pragmatic compromise dependent on parental instruction. All patients were receiving bolus feeds *via* their PEG during the study.

Patients were on the same medication during the pre- and post-PEG impedance procedure; eight were not on any medication influencing gastric pH or motility, one was on omeprazole and cisapride, and one was on ranitidine and Gaviscon®. There was no change in the parent/carer subjective impression of potential reflux-related events or symptoms and no change in the frequency of diagnosis of chest aspiration or infection.

The study protocol was approved by the Royal Free NHS Trust Ethical Review Committee. On the initial visit to the clinic informed consent was obtained from the parent or guardian.

An MII catheter (outer diameter 2 mm) with two pH-sensitive antimony electrodes and seven impedance electrodes (PRZ-062B00013, Sandhill Scientific, Inc., Colorado, USA) was used. Changes in intra-oesophageal impedance were measured along this catheter. The impedance was measured between seven adjacent electrodes (15 mm apart), thus enabling readings to be obtained from 6 impedance channels (6 adjacent electrode pairings). The catheter was passed transnasally and positioned by a height-derived formula^[22] with total measuring segments reaching from approximately 1.5 cm above the lower oesophageal sphincter (channel 6) to the upper oesophagus (channel 1). The pH sensors were situated at the level of channel 6, approximately 2 cm above the gastro-oesophageal junction, and at the level of channel 1. The catheter was connected to a Windows 98 personal computer, *via* voltage transducers (Z-Box) that continuously recorded impedance and pH events (Sandhill Scientific, Inc). Impedance and pH signals were sampled at a rate of 50 Hz per channel, as compared to 0.25 Hz in conventional pH-metry. Impedance and pH recordings were made for 12 h.

All impedance recordings were visually analysed for

the typical MII pattern of GOR. This was defined as any retrograde-passing bolus detected by channel 4 with a duration of more than 5 s. The reflux index was calculated for both proximal and distal pH sensors by (percentage of time < pH 4.0/total study duration). The clearance time for a liquid bolus may differ from the time taken for the acid environment to neutralise at the pH sensors. Thus the height reached by each bolus, as well as the volume- and acidity-clearance, was registered for each MII-defined GOR episode. Mean values for all episodes were calculated for each patient. As data from children ($n = 10$) in pre- and post-PEG groups was not normally distributed, medians are reported for between-group comparisons, and non-parametric tests (Wilcoxon Rank Sum) used in statistical analyses. *A priori* variables selected for comparison were: reflux index (proximal and distal pH), total reflux events, acid-reflux events, non-acid reflux events, bolus height, bolus clearance time and acid clearance time.

RESULTS

Prior to PEG placement a total of 183 reflux events were detected by the combined pH/II procedure. 156 (85.2%) were non-acidic and 27 (14.8%) were acidic. Post PEG placement a total of 355 reflux events were detected, of which 182 (51.3%) were non acid and 173 (48.7%) were acidic.

The total number of reflux events and acid reflux events were significantly lower before PEG placement ($P = 0.047$ and $P = 0.028$, respectively) (Table 1). Individual measurements are detailed in Tables 2 and 3. The average minimum distal pH was lower by 1.1 pH units post-PEG placement ($P = 0.05$) and the distal reflux index was significantly higher, but still within normal limits after the procedure [$P = 0.032$, reflux index (RI) 0.25% pre-PEG and 2.95% post-PEG].

The percentage of the GOR events reaching the uppermost impedance channel (channel 1) i.e. the pharyngeal space, pre-PEG placement was 56%. Post-procedure this increased significantly to 82%.

The median number of non-acid reflux events per hour, average reflux height, total distal and proximal acid clearance time and total bolus clearance time were all lower pre-PEG placement, but were not significantly different.

The average minimum proximal pH was also lower post-PEG placement but again, this was not statistically significant.

Nutritional improvement occurred between the pre- and post-PEG insertion with a median weight gain of 2.53 kg (range 0.8–7.24 kg).

Table 1 summarises the main results before and after PEG placement.

DISCUSSION

We describe the effect of PEG placement on GOR events

Table 1 Summary of pH and multiple intraluminal impedance results before and after percutaneous endoscopic gastrostomy placement

Parameter	Pre-PEG	Post-PEG	P-value
Total GOR events	183	355	
Median	17.50	39.50	0.047
Range	2-54	3-63	
Total number of GOR events reaching uppermost channel 1	103	290	
Median	9.00	27.00	0.022
Range	1-29	1-55	
Total non-acid GOR events	156	182	
Median	13.00	18.00	0.610
Range	2-49	3-38	
Total acid GOR events	27	173	
Median	1.00	13.00	0.028
Range	0-11	0-50	
Bolus clearance time (s)			
Median	13.45	13.00	0.445
Range	9-20	9-19	
Height (channel)			
Median	1.50	1.50	0.172
Range	1-3	1-2	
Proximal pH			
Median	5.70	5.15	0.058
Range	5-7	4-6	
Distal pH			
Median	5.30	4.20	0.050
Range	4-6	4-5	
Proximal acid clearance time (s)			
Median	17.00	20.10	0.715
Range	2-320	12-33	
Distal acid clearance time (s)			
Median	38.70	39.50	0.500
Range	10-130	26-92	
Proximal reflux index (%)			
Median	0.15	0.70	0.092
Range	0-0	0-11	
Distal reflux index (%)			
Median	0.25	2.95	0.032
Range	0-2	0-40	

GOR: Gastro-oesophageal reflux; PEG: Percutaneous endoscopic gastrostomy.

in neurologically impaired children with feeding difficulties by using a combined pH and intraluminal impedance measurement.

The use of combined pH and impedance allows detection of both acid ($\text{pH} < 4$) and non-acid ($\text{pH} \geq 4$) GOR episodes, as well as the height of the refluxate and the total acid clearance time. In our study 183 reflux events were detected by MII pre-PEG insertion, of which 85.2% were non-acid and would therefore have been undetected using the “gold standard” pH-metry. The total number of GOR episodes more than doubled after PEG insertion. Furthermore, the average distal oesophageal pH was significantly lower after PEG placement. This is clinically relevant as we have shown that an acid reflux event takes longer to clear than a non-acid bolus. Skopnik *et al*^[17] in a study of 17 infants using MII, detected that 90% of GOR episodes were non-acid and therefore undetectable by conventional pH-metry.

Table 2 Individual results of pH and multiple intraluminal impedance results before percutaneous endoscopic gastrostomy placement

Patient	Total GOR	Total GOR/h	Total non-acid GOR	Total non-acid GOR/h	Total acid GOR	Total acid GOR/h	Prox RI (%)	Dist RI (%)	Reflux events reaching ch 1
1	24	2.00	24	2.00	0	0.00	0.0	0.0	18
2	54	4.50	49	4.08	5	0.42	0.2	0.4	29
3	18	1.50	13	1.08	5	0.42	0.1	2.2	6
4	7	0.58	6	0.50	1	0.08	0.3	0.1	6
5	23	1.92	22	1.83	1	0.08	0.2	0.5	14
6	3	0.25	2	0.17	1	0.08	0.0	0.1	1
7	19	1.58	19	1.58	0	0.00	0.0	0.0	9
8	2	0.17	2	0.17	0	0.00	0.0	0.0	1
9	17	1.42	6	0.50	11	0.92	0.3	2.0	10
10	16	1.33	13	1.08	3	0.25	0.2	1.2	9

"Ch 1" refers to the most proximal impedance measurement and was located in the pharynx or most proximal 3 cm of oesophagus. GOR: Gastro-oesophageal reflux; RI: Reflux index.

Table 3 Individual results of pH and multiple intraluminal impedance results after percutaneous endoscopic gastrostomy placement

Patient	Total GOR	Total GOR/h	Total non-acid GOR	Total non-acid GOR/h	Total acid GOR	Total acid GOR/h	Prox RI (%)	Dist RI (%)	Reflux events reaching ch 1
1	57	4.8	7	0.58	50	4.17	10.6	39.9	55
2	48	4.0	38	3.17	10	0.83	2.4	3.2	41
3	63	5.3	25	2.08	38	3.17	1.2	5.6	42
4	14	1.2	11	0.92	3	0.25	0.1	0.4	12
5	3	0.3	3	0.25	0	0.00	0.0	0.0	1
6	33	2.8	17	1.42	16	1.33	0.2	3.6	20
7	46	3.8	27	2.25	19	1.58	0.1	2.0	34
8	49	4.1	19	1.58	30	2.50	6.7	10.1	47
9	21	1.8	21	1.75	0	0.00	0.0	0.0	18
10	21	1.8	14	1.17	7	0.58	1.5	2.7	20

"Ch 1" refers to the most proximal impedance measurement and was located in the pharynx or most proximal 3 cm of oesophagus. GOR: Gastro-oesophageal reflux; RI: Reflux index.

Likewise, Wenzl *et al*^[23] showed in 50 patients that only 14.9% of MII-determined reflux episodes were acidic, whilst non-acidic reflux events have clearly been temporally associated with unexplained respiratory phenomena^[24].

The diagnosis of GOR is more difficult in children with neurological impairment as the characteristic features may be absent. Although GOR may cause pain manifesting as restlessness, abnormal movements or food refusal, these symptoms may be subtle and frequently go undetected in the disabled child. Any operative procedure is a major undertaking with potential complications, particularly in an undernourished child who has recurrent respiratory infections and may have postural deformities. Therefore accurate assessment of GORD and careful patient selection is even more important in these children before PEG insertion, particularly as patients with moderate to severe GOR are already considered unsuitable for the procedure. For this reason, operative gastrostomies are frequently performed in conjunction with a fundoplication in those children with refractory or severe GOR. It has been previously reported^[24] that neurologically impaired children may have a reduction in the lower oesophageal sphincter (LOS) pressure, which may predispose not only to GOR, but

also to recurrent respiratory infections. This concept was supported by Wenzl *et al*^[25] who, in a study evaluating the link between reflux and respiratory phenomena in 22 infants, reported 78% of the reflux episodes causing apnoea to be non-acidic. Changes in gastric motility and lower oesophageal sphincter pressure following gastrostomy tube placement may also account for the observation that clinically significant GOR is detected in 75% of children after insertion of a gastrostomy tube^[9]. Therefore the increase in GOR events entering the pharyngeal space from 56% to 82% after the placement of a PEG may have significant clinical implications, suggesting an increased risk of aspiration pneumonia in this vulnerable cohort of children.

None of the patients selected for this study had an abnormal reflux index prior to PEG-placement. Although the post-procedure median reflux index still remained < 4%, the more than 10 fold increase in RI following the procedure suggests that less careful selection of patients for such an intervention may lead to a profound deterioration in GOR. In fact three patients had a RI > 4% post procedure. The detected increase in distal reflux index and total number of acid GOR events, along with the increased height of refluxate is likely to be the result of disordered gastric motility, and hence the delayed

emptying, that arises from the anchoring of the gastric wall to the abdominal wall by the PEG.

Mollitt *et al.*^[9] observed that, while a gastrostomy tube can greatly improve nutritional status and facilitate care of the neurologically disabled child, postoperative follow-up for the development of GOR was essential. Rehabilitation of nutrition in children with neurological impairment can be associated with an increase in mortality and morbidity secondary to GOR. MII has potentially significant implications in the diagnosis, patient selection and follow-up of neurologically impaired, gastrostomy-fed children who have an increase risk of developing GOR. Indeed, for the first time we are now able to accurately and objectively undertake a physiologically appropriate assessment of GOR which is of particular clinical significance in children with neurological impairment. A more accurate assessment of the pathophysiology of GORD and disturbed gastro-oesophageal motility may allow us to study the effects of treatment modalities such as feed thickeners, proton pump inhibitors and prokinetic agents, which may subsequently improve the efficacy of our therapeutic approach in this complex subgroup of children.

The placement of a PEG in neurologically compromised children needs careful consideration in view of the presented findings. The number and severity of GOR events are likely to increase after the procedure and may lead to significant morbidity in this group of children.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) technique or "non surgical opening in the stomach" has become increasingly popular for the provision of nutrition in disabled children particularly as several studies have demonstrated that PEG-fed children achieved better weight gain than those fed via a tube in their stomach via the nose (Nasogastric tubes). However, nutritional rehabilitation using a feeding gastrostomy of disabled children is associated with increased mortality and morbidity secondary to gastro-oesophageal reflux (GOR). So far, pH studies were helpful in diagnosing "acid reflux" in these children. However pH measurements cannot detect non acid reflux GOR due to the proximity to the physiological oesophageal pH and hence a pH-independent technique, known as multiple intraluminal impedance (MII) has been established.

Research frontiers

The aim of this study was to measure GOR in neurologically impaired children before and after insertion of a PEG using the combined pH/MI procedure.

Innovations and breakthroughs

For the first time the authors were now able to accurately and objectively undertake a physiologically appropriate assessment of acid and non-acid GOR.

Applications

Using combined pH/MI testing the authors could undertake a physiologically appropriate assessment of gastroesophageal reflux which is of particular clinical significance in children with neurological impairment

Terminology

MI is a technique which detects a typical decrease of electrical impedance (resistance) during the passage of a bolus through a measuring segment. The use of multiple segments along a catheter allows the analysis of movement, direction and height attained of the bolus, making it possible to distinguish antegrade and retrograde bolus movement.

Peer review

This is a nice contribution. Subjects were bolus fed during the day of their pH/MI. It is well written paper, with a clear message that PEG feeds in this paediatric subpopulation aggravate or promote reflux.

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S- Editor Wang JL **L- Editor** Rutherford A **E- Editor** Zheng XM

Polymorphisms in NF- κ B, PXR, LXR, PPAR γ and risk of inflammatory bowel disease

Vibeke Andersen, Jane Christensen, Anja Ernst, Bent A Jacobsen, Anne Tjønneland, Henrik B Krarup, Ulla Vogel

Vibeke Andersen, Department of Medical, Viborg Regional Hospital, 8800 Viborg, Denmark

Jane Christensen, Anne Tjønneland, Institute of Cancer Epidemiology, Danish Cancer Society, 2100 Copenhagen, Denmark

Anja Ernst, Henrik B Krarup, Department of Clinical Biochemistry, Aarhus University Hospital, 9000 Aalborg, Denmark

Bent A Jacobsen, Department of Medical Gastroenterology, Aarhus University Hospital, 9000 Aalborg, Denmark

Ulla Vogel, National Food Institute, Technical University of Denmark, 2860 Søborg, Denmark

Ulla Vogel, Institute for Science, Systems and Models, University of Roskilde, 4000 Roskilde, Denmark

Ulla Vogel, National Research Centre for the Working Environment, 2100 Copenhagen, Denmark

Author contributions: Andersen V and Vogel U designed and performed the research and wrote the paper; Andersen V, Ernst A, Jacobsen BA and Krarup HB conceived and designed the patient cohort; Christensen J and Tjønneland A performed the data analyses; all authors approved the manuscript.

Supported by This project was supported by the "Familien Erichsen Mindefond", the Lundbeck Foundation, the Danish Research Council, the Western Danish Research Forum for Health Science, the County of Viborg, the Danish Colitis-Crohn Association, "John M Klein og hustrus mindelegat", and the A.P. Møller Foundation for the Advancement of Medical Science

Correspondence to: Vibeke Andersen, PhD, Department of Medical, Viborg Regional Hospital, 8800 Viborg, Denmark. va9791@gmail.com

Telephone: +45-89272641 Fax: +45-89273484

Received: May 28, 2010 Revised: August 14, 2010

Accepted: August 21, 2010

Published online: January 14, 2011

1801282) and C1431T (rs 3856806); pregnane X receptor (PXR) (NR1I2) *PXR* A-24381C (rs1523127), C8055T (2276707), and A7635G (rs 6785049); and liver X receptor (LXR) (NR1H2) *LXR* T-rs1405655-C and T-rs2695121-C were assessed in a Danish case-control study of 327 Crohn's disease patients, 495 ulcerative colitis (UC) patients, and 779 healthy controls. Odds ratio (OR) and 95% CI were estimated by logistic regression models.

RESULTS: The *PXR* A7635G variant, the *PPAR γ* Pro12Ala and *LXR* T-rs2695121-C homozygous variant genotypes were associated with risk of UC (OR: 1.31, 95% CI: 1.03-1.66, $P = 0.03$, OR: 2.30, 95% CI: 1.04-5.08, $P = 0.04$, and OR: 1.41, 95% CI: 1.00-1.98, $P = 0.05$, respectively) compared to the corresponding homozygous wild-type genotypes. Among never smokers, *PXR* A7635G and the *LXR* T-rs1405655-C and T-rs2695121-C variant genotypes were associated with risk of IBD (OR: 1.41, 95% CI: 1.05-1.91, $P = 0.02$, OR: 1.63, 95% CI: 1.21-2.20, $P = 0.001$, and OR: 2.02, 95% CI: 1.36-2.99, $P = 0.0005$, respectively) compared to the respective homozygous variant genotypes. *PXR* A7635G (rs6785049) variant genotype was associated with a higher risk of UC diagnosis before the age of 40 years and with a higher risk of extensive disease (OR: 1.34, 95% CI: 1.03-1.75 and OR: 2.49, 95% CI: 1.24-5.03, respectively).

CONCLUSION: Common *PXR* and *LXR* polymorphisms may contribute to risk of IBD, especially among never smokers.

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Abstract

AIM: To investigate the contribution of polymorphisms in nuclear receptors to risk of inflammatory bowel disease (IBD).

METHODS: Genotypes of nuclear factor (NF)- κ B (NFKB1) *NF κ B* -94ins/del (rs28362491); peroxisome proliferator-activated receptor (PPAR)- γ (*PPAR γ*) *PPAR γ* Pro12Ala (rs

Key words: Crohn's disease; Genetic susceptibility; Single nucleotide polymorphisms; Smoking status; Transcription factors; Ulcerative colitis

Peer reviewer: María IT López, Professor, Experimental Biology, University of Jaen, araje de las Lagunillas s/n, Jaén 23071, Spain

Andersen V, Christensen J, Ernst A, Jacobsen BA, Tjønneland A, Krarup HB, Vogel U. Polymorphisms in NF- κ B, PXR, LXR, PPAR γ and risk of inflammatory bowel disease. *World J Gastroenterol* 2011; 17(2): 197-206 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/197.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.197>

INTRODUCTION

Chronic inflammatory bowel diseases (IBDs), ulcerative colitis (UC), and Crohn's disease (CD) are complex diseases that result from the interaction of numerous genetic and environmental factors^[1,2]. Recent studies have increased dramatically the number of genes known to be involved in IBD^[3-7]. However, the contribution of *NOD2* gene polymorphisms to IBD etiology in populations of Northern Europe is relatively small^[8-10], which has heightened interest in resolving the genetic determinants of IBD in these countries.

The rising incidence of IBD in the West suggests that environmental factors play a major role in its pathogenesis. The intestinal lumen contains a vast array of different substances that may interact with the host, such as dietary factors, microbial components, and environmental pollutants. Many of these stimuli interact with the transcription factor nuclear factor (NF)- κ B *via* activation of Toll-like receptors (TLRs) such as TLR4^[11,12]. Nuclear receptors are intracellular transcription factors that are activated by ligands^[13], which constitute a link between environmental factors and the regulation of many cellular processes, including inflammation^[14-16]. Thus, genetic variation in certain transcription factors may modify the regulation of relevant environmental factors and the associated risk of IBD.

Activation of NF- κ B leads to the induction of pro-inflammatory signal cascades^[13,17] and the resolution of intestinal inflammation^[18-20]. Studies on animal models of colitis^[21,22] and IBD patients^[23,24] suggest that impaired NF- κ B function leads to IBD. A polymorphism that involves deletion of four nucleotides in the NF- κ B promoter region, named -94ATTG ins/del, has been associated with attenuated promoter activity in luciferase reporter studies^[25]. The variant allele has been investigated as an IBD risk gene, but the results of these studies have been inconsistent^[24-31].

Activation of the nuclear receptors peroxisome proliferator-activated receptor (PPAR) γ , pregnane X receptor (PXR), and liver X receptor (LXR) leads to transcriptional regulation of pro-inflammatory target genes^[14,32,33] and inhibition of NF- κ B activity^[15,16,34,35], which results in a decrease in inflammation.

Studies of animal colitis models^[36-38] and IBD patients^[39] have suggested that impaired PPAR γ expression may confer IBD. The PPAR γ Pro12Ala variant allele is in tight linkage with the PPAR γ C1431T variant allele^[40], and the Pro to Ala substitution results in decreased transcriptional activation of target genes^[41]. Studies on the associa-

tion of the PPAR γ C1431T and Pro12Ala polymorphisms with a risk for IBD have demonstrated varying results^[42-44].

Loss of PXR function has been associated with intestinal inflammation in animal studies^[15], and low levels of PXR expression have been found in the intestine of UC patients^[45]. The PXR A7635G (rs6785049) homozygous variant genotypes and PXR C8055T (rs2276707) variant genotypes have been associated with a pronounced induction of a PXR target gene, *CYP3A4*, after treatment with rifampin^[46]. However, studies of PXR polymorphisms in relation to the risk for IBD have been inconsistent^[47-50].

Loss of LXR function compromised innate immunity in an animal model, which was attenuated after LXR administration^[14]. The LXR tag polymorphisms in intron 7 rs1405655 and intron 2 rs2695121 have been previously investigated as candidate gene targets involved in Alzheimer's disease^[51-53].

Tobacco smoke is a source of many exogenous compounds and induces inflammation^[54]. Moreover, smoking differentially affects the risk of CD and UC^[55], and the underlying mechanisms behind these effects are poorly understood^[56].

Accordingly, altered responses of NF- κ B, PPAR γ , PXR, and LXR to environmental pathogens may be involved in susceptibility to IBD. Hence, genetic variations in the transcription factors may modify the inflammatory response to environmental stimuli and affect the risk for IBD.

In the present study, we determined the allele and haplotype frequencies of polymorphisms in the genes that encode the transcription factors NF- κ B (*NFKB1*) -94ins/del (rs28362491); PPAR γ (*PPARG*) Pro12Ala (rs 1801282) and C1431T (rs 3856806); PXR (*NR1I2*) A-24381C (rs1523127), C8055T (rs2276707-T), and A7635G (rs 6785049); and LXR β (*NR1H2*) T-rs1405655-C and T-rs2695121-C. These polymorphisms were investigated together with the smoking status in a Danish cohort of 327 patients with CD, 495 patients with UC, and 779 healthy controls.

MATERIALS AND METHODS

Ethics

All subjects received written and oral information and provided written informed consent. The study was performed in accordance with the Declaration of Helsinki and was approved by the local Scientific Ethical Committees (VN2003/124).

Patients and controls

Diagnosis of CD or UC was based on clinical, radiological, endoscopic and histological examinations (infectious and other cases of IBD were excluded)^[56-58]. Patients were recruited from Viborg, Aalborg, and Herning Regional Hospitals from January 2004 to March 2005. Healthy blood donors recruited from Viborg Hospital served as controls. All subjects were Caucasian and older than 18 years of age. Data on the extent of the disease (CD: L1, L2, L3, UC: E1, E2, E3), family history, surgical treatment, advanced

medical treatment, age at diagnosis (under or over 40 years of age), and information on smoking habits at the time of diagnosis (patients) and at study entry (healthy controls) were collected.

Genotyping

Functional single nucleotide polymorphisms (SNPs) were selected based on the literature, except in the case of LXR with tag SNPs selected based on previous disease association^[51-53] because there were no available data on the functional effects. DNA was extracted from EDTA-stabilized peripheral blood samples from all patients and healthy controls using either a PureGene (Gentra Systems, Minneapolis, MN, USA) or Wizard Genomic (Promega, Madison, WI, USA) DNA purification kit, according to the manufacturers' recommendations.

Genotypes were determined by Taqman allelic discrimination (ABI 7500/7900HT, Applied Biosystems). DNA (20 ng) was analyzed in volumes of 4 μ L. Samples from cases and sub-cohort members were mixed during genotyping, and laboratory staff were blinded to the case or control status during analysis. Known genotype controls were included in each run. To confirm reproducibility, 10% of the samples were genotyped again. The genotypes exhibited 100 % identity.

NF κ B (NFKB1) ATTG ins/del (rs28362491) and PPAR γ (PPARG) Pro12Ala were genotyped as previously described^[59] and^[60], respectively). PPAR γ (PPARG) C1431T^[61], PXR (NR1I2) A-24381C (rs1523127), C8055T (rs2276707), and A7635G (rs6785049); and LXR- β (NR1H2) T-rs1405655C and T-rs2695121C were assessed using developed assays (Applied Biosystems).

Statistical analysis

Logistic regression was utilized to analyze the relationship between the investigated polymorphisms and IBD. The statistical analysis included only subjects with all necessary information available. Age was entered linearly in the model after verifying these data using a linear spline^[62]. Subgroup analyses were performed on polymorphisms in relation to the extent of the disease (CD: L1, L2, L3, UC: E1, E2, E3), family history, surgical treatment, advanced medical treatment, and age at diagnosis (above or below 40 years of age) for all cases. The haplotypes were inferred manually as described previously^[63].

Power analysis

The Genetic Power Calculator for case-control was utilized for power analysis of discrete traits^[64]. This study had greater than 80% power to detect a dominant effect with an odds ratio (OR) of 1.5 in either CD or UC, or 1.4 if CD and UC were combined.

RESULTS

Study population description

Characteristics of the Danish IBD patients and controls are shown in Table 1. Current smoking was more common among CD than UC patients, with incidences of

Table 1 Description of study participants *n* (%)

	CD (<i>n</i> = 327)	UC (<i>n</i> = 495)	Controls (<i>n</i> = 779)
Sex			
Male	129 (39)	239 (48)	400 (51)
Female	198 (61)	256 (51)	379 (49)
Age (yr)			
Median (5%-95%)	43 (23-76)	49 (24-76)	43 (23-60)
Age at diagnosis (yr)			
Median (5%-95%)	30 (15-64)	35 (17-68)	
Smoking habits			
Smokers	167 (51)	86 (17)	205 (26)
Never smokers	115 (35)	226 (46)	391 (50)
Former smokers	45 (14)	183 (37)	183 (23)
Location of UC			
Proctitis (E1)		207 (42)	
Left side (E2)		183 (37)	
Extensive (E3)		93 (19)	
Data not available		12 (2)	
Location of CD			
Colonic (L2)	151 (46)		
Ileal (L1)	74 (23)		
Ileocolonic (L3)	89 (27)		
Data not available	13 (4)		
Medication			
Advanced ¹	140 (43)	103 (21)	
No advanced medication ²	182 (56)	389 (79)	
Data not available	5 (2)	3 (1)	
Operation			
Yes	149 (46)	14 (3)	
No	171 (52)	472 (95)	
Data not available	7 (2)	9 (2)	

Disease location was classified according to the WGO Montreal classification. Statistical analyses included subjects for whom all information was available. ¹Azathioprine, 6-mercaptopurine, tumor necrosis factor inhibitors, or methotrexate; ²5-aminosalicylic acid, prednisolone. CD: Crohn's disease; UC: Ulcerative colitis.

51% and 17%, respectively. The genotype distributions among the controls did not deviate from Hardy-Weinberg equilibrium. The variant allele frequencies of the studied polymorphisms are shown in Table 2.

Associations between polymorphisms and disease phenotypes

The association between genotypes and the disease risk was analyzed separately for CD and UC (Table 3). The PXR A7635G (rs6785049) variant genotypes, PPAR γ Pro12Ala homozygous variant, and LXR T-rs2695121-C homozygous genotypes were associated with a higher risk of UC, as compared to the homozygous wild-type genotype (OR: 1.31, 95% CI: 1.03-1.66, *P* = 0.03, OR: 2.30, 95% CI: 1.04-5.08, *P* = 0.04, and OR: 2.41, 95% CI: 1.00-1.98, *P* = 0.05, respectively). No association was found between risk of CD and any genotype. Furthermore, no association was found between NF κ B -94 ins/del or PPAR γ C1431T polymorphisms and disease risk (Table 3).

Interaction between gene polymorphisms and smoking

The association between genotypes and disease risk was analyzed for current smokers, previous smokers, and never smokers. There was no interaction between smoking

Table 2 Allele frequencies for the gene polymorphisms in Crohn's disease and ulcerative colitis patients *n* (%)

	CD	UC	Controls
NF- κ B -94ins/del			
I	379 (58)	583 (59)	919 (59)
D	275 (42)	407 (41)	639 (41)
PPAR γ Pro ¹² Ala			
C	564 (86)	844 (85)	1315 (84)
G	90 (14)	146 (15)	243 (16)
PPAR γ C1431T			
C	560 (86)	832 (84)	1327 (85)
T	94 (14)	158 (16)	231 (15)
PXR rs1523127			
A	395 (60)	570 (58)	926 (59)
C	259 (40)	420 (42)	632 (41)
PXR rs2276707			
C	540 (83)	825 (83)	1275 (82)
T	114 (17)	165 (17)	283 (18)
PXR rs6785049			
A	426 (65)	615 (62)	1011 (65)
G	228 (35)	375 (38)	547 (35)
LXR rs1405655			
T	435 (67)	675 (68)	1079 (69)
C	219 (33)	315 (32)	479 (31)
LXR rs2695121			
T	292 (45)	430 (43)	727 (47)
C	362 (55)	560 (57)	831 (53)

NF- κ B: Nuclear factor κ B; PPAR γ : Peroxisome proliferator-activated receptor γ ; CD: Crohn's disease; UC: Ulcerative colitis; PXR: Pregnane X receptor; LXR: Liver X receptor.

status and gene polymorphisms in relation to the risk of CD or UC (data not shown). In general, there was an association between smoking status and the risk of CD and UC. The OR for risk of CD was high among smokers and low among former smokers, regardless of genotype status. In contrast, the OR for UC was high among former smokers and low among current smokers, regardless of genotype.

The ORs for associations between genotypes and the risk of CD, UC and combined IBD among individuals that had never smoked are shown in Table 4. The ORs were analyzed separately for CD and UC and for the combined groups to describe the risk of IBD because there was no heterogeneity between the two groups. The PXR A7635G (rs6785049) and LXR T-rs1405655-C and T-rs2695121-C variant genotypes were associated with a higher risk for IBD, as compared to the homozygous wild-type genotypes (OR: 1.41, 95% CI: 1.05-1.91, $P = 0.02$ and OR: 1.63, 95% CI: 1.21-2.20, $P = 0.001$, OR: 2.02, 95% CI: 1.36-2.99, $P = 0.0005$, respectively).

Haplotype analysis

Haplotype analysis among the healthy controls demonstrated that the PXR C8055T variant genotype was more frequent in carriers of the PXR A7635G variant allele than among carriers of the A7635G wild-type, which indicated that these two polymorphisms were linked. Moreover, the presence of the A-24381C variant allele seemed to be independent of the PXR C8055T and A7635G genotypes. No significant association of PXR haplotypes and disease risk

was determined (data not shown). Tables 5 and 6 show the minor allele frequencies of the PXR polymorphisms compared to those in other studies, and published associations between PXR polymorphisms and risk of IBD^[47-59].

Haplotype analysis in the healthy controls demonstrated that carriage of the LXR rs1405655 C variant allele was linked to the presence of the LXR rs2695121 C variant allele. Carriage of the LXR rs1405655 C allele in this instance did not add to the risk of IBD, compared to carriage of only the rs2695121 C allele. The OR for the association between the LXR haplotype that encompassed the T-rs2695121-C and the T-rs1405655-C variant allele was 1.17, 95% CI: 1.00-1.36 and 1.23, 95% CI: 1.00-1.52, compared to the compound wild-type haplotype, respectively (data not shown).

Haplotype analysis was not performed for the closely linked PPAR γ Pro12Ala and C1431T polymorphisms.

Subgroup analysis

Subgroup analysis revealed that the PXR A7635G (rs6785049) variant genotype was associated with a higher risk of UC diagnosis before the age of 40 years and with a higher risk of extensive disease (OR: 1.34, 95% CI: 1.03-1.75 and OR: 2.49, 95% CI: 1.24-5.03, respectively), and the LXR T-rs2695121-C variant genotype was associated with a higher risk of advanced medical treatment for UC (OR: 1.80, 95% CI: 1.08-2.99) as compared to the homozygous wild-type genotype (data not shown).

DISCUSSION

In the present case-control study of 822 IBD patients (327 CD and 495 UC) and 773 healthy controls, we determined that PXR and LXR variant allele carriers were at higher risk of UC than the homozygous wild-type carriers, and that the association was strongest among individuals that had never smoked and those with severe UC. An association between PPAR γ Pro12Ala and the risk of UC was determined based on only a few subjects. No associations were determined between gene polymorphisms and risk for CD or UC among previous or current smokers. Furthermore, no associations were found between the NF- κ B gene polymorphism and risk of CD or UC. The association between LXR C-rs1405655-T and T-rs2695121-C variant genotypes and the risk of IBD among individuals that had never smoked withstood Bonferroni correction for multiple testing, whereas the other associations were not validated by these analyses. The strengths and weaknesses of the present study must be considered^[65]. For instance, one strength of the present study is the well-characterized study subjects with information that included smoking status. There are various methods used to determine the control group with associated advantages and disadvantages^[66]. In this study, the control group consisted of blood donors, who were not a random sample of the population. However, confounding data is not a likely explanation of the association because both cases and controls were not aware of their genotypes, and geno-

Table 3 Odds ratio for the studied gene polymorphisms in Crohn's disease and ulcerative colitis patients

	CD	UC	Control	OR _{CD}	95% CI ¹	P value	OR _{UC}	95% CI ¹	P value
NF- κ B -94ins/del									
II	107	175	267	1.00	-		1.00	-	
ID	165	233	385	1.08	0.80-1.46	0.62	0.94	0.72-1.21	0.62
DD	55	87	127	1.21	0.81-1.81	0.36	1.04	0.73-1.47	0.83
ID and DD	220	320	512	1.11	0.83-1.48	0.48	0.96	0.75-1.23	0.76
PPAR γ Pro ¹² Ala									
CC	240	364	549	1.00	-		1.00	-	
CG	84	116	217	0.88	0.65-1.20	0.43	0.83	0.63-1.09	0.17
GG	3	15	13	0.48	0.13-1.77	0.27	2.30	1.04-5.08	0.04
CG and GG	87	131	230	0.86	0.64-1.16	0.33	0.90	0.69-1.17	0.42
PPAR γ C1431T									
CC	241	352	561	1.00	-		1.00	-	
CT	78	128	205	0.81	0.59-1.12	0.20	1.00	0.76-1.31	0.99
TT	8	15	13	1.36	0.54-3.42	0.52	1.95	0.90-4.27	0.09
CT and TT	86	143	218	0.85	0.62-1.15	0.29	1.05	0.81-1.37	0.69
PXR rs1523127									
AA	114	160	280	1.00	-		1.00	-	
AC	167	250	366	1.06	0.79-1.43	0.71	1.15	0.89-1.50	0.29
CC	46	85	133	0.89	0.59-1.35	0.59	1.11	0.78-1.56	0.57
AC and CC	213	335	499	1.02	0.77-1.35	0.91	1.14	0.89-1.46	0.30
PXR rs2276707									
CC	223	339	517	1.00	-		1.00	-	
CT	94	147	241	0.92	0.68-1.24	0.57	0.97	0.75-1.26	0.84
TT	10	9	21	1.25	0.56-2.76	0.58	0.67	0.30-1.51	0.33
CT and TT	104	156	262	0.94	0.71-1.26	0.69	0.95	0.74-1.22	0.68
PXR rs6785049									
AA	137	184	334	1.00	-		1.00	-	
AG	152	247	343	1.12	0.84-1.49	0.46	1.35	1.05-1.74	0.02
GG	38	64	102	0.91	0.58-1.40	0.66	1.18	0.81-1.71	0.39
AG and GG	190	311	445	1.07	0.81-1.40	0.65	1.31	1.03-1.66	0.03
LXR rs1405655									
TT	143	229	383	1.00	-		1.00	-	
CT	149	217	313	1.26	0.95-1.68	0.11	1.22	0.95-1.57	0.11
CC	35	49	83	1.12	0.71-1.78	0.62	1.01	0.67-1.51	0.97
CT and CC	184	266	396	1.23	0.94-1.62	0.13	1.18	0.93-1.49	0.17
LXR rs2695121									
TT	62	88	170	1.00	-		1.00	-	
CT	168	254	387	1.28	0.90-1.83	0.17	1.30	0.95-1.77	0.10
CC	97	153	222	1.21	0.82-1.79	0.34	1.41	1.00-1.98	0.05
CT and CC	265	407	609	1.26	0.89-1.76	0.19	1.34	0.99-1.79	0.06

Statistical analyses included subjects for whom all information was available. ¹Adjusted for age, sex and smoking status. NF- κ B: Nuclear factor κ B; PPAR γ : Peroxisome proliferator-activated receptor γ ; CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds ratio; PXR: Pregnane X receptor; LXR: Liver X receptor.

typing was performed blindly. Furthermore, stratification could theoretically result in the determined associations. However, this possibility is considered unlikely because the cohort was recruited from an area of Denmark with a homogeneous population^[67]. Minor allele frequencies of *PXR* polymorphisms in the present study and in other published studies on Caucasian populations are shown in Table 5. The allele frequencies of the present study did not deviate from previously determined frequencies^[47,49,50]. Therefore, heterogeneity or stratification in the control group is not a likely explanation for the determined associations in our study (Table 5).

The present study included 1600 participants, and power analysis determined that this study had more than 80% power to detect a dominant effect with an OR of 1.5 in relation to either CD or UC, and 1.4 when CD and UC were combined. Moreover, genetic determinants may be stronger among patients with extensive development of

the disease^[68,69] and disease onset at a younger age. However, the obtained results cannot be excluded as false positive.

An association of the *NF- κ B* -94 ins/del with UC, CD, or IBD was not determined in the present study. The variant allele has been associated with a risk of UC in a study that used the family-based association test and the transmission disequilibrium test in 131 IBD pedigrees with UC offspring, which was replicated in a second set of 258 UC and 653 healthy controls with an OR for the combined studies of 1.57 (1.14-2.16)^[25]. This study was further replicated in a small study of 127 UC patients and 155 healthy controls^[26], whereas larger studies have not indicated any association between the polymorphism and IBD^[27-29], UC^[30,31], or CD^[24]. Our results are in accordance with the latter studies^[27-31].

In the present study, a statistically significant (although modest) association was determined between the homozygous *PPAR γ* Pro12Ala variant genotype and an increased

Table 4 Odds ratio for the gene polymorphisms among Crohn's disease and ulcerative colitis never smokers

	NS-CD	NS-UC	NS-control	OR _{NS-CD}	95% CI ¹	P value	OR _{NS-UC}	95% CI ¹	P value	OR _{NS-IBD}	95% CI ¹	P value
NF-κB -94ins/del												
II	40	79	136	1.00	-		1.00	-		1.00	-	
ID	56	109	194	0.99	0.62-1.57	0.97	0.98	0.68-1.42	0.93	0.98	0.71-1.36	0.92
DD	19	38	61	1.07	0.57-2.00	0.83	1.09	0.67-1.79	0.72	1.09	0.70-1.68	0.71
ID and DD	75	147	255	1.01	0.65-1.56	0.97	1.01	0.72-1.43	0.95	1.01	0.74-1.37	0.96
PPAR γ Pro ¹² Ala												
CC	83	167	270	1.00	-		1.00	-		1.00	-	
CG	31	50	117	0.86	0.54-1.38	0.54	0.71	0.48-1.04	0.08	0.75	0.54-1.05	0.09
GG	1	9	4	0.80	0.09-7.29	0.84	3.99	1.20-13.32	0.02	2.77	0.85-9.00	0.09
CG and GG	32	59	121	0.86	0.54-1.37	0.53	0.81	0.56-1.17	0.26	0.82	0.59-1.13	0.21
PPAR γ C1431T												
CC	85	163	285	1.00	-		1.00	-		1.00	-	
CT	26	56	100	0.88	0.53-1.44	0.60	0.98	0.67-1.44	0.93	0.93	0.67-1.31	0.70
TT	4	7	6	2.16	0.59-7.90	0.24	2.05	0.67-6.26	0.21	2.06	0.75-5.67	0.16
CT and TT	30	63	106	0.95	0.59-1.53	0.83	1.04	0.72-1.51	0.82	1.00	0.72-1.39	0.99
PXR rs1523127												
AA	43	74	149	1.00	-		1.00	-		1.00	-	
AC	51	103	176	1.01	0.64-1.60	0.97	1.15	0.79-1.67	0.46	1.11	0.80-1.53	0.54
CC	21	49	66	1.10	0.60-1.99	0.77	1.52	0.95-2.41	0.08	1.36	0.90-2.05	0.15
AC and CC	72	152	242	1.03	0.67-1.59	0.89	1.25	0.88-1.77	0.21	1.17	0.87-1.59	0.30
PXR rs2276707												
CC	73	150	260	1.00	-		1.00	-		1.00	-	
CT	36	70	119	1.08	0.69-1.70	0.74	1.03	0.72-1.47	0.89	1.04	0.76-1.43	0.81
TT	6	6	12	1.76	0.64-4.86	0.27	0.86	0.31-2.34	0.76	1.16	0.51-2.63	0.73
CT and TT	42	76	131	1.14	0.74-1.76	0.55	1.01	0.71-1.43	0.96	1.05	0.77-1.43	0.75
PXR rs6785049												
AA	42	77	168	1.00	-		1.00	-		1.00	-	
AG	52	119	176	1.19	0.75-1.88	0.47	1.49	1.04-2.13	0.03	1.38	1.01-1.89	0.05
GG	21	30	47	1.79	0.97-3.31	0.06	1.40	0.82-2.39	0.22	1.53	0.97-2.43	0.07
AG and GG	73	149	223	1.31	0.85-2.02	0.21	1.47	1.04-2.07	0.03	1.41	1.05-1.91	0.02
LXR rs1405655												
TT	43	95	203	1.00	-		1.00	-		1.00	-	
CT	55	106	154	1.69	1.07-2.65	0.02	1.54	1.08-2.18	0.02	1.58	1.16-2.16	0.004
CC	17	25	34	2.32	1.19-4.55	0.01	1.66	0.93-2.95	0.09	1.85	1.12-3.07	0.02
CT and CC	72	131	188	1.80	1.18-2.77	0.01	1.56	1.11-2.17	0.01	1.63	1.21-2.20	0.001
LXR rs2695121												
TT	15	30	90	1.00	-		1.00	-		1.00	-	
CT	59	126	197	1.82	0.98-3.39	0.06	1.98	1.23-3.17	0.005	1.93	1.28-2.92	0.002
CC	41	70	104	2.37	1.23-4.57	0.01	2.09	1.25-3.49	0.005	2.18	1.39-3.41	0.0007
CT and CC	100	196	301	2.01	1.11-3.64	0.02	2.01	1.28-3.17	0.002	2.02	1.36-2.99	0.0005

Statistical analyses included subjects for whom all information was available. ¹Adjusted for age and sex. NS: Never smoker; NF-κB: Nuclear factor κB; PPAR γ : Peroxisome proliferator-activated receptor γ ; CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds ratio; PXR: Pregnane X receptor; LXR: Liver X receptor.

Table 5 Minor allele frequencies of pregnane X receptor polymorphisms in studied populations

	Controls	C-rs3814055-T	A-rs1523127-C	C-rs2276707-T	A-rs6785049-G	
Danish	779		0.41	0.18	0.35	Present study
Irish	336	0.433	0.452	0.142	0.406	Dring <i>et al</i> ^[47]
Scottish	334		0.394			Ho <i>et al</i> ^[49]
Spanish	550	0.382		0.192		Martínez <i>et al</i> ^[50]

Rs3814055 and rs1523127 are closely linked.

risk of IBD. This result cannot be excluded as random because of the small sample size. In a combined Dutch and Chinese study, the PPAR γ C1431T variant allele was associated with UC in the Chinese study group but not in the Dutch study group, and no associations were indicated with CD^[42]. No associations between PPAR γ Pro12Ala polymorphism and UC^[43] or CD^[44] have been demonstrated in two small studies. Therefore, these collective studies

have not yielded consistent data that supported involvement of PPAR γ in IBD.

PXR A7635G (rs6785049) variant allele carriers were at a higher risk of UC and IBD than homozygous wild-type carriers were. Furthermore, risk was highest among individuals that had never smoked. Table 6 shows the results of published association studies of PXR polymorphisms in IBD. The risk allele is indicated for positive

Table 6 Published associations between pregnane X receptor polymorphisms and inflammatory bowel disease risk

	Cases	Controls	C-25385T (rs3814055)	A-24381C (rs1523127)	C8055T (rs2276707)	A7635G (rs6785049)	
Danish ¹	822	779		Neg	Neg	Variant	Present study
Irish ²	422	336	Wild-type	Wild-type	Variant	Wild-type	Dring <i>et al</i> ^[47]
Scottish ²	715	334		Neg			Ho <i>et al</i> ^[49]
Spanish ²	696	550	Variant		Wild-type		Martínez <i>et al</i> ^[50]
Canadian ³	270	336		Neg		Neg	Amre <i>et al</i> ^[48]

No association is indicated by “neg”. The risk allele is indicated for positive associations between the pregnane X receptor polymorphisms and inflammatory bowel disease risk. ¹Associations adjusted for smoking status; ²Associations not adjusted for smoking status; ³Children with Crohn’s disease.

associations, whereas a null result is indicated as “neg” in Table 6. These results were inconsistent. No association was determined between the PXR A-24381C (rs1523127) polymorphism and IBD in the present study or in a previous Scottish study^[49]. In contrast, Irish and Spanish studies have indicated opposite associations between IBD and the closely linked PXR C-25385T (rs3814055) polymorphism^[47,50]. Furthermore, the A7635G (rs6785049) variant genotype was found to be associated with risk for UC in the present study, whereas this allele was indicated to be protective for IBD in the Irish study^[47]. Collectively, these results suggest that variable linkage disequilibrium between the investigated and biologically functional SNPs, and population heterogeneity may contribute to the inconsistent results.

Low levels of PXR were expressed in the intestine of UC patients, and high PXR activity ameliorated colitis in an animal IBD model^[70]. Thus, impaired PXR function may fail to suppress NF- κ B-induced intestinal inflammation^[13,71]. Moreover, attenuated activation of PXR target genes, such as the xenobiotic transporters *MDR1* (ABCB1) and *MRP2* (ABCC2), may lead to a less proficient epithelial barrier. Several lines of evidence support the role of impaired xenobiotic transport in IBD, including the development of colitis in *mdr1a*-deficient mice^[72], low *MDR1* expression levels in UC patients^[73], and a meta-analysis that indicated an association between an *MDR1* (ABCB1) polymorphism and the risk of UC^[74]. Therefore, impaired PXR function may lead to less effective induction of *MDR1* and export of harmful substances that originate from bacteria, diet, and pollutants.

The present investigation yielded strong associations between the LXR T-rs2695121-C homozygous variant allele and the risk of UC, and between both of the studied LXR variants and the risk of IBD among individuals that had never smoked. Haplotype analysis suggested a strong linkage between the two polymorphisms, and that carriage of the LXR T-rs1405655-C variant genotype coupled to the other LXR polymorphism does not add to the risk of IBD, compared to carriage of only the LXR T-rs2695121-C variant genotype. These polymorphisms have only been previously investigated in relation to Alzheimer’s disease^[53]. LXR seems to have anti-inflammatory properties, and LXR represses a set of inflammatory genes after activation by bacterial components or cytokines^[32]. Furthermore, LXR has been recently demon-

strated to upregulate xenobiotic transport proteins, such as *MDR1* (ABCB1)^[75] and *MRP2* (ABCC2)^[76]. Therefore, our results suggest the involvement of LXR in UC etiology.

Finally, the present study suggested that the associations between the PXR A7635G (rs6785049) and both of the studied LXR variant genotypes and UC were stronger among never smokers than among previous or current smokers. Therefore, the impact of the PXR and LXR gene polymorphisms on population disease risk may be larger in population with low frequencies of smokers than in those with many smokers. None of the associations indicated in the previously mentioned studies were adjusted for smoking status. Therefore, differences in relevant exposure may have contributed to the inconsistent results. We have previously found that inclusion of smoking status may be essential for evaluation of genetic predisposition to IBD (unpublished data, V. Andersen), and the present study is in accordance with our former study. Moreover, recently, passive smoking has been suggested to confer risk of IBD in children^[77,78].

Tobacco smoke contains > 3000 different chemical substances that have an impact on many biological pathways in relation to IBD^[55]. However, no interaction between smoking status and the studied polymorphisms was determined in the present study. Tobacco smoke suppresses NF- κ B activation in blood mononuclear cells^[58], and a similar mechanism may occur in the intestine.

In summary, the present study of 1600 individuals suggests that PXR and LXR are implicated in determining individual susceptibility to UC in the Danish high-incidence population. Furthermore, the conferred risk seems to be strongest among individuals that have never smoked. Clearly, further research is necessary to assess the overall role of inborn variants in PXR and LXR on UC susceptibility and the underlying biological mechanisms in relation to IBD etiology. Our results suggest that inclusion of smoking status may be essential for the evaluation of the role of genetic predisposition to IBD.

COMMENTS

Background

Environmental and genetic factors are involved in the etiology of the chronic inflammatory bowel diseases (IBDs), ulcerative colitis (UC), and Crohn’s disease. Furthermore, gene-environment interactions may result from variants in genes involved in the handling of environmental factors.

Research frontiers

The rising incidence of IBD in the West suggests that environmental factors play a major role in its pathogenesis. Nuclear receptors are intracellular transcription factors that constitute a link between environmental factors and the regulation of many cellular processes, including inflammation. In this study, the authors demonstrated that genetic variants in the nuclear receptors pregnane X receptor (PXR) and liver X receptor (LXR) may confer risk of UC. Furthermore, the conferred risk seems to be strongest among individuals that have never smoked.

Innovations and breakthroughs

Recent reports have highlighted the importance of genetic variations in the etiology in IBD. This study explores the contribution of genetic variations in nuclear factors to risk of IBD. This is the first study to suggest that LXR may confer risk of UC, and moreover, add to our knowledge of risk of UC associated with PXR variants. Next, this study substantiated the authors' previous findings that inclusion of smoking status may be essential for the evaluation of the role of genetic predisposition to IBDs.

Applications

By understanding the genetic contribution to risk of IBDs, this study adds further to our knowledge about the biological pathways that lead to disease, which is considered a prerequisite for development of new molecular targets for treatment.

Terminology

PXR, LXR and peroxisome proliferator-activated receptor γ (PPAR γ) are nuclear receptors, i.e. sensors of the environment, because they are activated by the binding of various compounds termed ligands, and next, in similarity with nuclear factor (NF)- κ B, they are transcription factors, i.e. they regulate transcription of their target genes. Thereby, nuclear factors may constitute a link between environmental factors and the regulation of inflammation.

Peer review

The authors examined the contribution of genetic variants in the nuclear receptors PXR, LXR and PPAR γ and the transcription factor NF- κ B to the risk of IBDs. The study revealed that variants in genes that coded for PXR and LXR confer risk of UC, especially among never smokers. Furthermore, the study demonstrates that inclusion of smoking status may be essential for the evaluation of the role of genetic predisposition to IBDs.

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S- Editor Sun H L- Editor Kerr C E- Editor Zheng XM

Acute diverticulitis in younger patients: Any rationale for a different approach?

Gil R Faria, Ana B Almeida, Herculano Moreira, João Pinto-de-Sousa, Pedro Correia-da-Silva, Amadeu P Pimenta

Gil R Faria, Ana B Almeida, Herculano Moreira, João Pinto-de-Sousa, Pedro Correia-da-Silva, Amadeu P Pimenta, Department of General Surgery, Hospital de São João, Al. Prof. Hernâni Monteiro, HSJ, 4200-319 Porto, Portugal

Gil R Faria, Herculano Moreira, João Pinto-de-Sousa, Amadeu P Pimenta, Faculty of Medicine of the University of Porto, Al. Prof. Hernâni Monteiro, HSJ 4200-319 Porto, Portugal

João Pinto-de-Sousa, Amadeu P Pimenta, Institute of Pathology and Molecular Immunology of the University of Porto - IPATIMUP - Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal

Author contributions: Faria GR and Almeida AB collected the data; Faria GR and Pinto-de-Sousa J analyzed the results; Faria GR and Moreira H wrote the article; Moreira H and Correia-da-Silva P designed the study; Pinto-de-Sousa J and Pimenta AP reviewed the article.

Correspondence to: Gil R Faria, MD, MSc, Department of General Surgery, Hospital de São João, Al. Prof. Hernâni Monteiro, HSJ, 4200-319 Porto, Portugal. gilfaria@netcabo.pt

Telephone: +351-2-25512100 Fax: +351-2-24112916

Received: March 30, 2010 Revised: May 5, 2010

Accepted: May 12, 2010

Published online: January 14, 2011

Abstract

AIM: To compare the natural history and course of acute diverticulitis in a younger age group with an older population and to evaluate whether younger patients should be managed differently.

METHODS: This study was a retrospective review of 157 patients treated with acute diverticulitis between January 1, 2004 and December 31, 2007. Diverticulitis was stratified according to the Hinchey classification. Patients were divided into 2 populations: group A \leq 50 years ($n = 31$); group B > 50 years ($n = 126$). Mean patient follow-up was 15 mo.

RESULTS: The median age was 60 years. A significantly higher proportion of patients in group B presented with complicated diverticulitis (36.5% vs 12.9%, $P = 0.01$). Recurrence was more frequent in group A

(25.8% vs 11.1%, $P = 0.03$) and the mean time-to-recurrence was shorter (12 mo vs 28 mo, $P = 0.26$). The most severe recurrent episodes of acute diverticulitis were classified as Hinchey stage I and none of the patients required emergency surgery. In multivariate analysis, only age ($P = 0.024$) was identified as an independent prognostic factor for recurrence.

CONCLUSION: Based on the results of this study, we recommend that diverticulitis management should be based on the severity of the disease and not on the age of the patient.

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Key words: Acute diverticulitis; Recurrence; Age factors; Severity; Surgical treatment

Peer reviewers: Imran Hassan, MD, Assistant Professor, Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States; Jon C Gould, MD, FACS, Associate Professor of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States

Faria GR, Almeida AB, Moreira H, Pinto-de-Sousa J, Correia-da-Silva P, Pimenta AP. Acute diverticulitis in younger patients: Any rationale for a different approach? *World J Gastroenterol* 2011; 17(2): 207-212 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/207.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.207>

INTRODUCTION

Diverticulosis of the colon is an acquired condition that results from herniation of the mucosa through defects in the muscular layer. Epidemiological and anatomic studies have revealed that diverticular formation of the colon occurs primarily in industrialized and Westernized countries^[1,2]. The true incidence of diverticulosis is difficult

to measure because most individuals are asymptomatic. It is believed that approximately 60% of individuals over the age of 60, living in Westernized countries, will develop colonic diverticula^[3]. Postmortem studies revealed that the disorder is rare prior to the fourth decade, but increases progressively, so that at age 80 approximately 65% of patients will have diverticula of the colon^[4,5]. Several epidemiological studies have demonstrated an association between diverticulosis and diets low in dietary fibers and high in refined carbohydrates^[6-9].

Most patients with colonic diverticula remain asymptomatic; only 10%-25% will develop diverticulitis and 1% will actually require surgery^[1,3,10,11]. The clinical presentation of acute diverticulitis varies widely from mild inflammation to full-blown perforation and peritonitis. In most patients however, the clinical presentation of diverticulitis is mild and medical treatment will achieve successful management of the acute episode^[12].

When treated medically, the risk of recurrent diverticulitis ranges from 7% to 45%^[13-15], with most recurrences (90%) occurring in the first 5 years^[13-16]. The fact that the severity of recurrent attacks is usually considered to be higher than the first episode^[13] has been questioned by several authors^[16-21].

In recent years, there has been conflicting information regarding the incidence and natural history of diverticulitis in younger patients (aged 50 years or younger). While earlier studies found that the incidence ranged between 2% and 7%^[22-26], more recent studies reported a higher incidence ranging between 18% and 34%^[17,27]. The natural course and outcome of diverticulitis in the younger patient has also been a matter of discussion. While earlier studies reported a more severe course with a higher rate of complications and recurrent episodes in younger patients with diverticulitis^[7,8,22,25,28], some recent reports suggested a milder course, not different from that observed in older patients^[12,19,21,27]. The aim of this retrospective study was to compare the natural history and the course of acute diverticulitis in a younger age group with that of an older population. Our purpose was to evaluate whether younger patients should be treated according to the same criteria as their older counterparts.

MATERIALS AND METHODS

One hundred fifty seven consecutive patients aged 24-93 years (median, 60 years) with documented acute sigmoid diverticulitis were treated at the University Hospital São João at Porto, with a catchment area of roughly 1 million patients, between 2004 and 2007.

For the patients diagnosed with diverticulitis, who had prior attacks, the clinical, radiological and analytical data of the first episode was retrieved and analyzed. Patients were stratified for age, according to the age at first episode.

The diagnosis of acute diverticulitis was based on lower abdominal pain (usually on the left side), fever and an increase in serum inflammatory parameters. Emergency surgical treatment was undertaken in patients with diffuse peritonitis or septic shock. Patients not undergoing emer-

gency surgery had an abdominal computed tomography (CT) scan and only those with characteristic findings were included in this study. CT scan findings were classified as uncomplicated (localized colonic wall thickening and/or infiltration of pericolic fat) or complicated (pericolic or abdominal abscess, localized or free extra luminal air or contrast, bowel obstruction, and fistula formation). The severity of complicated diverticulitis was graded according to Hinchey's criteria^[29]. Stage I patients had small or confined pericolic abscesses, Stage II patients had larger pelvic or distant intra-abdominal abscesses, Stage III patients had purulent diverticulitis and in Stage IV patients had fecal peritonitis.

After resolution of acute inflammation, all patients who had no previous endoscopic diagnosis of diverticular disease underwent elective colonoscopy to confirm the presence of colonic diverticula and to rule out the presence of other diseases, such as cancer or inflammatory bowel disease.

The patients were stratified into 2 groups, according to age at first acute episode: group A included patients aged 50 years or younger ($n = 31$) and group B included patients older than 50 years ($n = 126$).

Demographic (age, gender, ethnicity), clinical (presentation signs and symptoms, endoscopic findings), laboratory [white blood cell count and C-reactive protein (CRP) measurements] and radiological (CT scan) features were analyzed and compared between the 2 groups.

Emergency surgery was undertaken if patients had diffuse peritonitis, septic shock, or if the clinical course did not improve after 48-72 h of conservative medical management. In some patients, elective surgery was performed after resolution of the acute episode and all of these had bowel preparation and primary anastomosis. CT-guided percutaneous drainage was performed in patients with abscesses larger than 4 cm in diameter.

Follow-up (range, 6-54 mo; median, 15 mo) was available for 98% of the population and completed in June 2008.

Recurrence of diverticulitis was diagnosed if the patient presented with similar symptoms and clinical findings, confirmed by laboratory and radiological investigations. The rate and severity of recurrences was compared between both groups, as well as the need of emergency or elective surgery after an episode of recurrent diverticulitis.

For statistical analysis, the program SPSS (version 15.0 for Mac) was used. The differences in the distribution of cases according to several parameters being analyzed were compared by χ^2 test for categorical variables and the Student *t*-test for continuous variables. Multivariate analysis was performed using the logistic regression method in order to identify independent factors associated with recurrence. $P < 0.05$ were considered significant.

RESULTS

The median age of the 157 patients with acute sigmoid diverticulitis was 60 years (range, 24-93 years). The mean age of patients in group A was 40 years (median, 41 years)

Table 1 Clinical and laboratory features of the 2 groups of patients according to age *n* (%)

	Group A (≤ 50 yr)	Group B (> 50 yr)	<i>P</i>
Signs and symptoms			
Abdominal pain	30 (96.7)	117 (92.8)	NS
Fever	9 (29.0)	30 (23.8)	NS
Acute abdomen	10 (32.3)	30 (23.8)	NS
Vomiting	5 (16.1)	26 (20.6)	NS
Constipation	3 (9.7)	23 (18.2)	NS
Diarrhea	2 (6.4)	13 (10.3)	NS
Laboratory			
Leucocytosis ($> 11 \times 10^9$)	24 (82.7)	67 (55.8)	0.01
Mean WBC count ($\times 10^9$)	15.0	13.1	0.01
Mean CRP (mg/dL)	151.5	133.5	0.005

Group A (*n* = 31) and Group B (*n* = 126). WBC: White blood cell; CRP: C-reactive protein.

and for group B was 65 years (median, 62 years). Sixty-eight patients (43.3%) were male and 89 (56.7%) were female (M/F: 0.76/1). Thirty-one patients (19.7%) were 50 years old or younger. There was a striking male predominance in the younger age group (group A) with a male:female ratio of 1.8:1. The older age group (group B) showed a female predominance with a male:female ratio of 0.61:1. The difference between both groups according to gender was significant (*P* = 0.008).

The most common symptom present on admission was left lower quadrant abdominal pain, which was observed in 147 (93.6%) patients. No significant differences were observed between groups A and B regarding the symptoms and signs most commonly observed in acute episodes (Table 1).

In this series, younger patients had a significantly higher percentage of leucocytosis (82.7% *vs* 55.8%, *P* = 0.01) than patients older than 50 years. The mean value of leucocytosis was significantly higher in patients of group A (*P* = 0.012) as well as the mean value of CRP elevation (*P* = 0.005) (Table 1).

One hundred and seven patients (68.2%) had a mild course of diverticulitis with no complications. Fifty patients (31.8%) had a more severe and complicated course. The most common complications were abdominal abscesses, which were observed in 33 cases (21.0%). Five patients required guided percutaneous drainage of abdominal abscesses. These resolved in all patients with no need for further urgent surgery. There were 17 patients (10.8%) who developed free perforation and all underwent emergency surgery (Table 2). No patient died during hospitalization.

One hundred and forty seven patients underwent an abdominal CT scan. Ten patients presented with diffuse peritonitis and septic shock, and underwent emergency surgery without further radiological investigation. In these cases, diagnosis of complicated diverticulitis was done intraoperatively.

A significantly lower proportion of patients of group A presented with complicated diverticulitis, compared to patients of group B (12.9% *vs* 36.5%, *P* = 0.01), namely

Table 2 Severity of disease (Hinchey classification) and surgical treatment of the 2 groups according to age *n* (%)

	Group A (≤ 50 yr)	Group B (> 50 yr)	<i>P</i>
Disease severity			0.01
Uncomplicated	27 (87.1)	80 (63.5)	
Complicated	4 (12.9)	46 (36.5)	
Complication grading			0.04
Grade I / II	3 (9.7)	30 (23.8)	
Grade III / IV	1 (3.2)	16 (12.7)	
Surgical treatment			
Emergency surgery	1 (3.2)	18 (14.3)	0.03
Elective surgery	4 (16.1)	5 (4.0)	0.07

Table 3 Recurrence and severity of recurrent disease in the 2 groups according to age *n* (%)

	Group A (≤ 50 yr)	Group B (> 50 yr)	<i>P</i>
Disease recurrence			0.03
Non recurrent	23 (74.2)	112 (88.9)	
Recurrent	8 (25.8)	14 (11.1)	
Severity of recurrence			0.10
Non complicated	6 (75)	10 (71)	
Complicated (Grade I)	2 (25)	4 (29)	

Table 4 Multivariate analysis of recurrence factors in patients with diverticulitis

Factor	Binary regression logistics	
	Exp (B)	<i>P</i>
Male gender	1.13	0.827
Age ≤ 50 yr	0.26	0.024
White blood cell count ($\times 10^9$)	0.99	0.830
Severity index	1.08	0.798

abdominal abscesses (Hinchey stages I / II, 9.7% *vs* 23.8%) and free perforation (Hinchey stages III / IV, 3.2% *vs* 12.7%) (*P* = 0.04) (Table 2).

A significantly lower proportion of patients of group A underwent an emergency operation after the first episode of acute diverticulitis (3.2% *vs* 14.3%, *P* = 0.026) (Table 2). Seventeen patients undergoing emergency surgery had free perforation with diffuse peritonitis, and 2 patients had pericolonic abscesses.

A significantly higher proportion of patients of group A developed recurrence of diverticulitis (25.8% *vs* 11.1%, *P* = 0.03) (Table 3). The mean interval between the primary episode and the recurrent disease was 12 mo (range, 1-40 mo; median, 4 mo) in group A and 28 mo (range, 1-150 mo; median, 6 mo) in group B (*P* = 0.26). No significant differences were observed between the 2 groups regarding the severity of recurrent episodes (*P* = 0.1) (Table 3). The most severe recurrent episodes of acute diverticulitis were classified as Hinchey stage I and none of the patients required emergency surgery.

In multivariate analysis of this series, age (*P* = 0.024) was identified as an independent prognostic factor of recurrence of acute diverticulitis (Table 4).

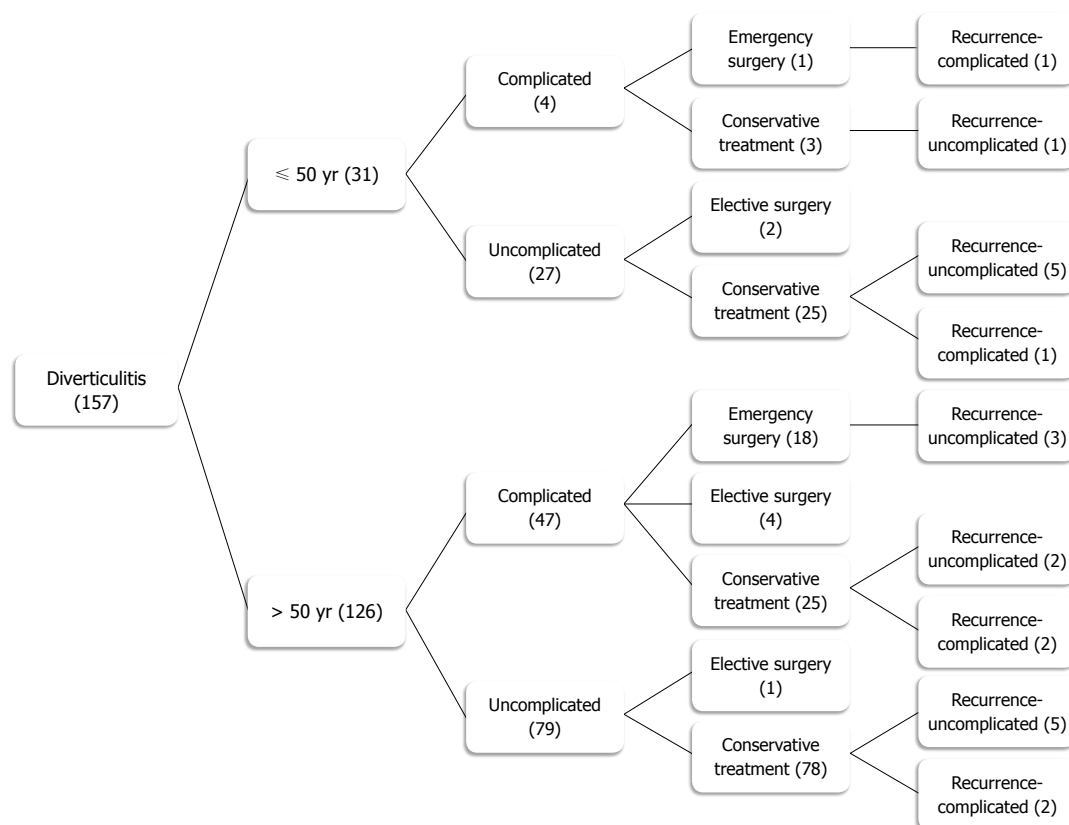


Figure 1 Flowchart of the clinical evolution of the patients with acute diverticulitis in our series.

DISCUSSION

For many years, diverticulosis was considered an old patient's disease with an incidence of 5% in patients younger than 50 years of age^[13]. Recently, the incidence of diverticulosis in younger patients has increased and has been reported to range from 18% to 34%^[17,19,21,27]. In our study, 19.7% of patients with diverticulitis were aged 50 years or younger.

We report a male predominance in younger patients with acute diverticulitis, which is in agreement with previous studies^[19,30].

Patients presenting with clinical signs, symptoms and laboratory findings suggesting acute diverticulitis underwent a CT scan of the abdomen at initial presentation. Abdominal CT is the diagnostic test of choice in acute diverticulitis. It has high sensitivity (approximately 93%-97%) and specificity approaching 100% for the diagnosis^[31,32]. It is the best method for grading the severity of inflammation, and it also enables the classification of diverticulitis into complicated or uncomplicated disease^[12,33].

In the present series, 17 patients (10.8%) with diffuse peritonitis and septic shock underwent emergency surgery. This observation is in agreement with other reports suggesting that 10% of patients admitted with acute diverticulitis will require surgical treatment during the same admission^[34].

The clinical course and severity of diverticulitis in younger patients have not yet been clearly defined. The most appropriate management of patients whose acute

diverticulitis resolves after medical treatment is still controversial. Diverticulitis in younger patients has been reported to have a more aggressive course or to require emergency surgery more frequently than in older patients^[7,8,13,21,24,28,35-37]. As such, it has been argued that all patients younger than 50 should undergo elective colon resection after an initial episode of acute diverticulitis, with the intention to prevent a recurrent attack, which could present with perforation and require a stoma^[11,17,36,37]. However, other studies published in the last decade failed to support this conclusion and suggest that the course of diverticulitis in younger patients is not as aggressive as once thought^[12,19,27,38-41]. These studies reported a course of disease in younger patients not different from the older age group and with a similar rate of complications. Our study found that complicated episodes of acute diverticulitis were significantly less frequent in younger patients, suggesting that diverticulitis in younger patients does not have a more aggressive course.

In our study, younger patients were significantly more prone to recurrent episodes, despite the relatively short follow-up period. Our data is consistent with earlier studies that reported a higher recurrence rate of diverticulitis among younger patients^[21,30].

The most appropriate timing for elective surgery following an episode of acute diverticulitis remains controversial. Parks first described medical management of diverticulitis in 1969^[13]. He stated that mortality rate for each subsequent attack of diverticulitis increased from 4.7% during the first admission to 7.8% during each subsequent

episode. He also reported that diverticulitis was less likely to respond to medical therapy as the number of acute episodes increased. Some retrospective studies suggested that the number of recurrences is associated with an increased need for a subsequent emergency surgical procedure^[19]. It was suggested^[42] that the likelihood of emergency surgery is increased by a factor of at least 2 with each subsequent hospitalization for diverticulitis. Thereafter, the American Society of Colon and Rectal Surgeons published a practice guideline for uncomplicated diverticulitis, which recommended definitive surgical treatment after 2 episodes of diverticulitis^[11] based on the data provided by Parks. In recent years, this finding has been questioned and in its most recent guidelines, the American Society of Colon and Rectal Surgeons, no longer considers the number of attacks as a definite indication for surgery^[41].

In this study, we have observed that in the subgroup of 18 patients, which developed acute recurrence after non-operative treatment (Figure 1), only 5 (27.8%) had complicated findings on CT scan, classified as Hinchey stage I. All patients that developed acute recurrence were successfully treated nonoperatively. These results suggest that recurrence is not associated with an increased rate of either complications or less successful medical management, and are in agreement with several recent publications that have questioned the practice of surgical resection after 2 attacks, as well as after complicated disease^[16-21,43,44].

According to our data, the great majority of younger patients (87.1%) had uncomplicated diverticulitis according to the CT scan criteria. Twenty-five of the younger patients (81%), after a first episode of uncomplicated diverticulitis, did not require surgery during follow-up. Although a significant amount of these patients developed recurrent episodes (24.0%), only one developed a recurrence with a pericolic abscess (Hinchey stage I) and none required emergency surgery. This data suggests that younger patients with uncomplicated diverticulitis may be managed safely without an operation after an initial episode of diverticulitis, which is in agreement with other recent reports^[45]. Three patients of group A (< 50 years old) with complicated diverticulitis did not require surgery during follow-up (Figure 1). One patient developed a non-complicated recurrence and none required an emergency operation. Although this may suggest that complicated diverticulitis may also be managed without an operation, the number of patients was too small to allow for any recommendation. These results are in agreement with another recent report^[46].

In conclusion, our study shows that acute diverticulitis is a mild disease with a low complication rate. In younger patients, there is a male predominance and the disease tends to be less severe than in the older age group, in spite of the higher recurrence rate. According to our results, the clinical course of uncomplicated acute diverticulitis is similar both in younger and older patients. Therefore the same guidelines should be used in the treatment of both groups of patients. The number of younger patients with complicated diverticulitis evaluated in this study was small but, should our results be confirmed in larger or even pro-

spective studies, it seems reasonable to recommend the adoption of a similar strategy in both age groups.

COMMENTS

Background

The management of patients with diverticular disease after resolution of an attack of acute diverticulitis, has been a matter of debate by colon and rectal surgeons. The reports of a more severe and virulent disease in younger patients has been questioned by several authors and although the incidence has increased in the last decades, these patients are increasingly managed conservatively.

Research frontiers

To compare the natural history and the course of acute diverticulitis in a younger age group with an older population and to evaluate whether younger patients should be managed differently.

Innovations and breakthroughs

In this retrospective study, the patients younger than 50 years at first presentation of diverticulitis had less severe disease but a higher recurrence rate (25.8% vs 11.1%). All the patients with recurrent disease were managed with conservative treatment and for the majority of patients, the most severe attack of diverticulitis was the first. On multivariate analysis, age was the only factor predictive of recurrence.

Applications

Based on the results of this study, the authors recommend that diverticulitis management should be based on the severity of the disease and not on the age of the patient.

Peer review

Overall this is a good observational study, with interesting conclusions. Its strength is that all patients had computed tomography-documented episodes of diverticulitis and good follow-up.

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S- Editor Tian L L- Editor Cant MR E- Editor Lin YP

Hemolysis results in impaired intestinal microcirculation and intestinal epithelial cell injury

Sebastiaan J Hanssen, Tim Lubbers, Caroline M Hodin, Frits W Prinzen, Wim A Buurman, Michael J Jacobs

Sebastiaan J Hanssen, Tim Lubbers, Caroline M Hodin, Wim A Buurman, Michael J Jacobs, Department of Surgery, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands

Sebastiaan J Hanssen, Frits W Prinzen, Michael J Jacobs, Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands

Sebastiaan J Hanssen, Michael J Jacobs, European Vascular Center Aachen-Maastricht, Department of Vascular Surgery, University Hospital Aachen, Pauwelsstraße 30, 52074 Aachen, Germany

Tim Lubbers, Caroline M Hodin, Wim A Buurman, Nutrition and Toxicology Research Institute Maastricht, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands

Frits W Prinzen, Department of Physiology, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands

Author contributions: Hanssen SJ, Lubbers T, Prinzen FW, Buurman WA and Jacobs MJ designed the study; Hanssen SJ, Lubbers T and Hodin CM performed the experiments; Prinzen FW, Buurman WA and Jacobs MJ provided financial support; Hanssen SJ, Lubbers T, Hodin CM and Prinzen FW analyzed the data; Hanssen SJ wrote the manuscript; Lubbers T, Hodin CM, Prinzen FW, Buurman WA and Jacobs MJ were involved in editing the manuscript.

Supported by The Profileringsfonds of the Maastricht University Medical Center (to Jacobs MJ and Buurman WA); and an AGIKO-stipendium 920-03-522 (to Lubbers T) from The Netherlands Organization for Health Research and Development

Correspondence to: Dr. Wim A Buurman, Department of Surgery, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht,

The Netherlands. w.buurman@maastrichtuniversity.nl

Telephone: +31-43-3881499 Fax: +31-43-3884154

Received: May 17, 2010 Revised: October 19, 2010

Accepted: October 26, 2010

Published online: January 14, 2011

hemoglobin (FHb) on intestinal microcirculation and intestinal epithelial injury in a rat model.

METHODS: To induce elevated intravascular circulating FHb, male Sprague-Dawley rats received water or FHb infusion. Microcirculatory changes in jejunum, ileum and colon were evaluated using fluorescent microspheres. Intestinal injury was quantified as plasmatic release of ileal lipid binding protein (iLBP) and verified by histological analysis of the ileum.

RESULTS: Water and FHb infusions resulted, when compared with saline infusion, in reduced intestinal microcirculation (after 30 min $P < 0.05$, or better; after 60 min FHb infusion $P < 0.05$ for jejunum and colon). Circulating FHb levels correlated significantly with release of iLBP (Spearman $r = 0.72$, $P = 0.0011$). Epithelial cell injury of the villi was histologically observed after water and FHb infusions.

CONCLUSION: This study shows that circulating FHb leads to a reduction in intestinal microcirculatory blood flow with marked injury to intestinal epithelial cells. These data support the hypothesis that circulating FHb contributes to the development of intestinal injury.

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Key words: Hemoglobin; Microcirculation; Organ injury; Vasoconstriction

Peer reviewer: Dr. Jianyuan Chai, PhD, MS, BS, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States

Hanssen SJ, Lubbers T, Hodin CM, Prinzen FW, Buurman WA, Jacobs MJ. Hemolysis results in impaired intestinal microcirculation and intestinal epithelial cell injury. *World J Gastroenterol* 2011; 17(2): 213-218 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i2/213.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.213>

Abstract

AIM: To study the effect of circulating cell-free oxy-

INTRODUCTION

Gastrointestinal complications following cardiovascular surgery are feared, as these complications are associated with high patient morbidity and mortality rates^[1-4]. The proposed pathophysiological mechanisms underlying intestinal complications include: (1) Ischemia/reperfusion and hypoperfusion injury as a result of redistribution of blood flow and increased oxygen demand; (2) Inflammatory mediated endothelial dysfunction and priming; and (3) Increased mesenteric vascular resistance^[5-7].

It is widely accepted that cardiovascular surgery is associated with considerable injury to red blood cells resulting in hemolysis. The use of extracorporeal circulation, donor blood transfusion and cell salvage devices inevitably leads to increased circulating levels of the hemolytic product cell-free oxyhemoglobin (FHb)^[8-10]. Such circulating FHb has been reported to scavenge endothelial nitric oxide (NO) in chronic hemolytic diseases, potentially perturbing microcirculatory blood flow which may result in organ injury and/or organ dysfunction^[11,12]. The effect of hemolysis on intestinal microcirculation and gut wall integrity remains unclear. We hypothesized that intravascular FHb compromises intestinal blood flow and consequently induces intestinal epithelial cell injury.

The present study aimed to evaluate intestinal blood flow and intestinal epithelial cell injury due to elevated circulating FHb levels. An animal model was developed with FHb plasma levels similar to those found during cardiovascular surgery. The influence of circulating FHb on intestinal microcirculation was studied using fluorescent microspheres and intestinal injury was evaluated both biochemically and histopathologically.

MATERIALS AND METHODS

Animals

The Animal Ethics Committee of the Maastricht University Medical Center approved the study. Male Sprague-Dawley rats, 450-500 g (Charles River Laboratories, Maastricht, The Netherlands) were housed under controlled conditions of temperature and humidity. Prior to the experiments, rats were fed standard rodent chow *ad libitum* and had free access to water.

Generation and measurement of FHb

To generate FHb-containing solution for infusion, heparinized blood was obtained from rats through aortic puncture 1 d prior to intervention. Red blood cells were isolated by centrifugation ($2750 \times g$, 15 min at 4°C). Supernatant and buffy-coat were carefully discarded and the remaining red blood cells were washed thrice in fresh sterile saline (1:3 v/v). Hemolysis was induced by freeze-thaw cycles. To remove all red blood cell membranes the solution was ultra-centrifuged ($20000 \times g$, 30 min at 4°C) and filtered (0.2 µm, Schleicher and Schuell, Dassel, Germany). Final FHb concentration was adjusted with sterile saline to 300 µmol/L. FHb concentration for infusion, as well as plasma values, were measured by derivative spectrophotometry^[13].

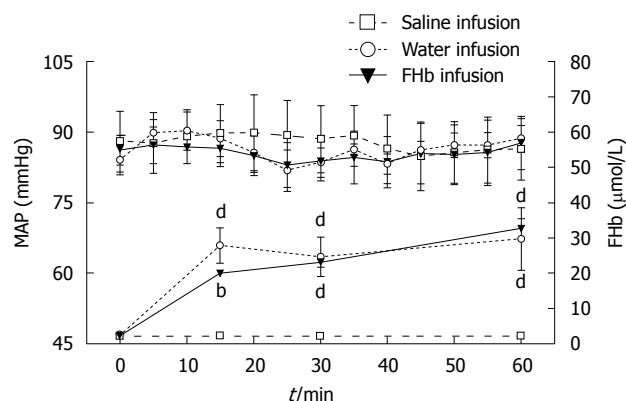


Figure 1 The effect of saline, water or free oxyhemoglobin infusion on mean arterial pressure and free oxyhemoglobin levels. During the study period the mean arterial pressure (MAP) (left Y-axis) remained unchanged in all interventional groups. Plasma free oxyhemoglobin (FHb) levels (right Y-axis) were significantly elevated in the group with water infusion and in the group with FHb infusion after 15 min and onwards. ^b $P < 0.01$, ^d $P < 0.001$.

Experimental design

After induction with 4% isoflurane, anesthesia was maintained at 2% during the whole study protocol. To calculate mean arterial pressure (MAP) a cannule (polyethylene tubing, PE-10) was placed in the left femoral artery and connected to an external pressure transducer (Uniflow®; Baxter, Utrecht, The Netherlands). Microspheres were infused *via* a cannule (PE-10, 11 cm) that was placed in the aortic arch *via* the right femoral artery. The left femoral vein was used to infuse saline, water or FHb.

Three groups ($n = 6$ per group) were included in the study. To induce intravascular hemolysis and yield circulating FHb, the first group received sterile pyrogen-free water infusion (prime 0.6 mL/100 g BW, continuous infusion of 2 mL/100 g BW per hour); the second group received FHb infusion (prime 0.65 mL/100 g BW, continuous infusion 1.3 mL/100 g BW per hour). The control group received saline infusion in the same volume as the water infusion group.

Assessment of microcirculatory blood flow using microspheres

To evaluate the intestinal microcirculatory blood flow pre-infusion, and after 15, 30 and 60 min, fluorescent microspheres with different colors were used (yellow, lemon, orange or persimmon; diameter 15 µm, 1×10^6 microspheres/mL; Dye-Trak®, Triton Technology, San Diego, CA). Infusion of microspheres (0.25-0.3 mL) and calculation of organ blood flow were performed as described previously^[14].

Evaluation of intestinal injury

To evaluate enterocyte damage, the release of intestinal ileal lipid binding protein (iLBP) was measured. For iLBP assessment, arterial blood samples (600 µL) were collected before infusion and after 15, 30 and 60 min of infusion. ILBP was measured in plasma by an Enzyme Linked Immuno Sorbent Assay (ELISA, detection limit 1.28 ng/mL), kindly provided by Hycult biotechnology (Hbt, Uden, The Netherlands).

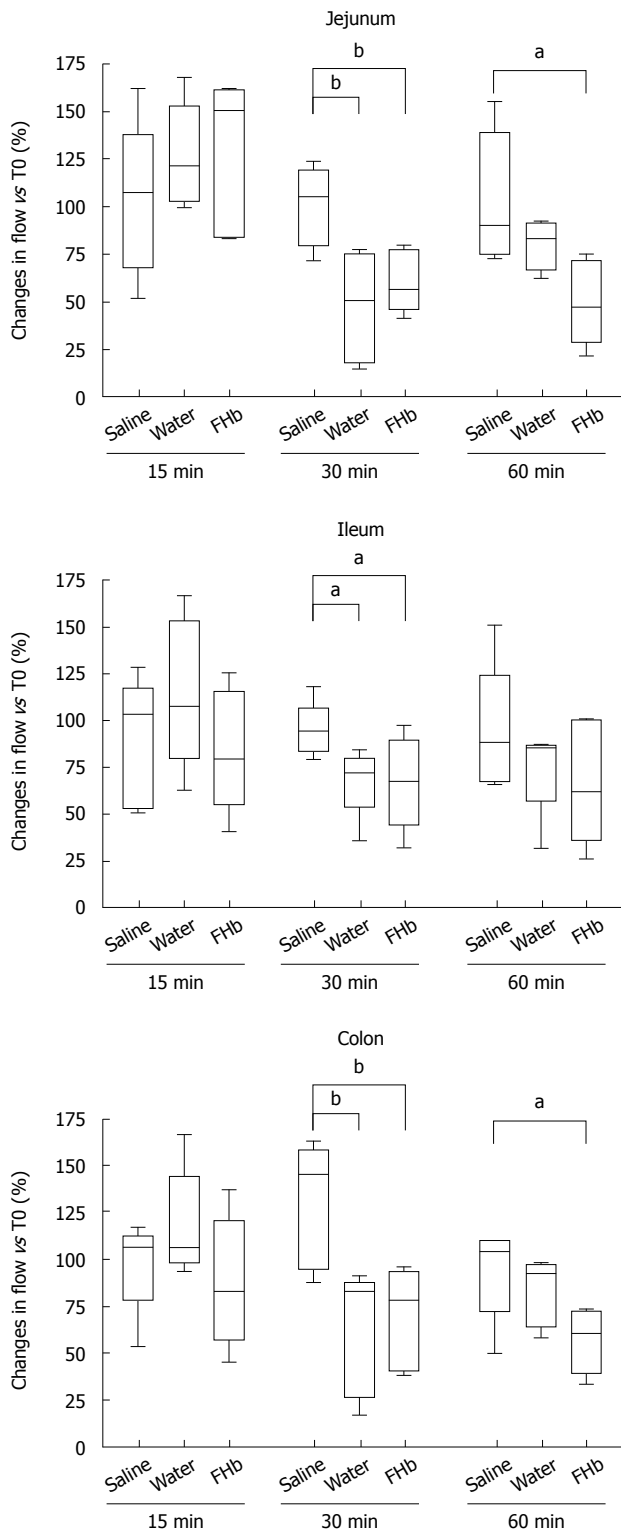


Figure 2 Decreased intestinal microcirculatory blood flow after water and free oxyhemoglobin infusion. The gut was divided into three sections: jejunum, ileum and colon. Basal flow was set at 100% for all groups at T0. In both the water infusion group as well as in the free oxyhemoglobin (Fhb) infusion group the microcirculation decreased. After infusion of saline, the microcirculatory blood flow remained around 100% throughout the study. Changes in time in microcirculatory blood flow are presented at T15, T30 and T60 and compared with the saline infusion group. ^a $P < 0.05$, ^b $P < 0.01$.

After sacrifice, ileum tissue samples were fixed in neutral buffered formaldehyde and embedded in paraffin

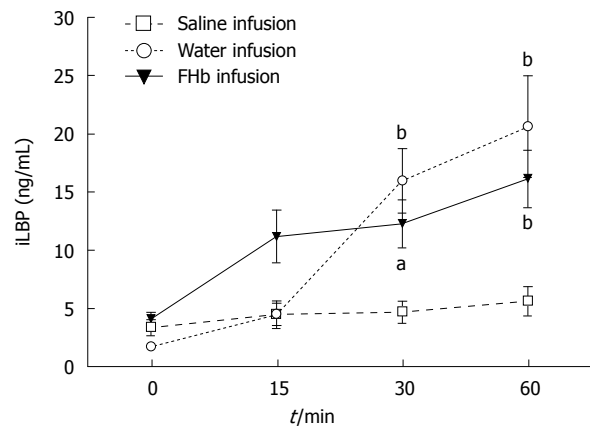


Figure 3 Hemolysis is associated with intestinal injury. The infusion of water and free oxyhemoglobin (Fhb) led to a rapid and significant release of ileal lipid binding protein (iLBP), a cytosolic protein mainly present in the ileum and expressed in mature enterocytes. ^a $P < 0.05$, ^b $P < 0.001$.

wax. For morphological evaluation, deparaffinized 3 μ m sections were either stained with hematoxylin and eosin or anti-iLBP, using a polyclonal rabbit anti-mouse iLBP (cross-reactive with rat, Hbt, Uden, The Netherlands) and a biotinylated polyclonal antibody swine anti-rabbit, a streptavidin-biotin system (Dako, Glostrup, Denmark), and visualized by applying 3-amino-9-ethylcarbazole (AEC, Sigma, St. Louis, MO). A Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F was used to capture images.

Statistical analysis

To test for differences in plasma levels of Fhb and iLBP, two-way analysis of variance with Bonferroni post-tests was used. To test for differences between changes in microcirculatory blood flow, two-tailed unpaired t -test was used. To evaluate an association between plasma Fhb and plasmatic iLBP release, Spearman correlation analysis for nonparametric data was used on area under the curve (AUC) between Fhb and iLBP for each individual subject of every group.

RESULTS

Hemolysis, Fhb and MAP

Plasma levels of Fhb before intervention were comparable between all groups (Figure 1). Water and Fhb infusions resulted in significantly elevated plasma levels of Fhb [peak values 29.6 (8.9) μ mol/L and 32.6 (2.7) μ mol/L, respectively, $P < 0.001$]. These levels are comparable to those found in patients during cardiovascular surgery in our University Medical Center, whereas infusion of saline did not result in elevated plasma Fhb levels. Infusion of either solution did not lead to changes in MAP.

Changes in intestinal microcirculation during hemolysis

The microcirculation was evaluated in the jejunum, ileum and colon (Figure 2). Whereas after 15 min of infusion no differences in blood flow occurred, at 30 min a significant decrease in microcirculatory blood flow of the

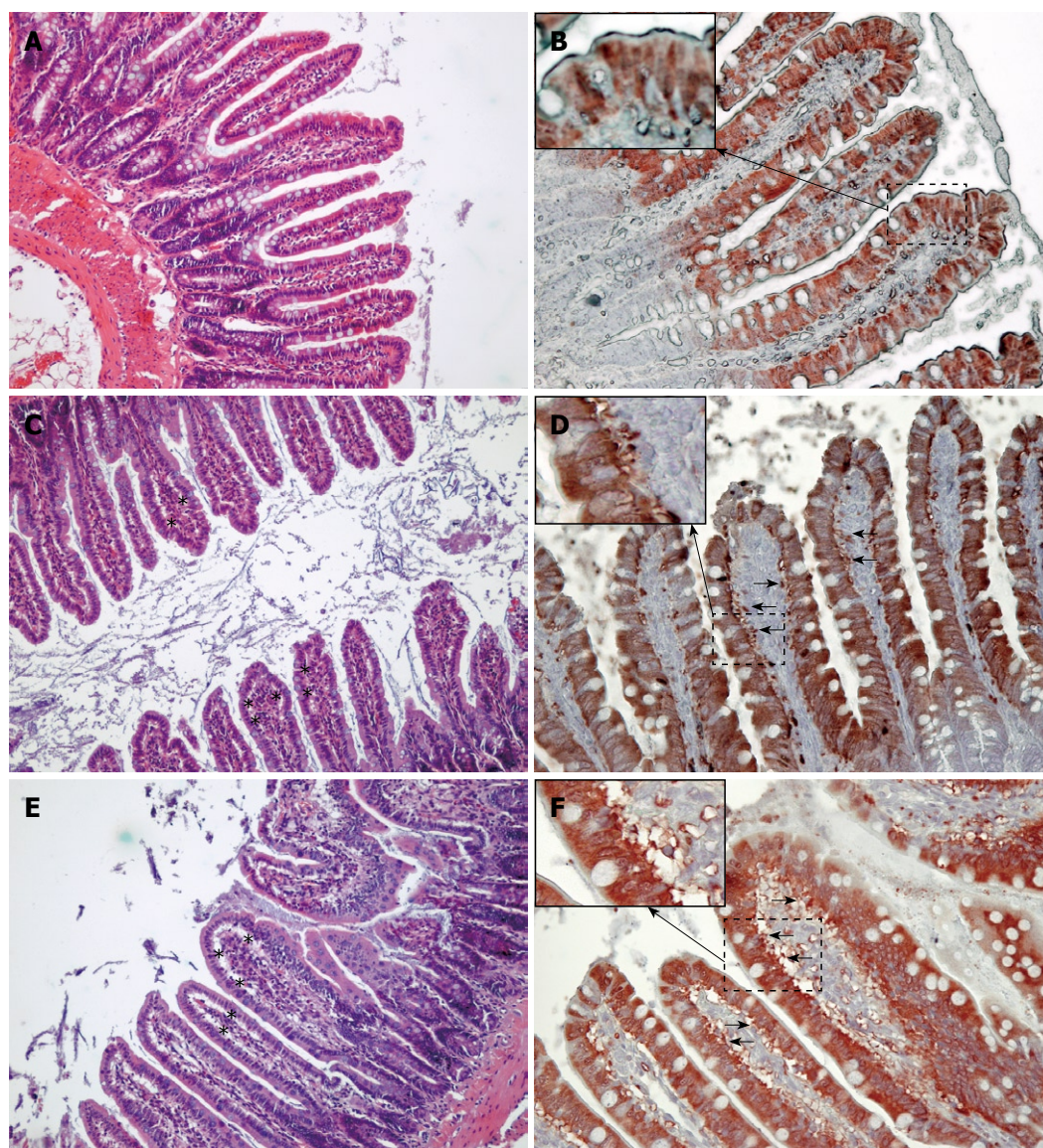


Figure 4 Histological and immunohistochemical evaluation of intestinal injury. Histological evaluation of the ileum was performed using HE staining (A, C and E, $\times 100$). When compared to the saline group (A, B), water (C, D) and free oxyhemoglobin (FHB) (E, F) infusions led to the development of subepithelial spaces (asterisks) in the villi. In support of the findings of plasmatic release of ileal lipid binding protein (iLBP), immunohistochemical analysis of the ileum shows cytosolic staining for iLBP in the epithelial cells of the upper part of the villi (B, $\times 200$). Leaking of iLBP in the subepithelial spaces (arrows) indicates intestinal epithelial cell injury after water or FHB infusion (D, F, $\times 200$). Insets show 400 \times magnification of selected areas where staining of iLBP can be seen outside the intestinal epithelial cellular membrane, indicating epithelial cellular injury (D, F).

jejunum, ileum and colon was seen, when compared to the saline group ($P < 0.05$ or better). After 60 min, the jejunal and colonic microcirculation was still significantly reduced in the FHB infusion group (jejunum and colon: FHB *vs* saline infusion, $P < 0.05$). These data indicate a deleterious effect of FHB on intestinal microcirculatory blood flow.

Intestinal injury during elevated circulating FHB levels

Release of iLBP: iLBP levels before infusion were comparable in all groups (Figure 3). Interestingly, at the time of reduced microcirculatory blood flow, at 30 and 60 min, plasma iLBP levels were significantly elevated in both the water and FHB infusion groups (peak values 20.6 (4.4) ng/mL and 16.1 (2.5) ng/mL, respectively). Moreo-

ver, the AUC for plasma FHB levels correlated significantly with the AUC for iLBP release ($r = 0.72$, $P = 0.0011$), indicating a strong association between hemolysis and intestinal injury.

Histological analysis for intestinal injury

Histological analysis (Figure 4) showed subepithelial spaces and injury at the tip of the villi in both the water infusion and FHB infusion groups, but not in the saline infusion group. Immunohistochemical staining of the ileum showed subepithelial spaces positive for iLBP in both the water and FHB infusion groups, indicating that iLBP had leaked from epithelial cells. No staining was observed in control sections (data not shown). These data indicate epithelial cell injury of the gut.

DISCUSSION

Taken together, these data show that elevated plasma levels of circulating FHB, as occurs during intravascular hemolysis, decrease intestinal microcirculation and result in intestinal injury, as demonstrated by the release of iLBP and confirmed immunohistochemically.

Our animal model was designed after a canine model developed by Minneci *et al.*^[11] to study the effects of water infusion-induced intravascular hemolysis on NO bioavailability and hemodynamic variables serving as a model for chronic hemolysis, such as occurs in sickle-cell anemia. Our infusion protocol was adjusted in order to attain plasma FHB levels comparable to those found during cardiovascular surgery in our university medical center (Figure 1)^[8]. The observed decreased microcirculatory blood flow at 30 min (Figure 2) suggests a rapid exhaustion of FHB scavenger proteins (haptoglobin, CD 163)^[15]. The reduction in microcirculatory blood flow was less pronounced after 60 min in both the water and FHB infusion groups. This might be explained by the gradual upregulation of heme-catabolizing enzymes (HO-1, biliverdin reductase)^[16,17] or enhanced NO-generating systems (NOS I, II and III)^[18,19], through either enhancement of FHB clearance or counteraction of the deleterious effects of FHB, respectively.

Intestinal damage, assessed by intestinal fatty acid-binding proteins such as iLBP (Figure 3), has been reported previously in cardiovascular surgical patients^[2,5]. To the authors' best knowledge, so far no studies have been performed to evaluate a possible correlation between FHB and gastrointestinal injury in clinical or experimental studies. Recently, we have shown that intestinal epithelial cell injury occurring during and after major cardiovascular surgery is associated with a systemic inflammatory response^[20]. It is tempting to speculate that FHB plays a role in the development of intestinal and also renal injury in such patients, where levels of circulating FHB are found comparable or even higher than those reached in this study^[20,21]. Acute gastrointestinal complications after cardiovascular surgery are known to result in a complicated postoperative period and are associated with high mortality rates. In a current study, we show that the concentrations of plasma FHB and urine N-acetyl glucosaminidase (NAG) levels increase during extracorporeal bypass-assisted cardiovascular surgery, which indicates that hemolysis and tubular epithelial injury have occurred. Interestingly, we have found that the total release of plasma FHB is independently correlated with urine NAG, which in turn is independently associated with the postoperative increase in serum creatinine^[21] used as a marker for acute kidney injury.

Taken together, our data suggest that circulating FHB is not only a health risk in chronic diseases such as sickle-cell anemia, thalassemia and malaria, but also may be a cause for concern in patients subject to extracorporeal circulation, blood transfusion and cell salvage devices resulting in substantial acute hemolysis. Therefore, circulating FHB levels should be closely monitored in clinical practice

when treating these patients, who are at risk of developing gastrointestinal complications. Future studies are needed to develop means to counteract the deleterious effects of circulating FHB.

ACKNOWLEDGMENTS

The authors wish to thank Dian Kuipers for laboratory assistance and Dr. Jan Serroyen, Department of Methodology and Statistics, Maastricht University Medical Center, Maastricht, The Netherlands, for critical revision of the statistical analyses used in this study.

COMMENTS

Background

Gastrointestinal complications after cardiovascular surgery are feared, while their pathophysiology is often unclear. Cardiovascular surgery is associated with considerable injury to red blood cells, resulting in hemolysis with concomitant release of cell-free hemoglobin. Recently, studies in patients experiencing elevated levels of cell-free hemoglobin have shown a disturbed microcirculatory blood flow.

Research frontiers

Hemolysis is not uncommon during cardiovascular surgery, resulting in elevated circulating levels of cell-free hemoglobin. The effect of hemolysis on intestinal microcirculation and gut wall integrity is unclear, so far. In this study, the authors demonstrate that hemolysis decreases intestinal microcirculatory blood flow, and that hemolysis, in particular the release of cell-free hemoglobin, is associated with intestinal damage.

Innovations and breakthroughs

Recent studies have emphasized the deleterious effects of hemolysis on microcirculatory blood flow and possible induction of organ injury. Moreover, the release of cell-free hemoglobin appears to play a pivotal role through its interference with vascular endothelial nitric oxide. This is the first study to show a decreased intestinal microcirculatory blood flow during elevated levels of circulating cell-free hemoglobin, resulting in intestinal damage. Moreover, a strong correlation between elevated circulating cell-free hemoglobin and intestinal injury is revealed.

Applications

By identifying hemolysis, and in particular the release of cell-free hemoglobin, as a potential initiator of intestinal damage, future studies can now address its specific role in the pathophysiology of gastrointestinal complications in the clinical setting of cardiovascular surgery. Furthermore, studies evaluating therapy options focussed on counteracting the deleterious effects of cell-free hemoglobin are needed.

Terminology

Intravascular hemolysis results in elevated levels of circulating cell-free hemoglobin. Due to its nitric oxide scavenging properties (nitric oxide being the most potent endothelial vasodilator known), the intravascular release of cell-free hemoglobin consequently might result in decreased tissue microcirculation and cause tissue damage. In this study, intestinal injury was measured by evaluating release of ileal lipid binding protein, a protein expressed mainly in the ileum, in mature enterocytes only.

Peer review

The study examined the effects of free hemoglobin and H₂O infusion on artery pressure, intestinal blood flow, and ileal lipid binding protein release in rats, and found that both factors can induce intestinal epithelial injury. The topic is interesting, study is well designed and carefully carried out, manuscript is well written, and results are convincing.

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S- Editor Tian L L- Editor Logan S E- Editor Lin YP

Angiogenic markers endoglin and vascular endothelial growth factor in gastroenteropancreatic neuroendocrine tumors

Patricia Kuiper, Lukas JAC Hawinkels, Eveline SM de Jonge-Muller, Izäk Biemond, Cornelis BHW Lamers, Hein W Verspaget

Patricia Kuiper, Eveline SM de Jonge-Muller, Izäk Biemond, Cornelis BHW Lamers, Hein W Verspaget, Department of Gastroenterology and Hepatology, Leiden University Medical Centre, 2300 RC Leiden, The Netherlands
 Lukas JAC Hawinkels, Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Centre, 2300 RC Leiden, The Netherlands

Author contributions: Kuiper P and Verspaget HW designed the research; Kuiper P, de Jonge-Muller ESM and Hawinkels LJAC performed the research; Kuiper P, Biemond I, Hawinkels LJAC, Lamers CBHW and Verspaget HW analyzed the data; Kuiper P and Verspaget HW wrote the paper; Biemond I, Lamers CBHW and Verspaget HW supervised the research.

Supported by Centre for Biomedical Genetics and Dutch Cancer Society RUL2005-3371 (Hawinkels LJAC)

Correspondence to: Patricia Kuiper, MD, Department of Gastroenterology and Hepatology, Leiden University Medical Center, Building 1, D4-29, PO Box 9600, 2300 RC Leiden, The Netherlands. p.kuiper@lumc.nl

Telephone: +31-71-5265718 Fax: +31-71-5248115

Received: August 26, 2010 Revised: September 9, 2010

Accepted: September 16, 2010

Published online: January 14, 2011

four-fold higher tissue levels of endoglin and VEGF were seen in tumors compared to associated normal tissue. This increased endoglin tissue expression in tumors was significantly related to tumor size ($P < 0.01$), presence of metastases ($P = 0.04$), and a more advanced tumor stage ($P = 0.02$), whereas expression of VEGF was not.

CONCLUSION: We suggest that endoglin is a potential marker to indicate and predict metastases, which might be useful in the post-resection therapeutic approach of patients with GEP-NETs.

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Key words: Neuroendocrine tumor; Carcinoid tumor; Angiogenesis factors; Endoglin; Vascular endothelial growth factor

Peer reviewer: De-Liang Fu, Professor, Department of General Surgery, Pancreatic Disease Institute, 12 Wulumuqi Road, Shanghai 200040, China

Abstract

AIM: To investigate the expression and potential prognostic role of vascular endothelial growth factor (VEGF) and endoglin in gastroenteropancreatic neuroendocrine tumors (GEP-NETs).

METHODS: Microvessel density (MVD) in GEP-NETs was evaluated using endoglin and CD31 immunohistochemistry. In addition, tissue levels of endoglin and VEGF were determined in homogenates by ELISA.

RESULTS: Endoglin was highly expressed on tumor endothelial cells. CD31 MVD in GEP-NETs was significantly higher compared to endoglin MVD ($P < 0.01$). Two- to

Kuiper P, Hawinkels LJAC, de Jonge-Muller ESM, Biemond I, Lamers CBHW, Verspaget HW. Angiogenic markers endoglin and vascular endothelial growth factor in gastroenteropancreatic neuroendocrine tumors. *World J Gastroenterol* 2011; 17(2): 219-225 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/219.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.219>

INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs), including gastrointestinal carcinoids and pancreatic neuroendocrine tumors (PNETs), comprise a very heterogeneous group of neoplasia, with respect to tumor biology, histocytology and prognosis^[1]. Despite

a slow-growing nature, they are primarily malignant^[2]. Angiogenesis, the formation of new blood vessels from the existing vascular bed, is a crucial process in tumor progression. When tumors reach a size of 1–2 mm, they become dependent on neovascularization, not only to provide them with nutrients and oxygen, but also as an exit route for metabolic waste products, further growth of the primary tumor, and eventually, metastatic spread^[3]. One of the key factors in angiogenesis is vascular endothelial growth factor (VEGF), which has numerous effects on endothelial cells (ECs), including induction of migration and differentiation^[4]. Several studies have addressed the prognostic implications of VEGF in patients with GEP-NETs, and trials investigating the action of the anti-VEGF antibody bevacizumab in patients with GEP-NETs are ongoing^[5,6].

Another important growth factor, with a pivotal role in angiogenesis is transforming growth factor (TGF)- β 1, a multifunctional cytokine that is involved in numerous physiological and pathological processes^[7]. Endoglin (CD105) is a co-receptor for TGF- β 1. As a result of its principal expression on ECs of newly formed blood vessels, several studies have suggested that endoglin is a specific marker of neovascularization in various cancer types^[8–10]. In pancreatic carcinomas, high endoglin microvessel density (MVD) has been found to be related to shorter survival, and therefore, is suggested to be a prognostic marker^[11]. In colorectal cancer, the vessel count by positive endoglin staining is able to identify patients at high risk of metastases^[12].

In the present study, we assessed the tissue expression and levels of two key players in the process of angiogenesis, namely endoglin and VEGF, to assess their potential clinical implications in patients with GEP-NETs.

MATERIALS AND METHODS

Patients

After surgical removal, tumor tissues were collected at the Department of Gastroenterology, Leiden University Medical Centre (LUMC), Leiden, and either frozen at -80°C for protein extraction and/or embedded in paraffin for immunohistochemical staining.

Sixty-eight homogenates (27 tumor samples and 41 normal samples) of 27 patients were available for the determination of tissue levels of endoglin. For the measurement of VEGF levels, one tumor sample was exhausted, therefore, the total number of tumor samples comprised 26. For CD31 and endoglin immunostaining, 50 and 49 samples, respectively, of 39 patients, were available. For most patients, but not all, both homogenates and paraffin slides were available. In total, 41 patients with GEP-NETs were included. GEP-NETs comprised PNETs and gastrointestinal neuroendocrine tumors, which were also referred to as carcinoids.

Clinicopathological information was obtained by evaluation of patients' medical files and pathology reports, when available. According to the classification of the World Health Organization for GEP-NETs, tumors were

categorized into well-differentiated neuroendocrine tumor (NET), well-differentiated neuroendocrine carcinoma (NEC), or poorly differentiated NEC^[13]. From some patients, the WHO classification was not assessable due to lack of specified classification. All studies were performed according to the guidelines of the LUMC medical ethics committee, in compliance with the Helsinki Declaration.

Immunohistochemistry

Immunohistochemistry was performed as follows. Tissues were fixed in formalin, embedded in paraffin, and cut into 5- μm sections. After deparaffinization and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% H_2O_2 (Merck, Darmstadt, Germany). Antigen retrieval was performed by boiling in 0.01 mol/L citrate buffer, pH 6.0, for 10 min. Slides were incubated overnight at room temperature (RT) with primary antibodies: biotinylated goat anti-human endoglin (1:200; R&D Systems Europe, Abingdon, UK), or mouse monoclonal anti-CD31 (1:400; Dako, Glostrup, Denmark) diluted in PBS with 1% bovine serum albumin (BSA), as described previously^[14]. Immunodetection was performed with a biotinylated goat anti-mouse antibody (for CD31) and horseradish peroxidase (HRP)-streptavidin complex (both Dako) for 30 min at RT. Staining was visualized using 0.05% 3,3'-diaminobenzidine (Sigma, Darmstadt, Germany) that contained 0.0038% H_2O_2 . Colon carcinomas were used as positive controls. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with an Olympus BX-51TF microscope equipped with a DP23-3-5 camera.

The endoglin and CD31 MVD in the tumor-bearing area were quantified by computerized analysis. Four representative tumor areas for either endoglin or CD31 were selected and photographed at $100\times$ magnification. Images were binarized and the extent of staining was quantified using ImageJ 1.43u (National Institutes of Health, Bethesda, MD, USA). Finally, the average MVD out of four photographs was taken. The microvessel quantification was performed blinded, that is, without knowledge of patients or tumor characteristics, and expressed as the number of pixels per field $\times 1000$.

Quantitative human endoglin and VEGF determinations in tissue samples

Tissues were homogenized and protein concentrations were determined according to Lowry *et al.*^[14,15]. Endoglin levels were determined in tissue homogenates, using a commercially available quantitative immunoassay (ELISA) for human endoglin, performed according to the manufacturer's instructions (R&D Systems), as described before^[14]. VEGF tissue levels were determined using a commercially available duoset (R&D Systems) as described before^[16].

Statistical analysis

Statistical analysis was performed using SPSS version 16 and GraphPad Prism version 5. Unpaired *t* test and one-

Table 1 Patient and tumor characteristics *n* (%)

Patients (<i>n</i> = 41)	
Age (yr)	
mean ± SD	47 ± 14
Range	20-77
Sex	
Male	17 (41.5)
Female	24 (58.5)
Tumor type	
Carcinoid	12 (29.3)
Functional PNET	19 (46.3)
Non-functional PNET	10 (24.4)
Tumor grade	
Well-differentiated NET	13 (31.7)
Well-differentiated NEC	26 (63.4)
Poorly differentiated NEC	1 (2.4)
Unknown	1 (2.4)
Metastases	
Present	26 (63.4)
Lymph node only	9 (34.6)
Liver only	7 (26.9)
Both	10 (38.5)
Absent	15 (36.6)
Tumors (<i>n</i> = 60)	
Primary or metastatic tissues	
Primary	45 (75.0)
Metastasis	15 (25.0)
Angio-invasion	
Present	11 (18.3)
Absent	49 (81.7)
Tumor size (mean ± SD, cm)	
Carcinoids	3.4 ± 2.7
Functional PNETs	1.9 ± 1.7
Non-functional PNETs	3.6 ± 2.4

PNETs: Pancreatic neuroendocrine tumors; NET: Neuroendocrine tumor; NEC: Neuroendocrine carcinoma.

way ANOVA were used to compare mean levels of endoglin and VEGF between various data sets. Orthogonal regression analysis and Pearson's correlation (*r*) were used to explore the relationship between two variables. Survival curves were plotted using the method of Kaplan and Meier. Results are reported as mean ± SE. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Overall, 41 patients with NETs were included (Table 1), of which, the majority were female. Most patients (28/41) had a solitary primary tumor, while 13/41 patient had multiple primaries. Primary tumors of 23/41 patients were localized in the pancreas, 5/41 in the duodenum, 10/41 in the small bowel, 1/41 in the appendix, 1/41 in the sigmoid, and in one patient, the exact primary tumor location was unknown. Functional tumors were mainly insulinomas (42.1%) and gastrinomas (52.6%). Tumor size was significantly different between the groups (*P* = 0.01), with a smaller tumor size for functional PNETs. Metastases were seen in the majority of patients, with an almost equal distribution of lymph node or liver location. Angio-invasion was present in only 18.3% of the tumors.

Endoglin and VEGF tissue levels were measured in 27

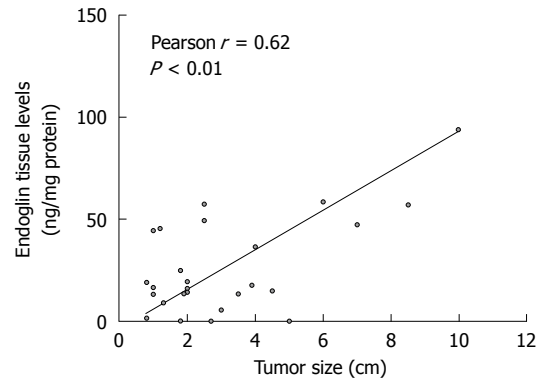


Figure 1 Orthogonal regression analysis of endoglin tissue levels and tumor size (*n* = 26) in 17 patients. (In one patient, information about tumor size was missing, so this patient was not included in this analysis). Increasing endoglin levels in tumors were significantly correlated with greater tumor size.

tumor samples from 18 patients with GEP-NETs. Endoglin and VEGF levels were significantly increased in tumors compared to (associated) normal tissues (Table 2). However, among the various types of GEP-NETs, both endoglin and VEGF levels were comparable. Metastatic tumors showed significantly higher endoglin levels compared to those in primary lesions. VEGF levels were also increased in metastases, although not significantly. Furthermore, well-differentiated NECs showed significantly higher endoglin levels compared to well-differentiated NETs. Again, this difference in VEGF levels was not statistically significant, although levels in well-differentiated NECs were also increased. Of particular interest, we observed that primary tumor tissues of patients who had developed lymph node or liver metastases displayed significantly higher endoglin levels than from those without metastases. Neither endoglin nor VEGF levels were significantly related to other clinicopathological parameters, including patients' age, sex, hormonal status (i.e. functional or non-functional) of the PNETs, or the presence of angio-invasion. Endoglin tissue levels, but not tissue levels of VEGF, were found to increase with tumor size (Figure 1). Finally, endoglin tumor levels showed no significant correlation with VEGF tumor levels (*r* = 0.11 with *P* = 0.59).

The immunohistochemical expression of endoglin and CD31 was analyzed in 39 patients with GEP-NETs. All tumors showed expression for CD31 and endoglin on intratumor vascular ECs. Endoglin expression was mainly observed on ECs of small tumor-associated blood vessels, whereas its expression in normal, non-tumorous tissue was weak or negative, in contrast to CD31 staining (Figure 2). The CD31 MVD was found to be significantly higher than the endoglin MVD in 73% of the tumor samples (*P* < 0.01). No significant differences in endoglin and CD31 MVD were observed between carcinoids and PNETs (Table 3). Furthermore, endoglin and CD31 MVD were not significantly related to clinicopathological parameters such as patients' age, sex, tumor size, functionality, and angio-invasion.

Endoglin and CD31 MVD were significantly corre-

Table 2 Mean endoglin and vascular endothelial growth factor levels in gastroenteropancreatic neuroendocrine tumors in relation to clinicopathological parameters

	Endoglin (ng/mg)				VEGF (pg/mg)			
	<i>n</i>	mean	SE	<i>P</i>	<i>n</i>	mean	SE	<i>P</i>
Tissues								
Normal	38	12.1	2.0	< 0.01 ²	38	75.0	9.5	< 0.01 ²
Tumor	27	26.8	4.5		26	316.8	46.0	
Tumor type								
Carcinoid	8	35.3	11.4	0.37	8	354.9	72.0	0.67
Functional PNET	14	25.4	4.7		13	274.4	46.7	
Non-functional PNET	5	16.8	8.7		5	366.2	186.8	
Origin								
Primary tumors	19	18.8	3.9	< 0.01 ²	18	293.2	52.0	0.45
Metastatic tumors	8	45.7	9.0		8	369.9	95.8	
WHO classification								
Well-differentiated NETs	6	7.6	5.2	0.02 ^{1,2}	6	200.2	52.8	0.21 ¹
Well-differentiated NECs	20	32.9	4.0		19	328.5	60.2	
Poorly-differentiated NECs	1	19.0	ND		1	795.0	ND	
Primary tumors: metastases								
Present	12	24.8	5.2	0.04 ²	11	339.5	76.4	0.28
Absent	7	8.5	3.5		7	220.6	54.8	

¹Result of unpaired *t* test to compare well-differentiated neuroendocrine tumors (NETs) with well-differentiated neuroendocrine carcinomas (NECs); ²*P* values are considered statistically significant. VEGF: Vascular endothelial growth factor; PNET: Pancreatic neuroendocrine tumor; ND: Not described.

Table 3 Microvessel density scores in gastroenteropancreatic neuroendocrine tumors in relation to clinicopathological parameters

	MVD-endoglin				MVD-CD31			
	<i>n</i>	mean ¹	SE ¹	<i>P</i>	<i>n</i>	mean ¹	SE ¹	<i>P</i>
Tumor type								
Carcinoid	11	55	107	0.30	13	123	23	0.75
Functional PNET	24	65	8		23	106	18	
Non-functional PNET	14	85	18		14	100	17	
Origin								
Primary tumors	36	66	8	0.58	37	111	13	0.69
Metastatic tumors	13	75	15		13	101	24	
WHO classification								
Well-differentiated NETs	13	69	18	0.93 ²	13	76	12	0.08 ²
Well-differentiated NECs	33	67	7		34	121	15	
Poorly-differentiated NECs	1	212			1	82		
Primary tumors: metastases								
Present	19	66	9	0.96	20	138	18	0.05 ³
Absent	17	67	14		17	88	15	

¹Values × 1000 pixels per area; ²Result of unpaired *t* test to compare well-differentiated neuroendocrine tumors (NETs) with well-differentiated neuroendocrine carcinomas (NECs); ³*P* values are considered statistically significant. MVD: Microvessel density; PNET: Pancreatic neuroendocrine tumor.

lated with endoglin tumor levels; $r = 0.64$ with $P < 0.01$ (Figure 3) and $r = 0.58$ with $P < 0.01$, respectively. VEGF tumor levels were not correlated with endoglin MVD ($r = 0.28$ with $P = 0.25$), but were borderline significantly correlated with CD31 MVD ($r = 0.43$ with $P = 0.07$).

To evaluate the prognostic potential of endoglin and VEGF tissue levels, Kaplan-Meier survival analysis was performed (Figure 4) by dividing the patients into two groups (i.e. low vs high) using the mean value of endoglin and VEGF tumor levels (Table 2). Both endoglin and VEGF tissue levels were not significantly related to patient survival. Furthermore, patients were divided into two groups based on the MVD of endoglin and CD31. Both

parameters were not significantly correlated with overall survival of these patients.

DISCUSSION

In this study, we observed that the expression of the angiogenic cell marker endoglin was related to tumor size, aggressiveness and metastatic potential in patients with GEP-NETs, whereas expression of another key player in angiogenesis, namely VEGF, was not.

In general, GEP-NETs are highly vascularized. In recent years, it has become clear that angiogenesis has important effects on tumor progression in several cancers,

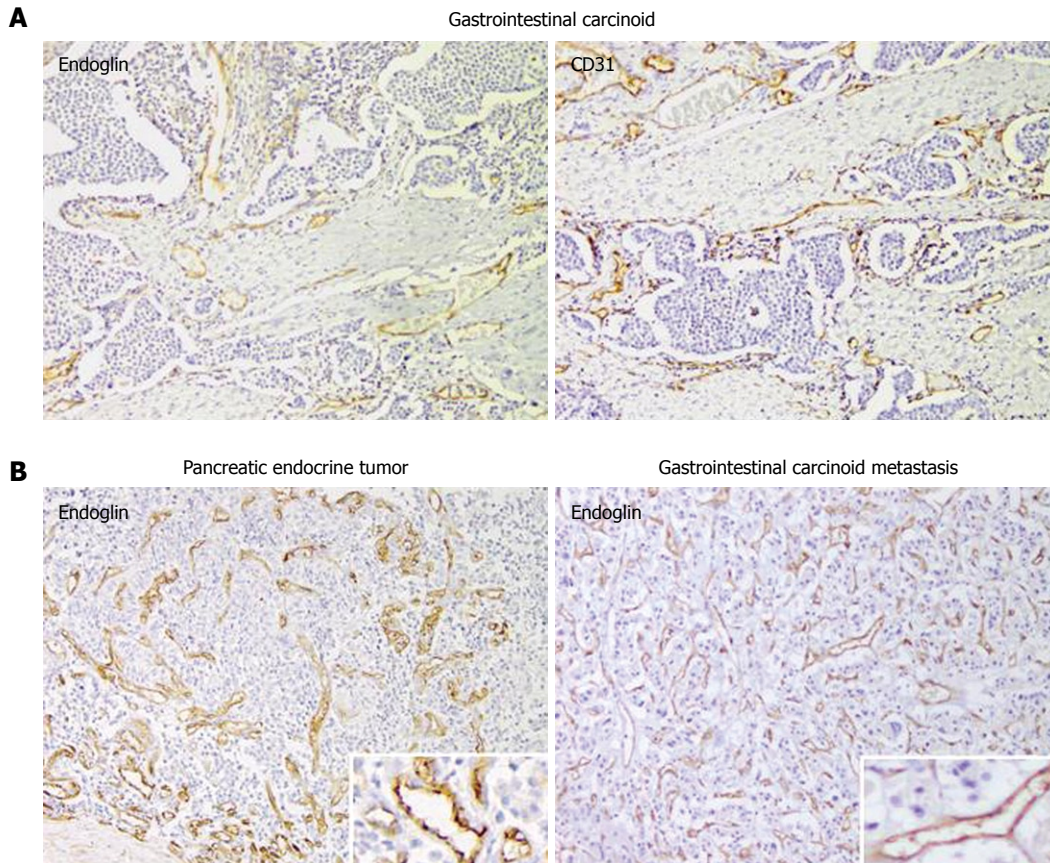


Figure 2 Immunostaining of endoglin and CD31 on peritumoral and intratumoral vessels in gastroenteropancreatic neuroendocrine tumors. A: Endoglin staining was limited to angiogenic vessels, whereas CD31 stained both old and new blood vessels in tumor tissue. Magnification 100 ×; B: Representative endoglin staining in a pancreatic neuroendocrine tumor and a gastrointestinal carcinoid metastasis (small bowel mesentery). Magnification 100 ×. Inserts show a higher magnification at 200 ×.

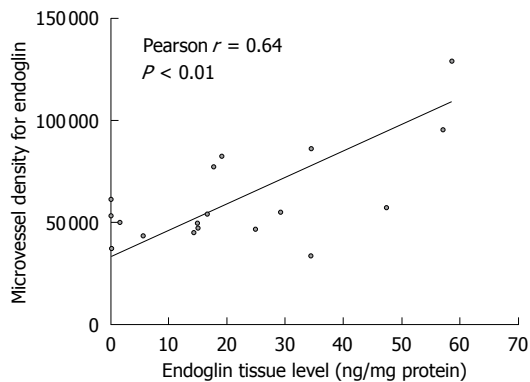


Figure 3 Correlation analysis of the endoglin microvessel density and endoglin tissue levels in tumors ($n = 17$). For one patient in whom endoglin tissue levels were assessed, no paraffin slides for microvessel density (MVD) determination were available. Endoglin MVD was significantly correlated with tumor levels of endoglin.

and the therapeutic role of angiogenesis inhibitors in the treatment of cancers is increasing^[17,18]. In this study, we investigated whether endoglin and VEGF were related to any clinicopathological characteristics of GEP-NETs, and evaluated their potential prognostic implications.

By immunohistochemistry, we observed high endoglin expression on vascular ECs in tumor tissues of GEP-NETs. In contrast to CD31, immunopositivity of endog-

lin was mainly observed on newly formed blood vessels, which indicates that endoglin is more representative of tumor neovascularization than the pan-endothelial marker CD31.

Furthermore, we found that endoglin tissue levels were significantly higher in tumors compared to normal tissues. We observed that increased endoglin expression was indicative of metastatic disease. Endoglin levels were higher in metastases compared to primary tumors, and primary tumors with metastases showed higher endoglin levels compared to tumors without metastases. Additionally, endoglin levels were increased in well-differentiated NECs compared to well-differentiated NETs, and higher endoglin levels were related to larger tumor size in patients with GEP-NETs. In several cancers, the extent of tumor angiogenesis was shown to reflect their potency to become invasive and form metastases^[19,20]. Our data indicate that tissue endoglin can serve as a potential assessment marker for tumor aggressiveness (i.e. NEC *vs* NET) and the presence of metastases following tumor resection. In the context of anticancer therapy, anti-endoglin treatment might provide a new effective anti-angiogenic strategy for GEP-NETs, but more research is needed. However, several promising *in vivo* and *in vitro* studies using anti-endoglin antibodies for anti-cancer treatment have recently been published^[21].

In the present study, we did not evaluate the immuno-

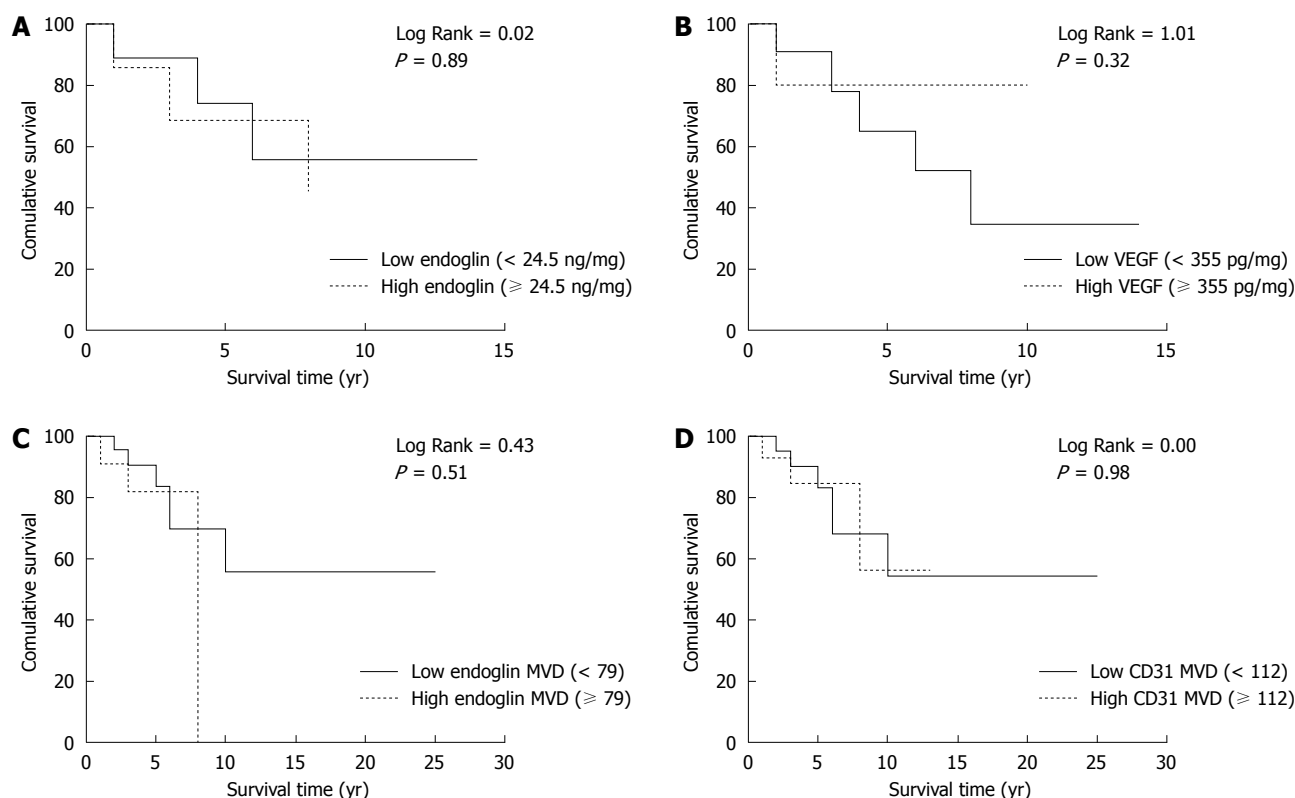


Figure 4 Kaplan-Meier survival analysis for endoglin tumor levels (A), vascular endothelial growth factor tumor levels (B), endoglin microvessel density (C) and CD31 microvessel density (D). Patients were divided into two groups based on mean tumor levels (A and B) or mean microvessel density (MVD) scores (C and D). None of the parameters showed a significant correlation with patient survival. VEGF: Vascular endothelial growth factor.

histochemical expression of VEGF. High immunoeexpression of VEGF on GEP-NETs has already been shown by others, but opposing results regarding the prognostic role of VEGF in these tumors have been reported. Takahashi *et al.*^[22] found no correlation of VEGF-A immunoeexpression with growth of blood vessels, hematogenous spread or tumor growth in pancreatic endocrine tumors. In contrast, Zhang *et al.*^[23] have revealed that strong expression of VEGF was associated with increased angiogenesis and poor prognosis in patients with GEP-NETs. However, we determined tissue VEGF expression in GEP-NETs and found that VEGF tissue levels showed a similar pattern to endoglin, but were not significantly related to any clinicopathological parameter. Therefore, we assume that, although VEGF is most likely to be involved in the process of neoplastic blood vessel formation in GEP-NETs, this key mediator of angiogenesis is not the appropriate prognostic marker in these tumors. In contrast, our data suggest that endoglin can function as a predictive marker for the development of metastases in GEP-NETs. Endoglin is a co-receptor for TGF- β 1. Among the various members of the TGF- β family, TGF- β 1 is mostly involved in cancer, and has been shown to stimulate angiogenesis^[24]. Endoglin is an important modulator of the TGF- β response; particularly in tumor pathogenesis^[25]. In another study by our group, strongly increased tissue levels of endoglin were observed in colorectal cancer, whereas premalignant lesions displayed endoglin levels comparable to those in normal tissues, which supports the pivotal role of endoglin in tumor progression^[14].

The fact that neither endoglin nor VEGF levels were associated with patient survival might be due to the relatively good prognosis of the patients. Gastrointestinal carcinoids show a 5-year survival rate of about 70%, whereas PNETs have a reported 5-year survival rate ranging from 25% to 100%, even in the case of (unresectable) liver metastases^[26,27]. In our study cohort, 10/18 patients in whom endoglin or VEGF levels were determined were still alive at the end of the study (median survival 8 years), which makes it unlikely to use one of these parameters as a predictor of outcome or survival marker. However, our data support a role for endoglin in identifying patients with GEP-NETs at risk for metastasis.

It is worth reiterating that the current study involved a relatively small number of patients. Nevertheless, GEP-NETs are a rare disease with a low incidence, which leads to general scarcity of patients and samples. However, we believe that the significant differences observed here are representative and illustrate the differential expression pattern of endoglin and VEGF among GEP-NETs.

In conclusion, we suggest that endoglin is a potential marker to predict present and future metastases, which might help to optimize the therapeutic approach in patients with GEP-NETs.

COMMENTS

Background

Angiogenesis is required for tumor growth and progression and development of metastases. Vascular endothelial growth factor (VEGF) and endoglin both

play an important role in angiogenesis. Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are rare and heterogeneous. Although markers for GEP-NETs exist, sensitive and specific markers that indicate tumor growth and behavior are lacking.

Research frontiers

The aim of the present study was to evaluate the expression and potential prognostic role of VEGF and endoglin in GEP-NETs.

Innovations and breakthroughs

From other studies it is already known that GEP-NETs are highly vascularized tumors. Although several studies have investigated the immunohistochemical expression VEGF in GEP-NETs, VEGF tissue levels or endoglin expression have not been studied in these tumors before. Therefore, this study is believed to be the first to investigate tissue expression and levels of VEGF and endoglin in GEP-NETs, to determine the clinical impact of these angiogenic factors in patients with GEP-NETs.

Applications

Based on our findings, we suggest that endoglin is a potential marker to indicate the presence of metastases in GEP-NETs. By demonstrating that increased endoglin expression on tumors is related to tumor aggressiveness (including grade of differentiation, size and presence of metastases), this study could present a future target for post-resection therapeutic intervention in the treatment of patients with GEP-NETs.

Terminology

Angiogenesis is the process of new blood vessel formation. This process is induced by several growth factors, including VEGF, and transforming growth factor (TGF)- β 1. Endoglin is a co-receptor for TGF- β 1 and a marker for angiogenic endothelial cells.

Peer review

This is a well-written paper that describes a study that evaluated the expression and potential prognostic role of VEGF and endoglin in a small sample of GEP-NETs patients, and is of considerable interest.

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S- Editor Sun H L- Editor Kerr C E- Editor Zheng XM

Evaluation of small bowel blood flow in healthy subjects receiving low-dose aspirin

Urara Nishida, Mototsugu Kato, Mutsumi Nishida, Go Kamada, Takeshi Yoshida, Shouko Ono, Yuichi Shimizu, Masahiro Asaka

Urara Nishida, Go Kamada, Takeshi Yoshida, Yuichi Shimizu, Masahiro Asaka, Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8648, Japan

Mototsugu Kato, Shouko Ono, Division of Endoscopy, Hokkaido University Hospital, Sapporo, Hokkaido, 060-8648, Japan
 Mutsumi Nishida, Department of Clinical Laboratory and Transfusion, Hokkaido University Hospital, Sapporo, 060-8648, Japan

Author contributions: Nishida U, Kato M and Nishida M contributed equally to this work; Kamada G, Yoshida T, Ono S, Shimizu Y and Asaka M supported this work; Nishida U and Kato M analyzed the data; Nishida U and Kato M wrote the paper.

Supported by Hokkaido University Hospital

Correspondence to: Mototsugu Kato, MD, PhD, Division of Endoscopy, Hokkaido University Hospital, North 14, West 5, Kita-ku, Sapporo, Hokkaido, 060-8648, Japan. m-kato@med.hokudai.ac.jp

Telephone: +81-11-7161161 Fax: +81-11-7067867

Received: August 18, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: January 14, 2011

= 0.0194) in the placebo group and -152.7 (95% CI: -1604.2 to 641.6, $P = 0.8172$) in the rebamipide group. Peak values of time intensity curves were -148.0 (95% CI: -269.4 to -26.2, $P = 0.0225$) in the placebo group and 28.3 (95% CI: -269.0 to 325.6, $P = 0.8343$) in the rebamipide group. Capsule endoscopy showed mucosal breaks only in the placebo group.

CONCLUSION: Short-term administration of low-dose aspirin is associated with small bowel injuries and blood flow.

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Key words: Small-intestine; Capsule endoscopy; Low-dose aspirin; Contrast-enhanced ultrasonography; Rebamipide

Peer reviewer: Dr. Uday C Ghoshal, MD, DNB, DM, FACP, Additional Professor, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow 226014, India

Abstract

AIM: To investigate the relationship between low-dose aspirin-induced small bowel mucosal damage and blood flow, and the effect of rebamipide.

METHODS: Ten healthy volunteers were enrolled in this study. The subjects were divided into two groups: a placebo group given low-dose aspirin plus placebo and a rebamipide group given low-dose aspirin plus rebamipide for a period of 14 d. Capsule endoscopy and contrast-enhanced ultrasonography were performed before and after administration of drugs. Areas under the curves and peak value of time-intensity curve were calculated.

RESULTS: Absolute differences in areas under the curves were -1102.5 (95% CI: -1980.3 to -224.7, P

Nishida U, Kato M, Nishida M, Kamada G, Yoshida T, Ono S, Shimizu Y, Asaka M. Evaluation of small bowel blood flow in healthy subjects receiving low-dose aspirin. *World J Gastroenterol* 2011; 17(2): 226-230 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i2/226.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.226>

INTRODUCTION

There have been several reports recently on the incidence of small bowel complications induced by non-steroidal anti-inflammatory drugs (NSAIDs)^[1,2]. However, there have been few investigations of low-dose acetylsalicylic acid (ASA)-induced small bowel damage. Endo *et al*^[3] reported that small bowel pathology was observed in 80%

of 10 healthy subjects after 2 wk with low-dose ASA but that there was no significant difference in mucosal break in subjects who used low-dose ASA and those who did not (with ASA: 30%, without: 0%). Smecuol *et al*^[4] reported that 3 cases of erosion and 1 case of bleeding were observed in 4 of 20 healthy subjects, and these cases were thought to have been caused by increase in permeation of the small intestinal mucosa (increased sucrose urinary excretion: 107.0 mg; range, 22.9–411.3, $P < 0.05$). These results indicated that mucosal breaks caused by taking low-dose ASA occurred not only in the upper gastrointestinal tract (GI) but also in the lower GI tract. However, the cause of small bowel injury is not clear.

Bjarnason *et al*^[5] reported that NSAIDs-induced small intestinal damage is caused by a lack of mucosal prostaglandins, decrease in blood flow, induction of nitric oxide and increased permeability. There are several key words for this hypothesis and we should investigate them, such as permeability, blood flow, free radicals, nitric oxide, and inflammation.

There are various techniques for measurement of blood flow in organs. Nishida *et al*^[6] reported that measurement of liver blood flow by using contrast-enhanced ultrasonography (CE-US) was useful for diagnosis of pancreatic carcinoma, and there have been some reports on evaluation of gastric blood flow using CE-US^[7,8].

We focused on blood flow in the small bowel as a possible cause of ASA-derived small bowel complications. There is no therapeutic strategy for the prevention of these complications. We wanted to investigate a candidate drug. Rebamipide is an anti-ulcer drug^[9]. Its actions include increasing endogenous prostaglandin^[10], scavenging free radicals, suppressing permeability, and elevating blood flow in the stomach^[11]. In this study, we investigated the relationship between low-dose ASA-induced small bowel mucosal damage and small bowel blood flow, and we also evaluated the preventive effect of rebamipide against small bowel damage and the effect of rebamipide on blood flow.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Hokkaido University Hospital. Written informed consent was given by all participants.

Inclusion and exclusion criteria

Inclusion criteria were absence of upper and lower GI injuries, such as erosion, ulcer, and bleeding on endoscopy. The subjects enrolled in this study were aged from 20 to 50 years. Subjects who were taking some drugs were excluded.

Study design

All of the participants in this study were healthy. A randomized, double-blind, cross-over, placebo-controlled trial using rebamipide was performed.

The study design is shown in Figure 1. The subjects were divided into two groups: a placebo group with low-

dose ASA (100 mg once daily) plus placebo (three times daily) and a rebamipide group with low-dose ASA (100 mg once daily) plus rebamipide (100 mg three times daily). The first period lasted 14 d. After a wash-out period of more than 14 d and the second period lasted a further 14 d. All subjects underwent capsule endoscopies and CE-US before and after each administration period.

The placebo was prepared by Yamanami Pharmacy. Rebamipide (100 mg) and the placebo were each contained in a soft colored capsule.

Randomization

Subjects were recruited for the treatment sequences in a random fashion according to a randomization schedule for the treatment period. A randomization number that was associated with a specific treatment, either rebamipide or placebo, was assigned to each subject. Randomized numbers were generated by the SAS program.

Capsule endoscopy

We used the Given video capsule system (PillCam®, Given Imaging Ltd., Yoqneam, Israel) in this study. The capsule endoscopy procedure and methodology for the review of images were conducted as previously described. All video images were analyzed by skilled reviewers (Nishida U and Kato M) who remained blinded to the subjects' treatment protocol. All images were saved for final comprehensive analysis upon completion of post-treatment capsule endoscopies.

Contrast-enhanced ultrasonography

The longitudinal view of the small bowel was imaged in the left upper abdomen. Baseline CE-US and contrast-enhanced CE-US were performed with a 7.5 MHz center frequency linear transducer using an ultrasound unit SSA-790A (AplioXG™, Toshiba Medical Systems Co., Otawara, Japan). The imaging mode was pulse subtraction. Prior to CE-US, 20 mg of scopolamine butylbromide was injected intravenously to suppress peristalsis. Perflubutane microbubbles (Sonazoid; GE Healthcare), a lyophilized preparation reconstituted for injection, were injected intravenously at a concentration of 0.015 mL/kg. Ten seconds after contrast medium injection, enhanced signals from blood flow in the small intestinal wall were captured until 45 s on cine clips equipped with the ultrasound unit. Regions of interests of fixed sizes were placed in the mucosal area of the small bowel at three regions (Figure 2). A time intensity curve (TIC) of blood flow enhancement signal in the small bowel was plotted from recorded ultrasonographic images using ImageLab software, which was developed by C++ software dedicated to CE-US images obtained by AplioXG. Area under the curve (AUC) and TIC peak value (maximum intensity) were calculated from the TIC. These values were used to estimate small bowel blood flow inside the mucosal layer.

Evaluation

The primary end point was evaluation of the change of

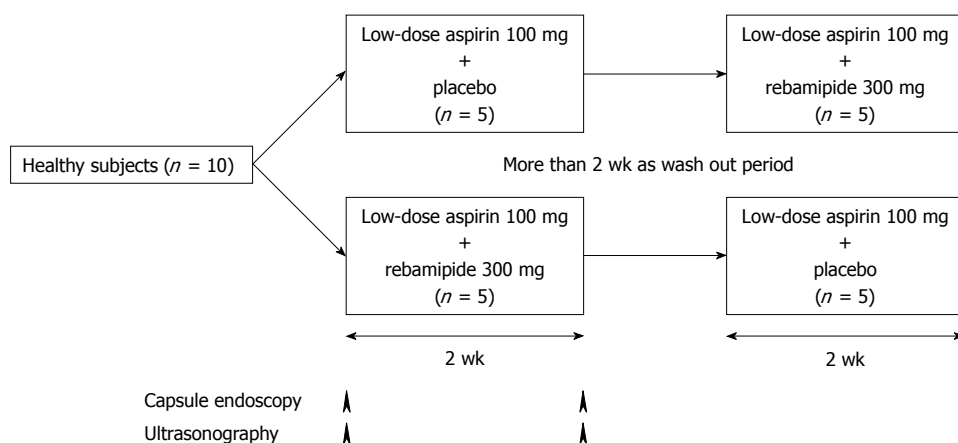


Figure 1 Study design. A randomized placebo-controlled double-blinded cross-over study was performed using rebamipide. Capsule endoscopy and contrast-enhanced ultrasonography were performed before and after administration of drugs.

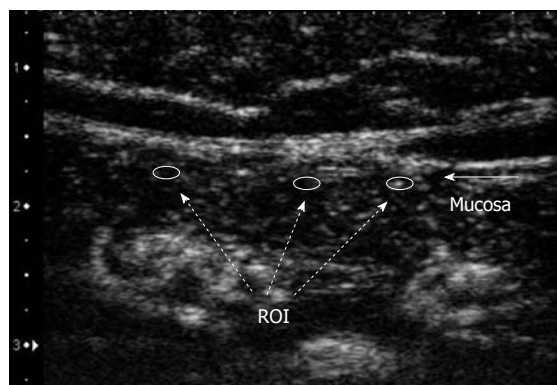


Figure 2 Image of contrast-enhanced ultrasonography. Regions of interests (ROI) were placed in the mucosal area of the small bowel at three regions. A time intensity curve of blood flow enhancement signal was plotted from recorded ultrasonographic images.

low-dose ASA-induced small bowel blood flow. Blood flow was measured by CE-US. TIC for blood flow in the small bowel was plotted from recorded CE-US images with Image Lab software. The secondary end point was evaluation of the preventive effect of rebamipide with low-dose ASA-related small bowel damages such as erosion, erythema, and petechiae.

In this study, a mucosal break of the small bowel was defined as erosion, ulcer, bleeding or perforation.

Safety assessment

Symptoms and other adverse events were recorded through this study period. If these events occurred, they were treated appropriately.

Statistical analysis

The primary end point was to evaluate changes in small bowel blood flow. Blood flow was estimated by AUC and TIC. They were analyzed by absolute differences between before and after each administration periods. They were evaluated by 95% confidential intervals. The secondary end point was to evaluate the preventive ef-

fect of rebamipide. Preventive effect was evaluated using small bowel mucosal injuries and subject numbers that got mucosal breaks. Small bowel injuries (ulcer, erosion and erythema) were described by mean \pm SD. They were analyzed by Fischer's exact test. Findings of $P < 0.05$ were considered significant. Statistical analyses were performed using SAS[®] version 8.2 (SAS Institute, Cary, NC).

RESULTS

Subjects

Ten males were enrolled in this study. The mean age of the subjects was 29 ± 5 years. Two subjects were infected with *Helicobacter pylori*.

Evaluation of blood flow

The values of AUC in the placebo group and rebamipide group were 464.2 ± 381.8 and 1414.1 ± 1340.3 , respectively. The absolute difference is shown in Table 1. In the placebo group, there was a significant difference in the values of AUC before and after taking ASA (difference: -1102.5 , 95% CI: -1980.3 to -224.7 , $P = 0.0194$). The difference in the rebamipide group, -152.7 , was not statistically significant (95% CI: -1604.2 to 641.6 , $P = 0.8172$).

Peak values of the TIC in the placebo group and the rebamipide group were 226.2 ± 251.4 and 402.5 ± 283.9 , respectively. In the placebo group, the difference in the peak values of TIC before and after taking ASA was -148.0 (95% CI: -269.4 to -26.2 , $P = 0.0225$), which was statistically significant. The difference in the rebamipide group, 28.3 , was not statistically significant (95% CI: -269.0 to 325.6 , $P = 0.8343$). The differences are shown in Table 1.

Small intestinal injuries

Changes in the numbers of erosions, petechiae and erythemas are shown in Table 2. Differences in the numbers of erosions, petechiae and erythemas in the placebo group were 0.5 ± 1.0 , 10.1 ± 53.6 and -1.0 ± 2.3 , respectively, and those in the rebamipide group were 0.0 ± 0.0 , -8.6 ± 15.4 and -0.6 ± 1.0 , respectively.

Table 1 The evaluation of area under the curve and time intensity curve by ultrasonography before and after taking low-dose aspirin ($n = 10$)

	Placebo			Rebamipide		
	A.D.	95% CI	P-value	A.D.	95% CI	P-value
AUC	-1102.5	-1980.3 to -224.7	0.0194	-152.7	-1604.2-641.6	0.8172
TIC	-148.0	-269.4 to -26.2	0.0225	28.3	-269.0-325.6	0.8343

A.D.: Absolute differences; AUC: Area under the curve, TIC: Time intensity curve.

Table 2 The evaluation of the preventive effect of rebamipide on low-dose aspirin-induced worsened small bowel injury compared with placebo (mean \pm SD)

	Placebo	Rebamipide
Erosion	0.5 \pm 2.7	0.0 \pm 0.0
Petechiae	10.1 \pm 53.6	-8.6 \pm 15.4
Erythema	-1.0 \pm 2.3	-0.6 \pm 1.0

Table 3 Number of subjects with small intestinal mucosal breaks

	Placebo	Rebamipide
Mucosal breaks	2	0

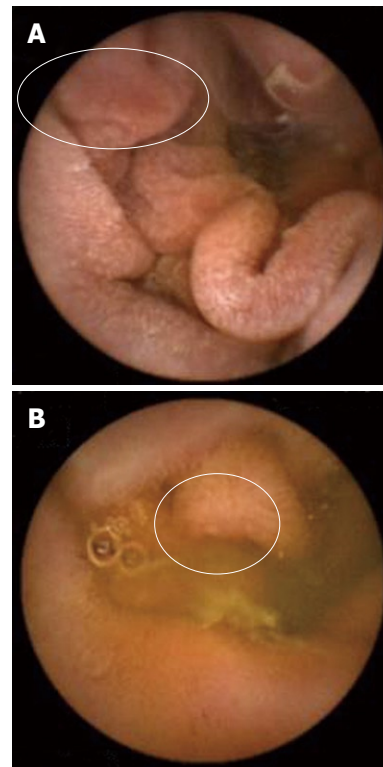
No ulcer, bleeding or perforation was observed. There were 2 cases of mucosal break in the placebo group but no cases in the rebamipide group (Table 3). Two cases of erosions were localized in the ileum region (Figure 3).

Safety assessment

No adverse events were observed throughout the study period.

DISCUSSION

In the present study, treatment with low-dose ASA resulted in a decrease in small bowel blood flow, with changes in AUC of -1102.5 (95% CI: -1980.3 to -224.7, $P = 0.0194$) and peak value of TIC of -148.0 (95% CI: -269.4 to -26.2, $P = 0.0225$) (Table 1). Low-dose ASA also caused small bowel damage. Treatment with ASA induced small bowel erosion in 2 cases and increased petechiae (Tables 2 and 3). These results indicated that low-dose ASA-induced small bowel injury is correlated with decreasing small bowel blood flow. Bjarnason *et al*^[5] proposed a cascade as the mechanism of NSAIDs-induced small bowel injury: NSAIDs induce a decrease in prostaglandin, the decrease in prostaglandin leads to a decrease in small bowel blood flow, and the decrease in small bowel blood flow leads to an increase in small bowel inflammation and injury. In another pathway, increasing permeability also leads to small bowel injury. Our results support their hypothesis; blood flow was one of the mechanisms of NSAIDs-related small bowel injury. However, small bowel damage was slight in our subjects. This might be due to the short-term use of ASA and the fact that the subjects were young and healthy.

**Figure 3** Image of capsule endoscopy. Low-dose aspirin induced mucosal breaks such as denuded areas and erosions were detected in the placebo group. A: Denuded area; B: Erosion.

Moreover, most patients who take low-dose ASA are chronic users, and decrease in small bowel blood flow may continue for a long time. Therefore, long-term observation of small bowel blood flow is needed.

On the other hand, low-dose ASA-induced small bowel erosions in the placebo group and the rebamipide group were 20% and 0%, respectively.

Small bowel blood flow did not decrease in the rebamipide group; changes in the AUC and TIC were -152.7 and 28.3 (not statistically significant). Kim *et al*^[11] reported that rebamipide did not decrease upper GI blood flow compared with placebo in healthy subjects taking ibuprofen. Our results suggested that rebamipide prevents a decrease in lower GI blood flow as well as a decrease in upper GI blood flow.

Recently, there have been three reports on the usefulness of drugs for low-dose ASA and NSAIDs-induced small bowel complications. Fujimori *et al*^[12] reported that misoprostol, a prostaglandin analogue, prevented

diclofenac-induced small bowel complications in healthy subjects. Niwa *et al.*^[13] reported that rebamipide prevented diclofenac-induced small bowel injury in healthy subjects. Shiotani *et al.*^[14] reported that geranylgeranylacetone (GGA: Teprenone) did not prevent low-dose ASA-induced small bowel damage. These drugs are cytoprotective drugs for gastric ulcer and gastritis. A proton pump inhibitor is useful for upper GI tract. However, it is not effective for the lower GI tract because of a lack of acid secretion. Further investigation is therefore needed to establish a novel therapeutic strategy for chemical-induced lower GI complications. These three reports may suggest that tentative drug for small bowel is needed to have the action for increasing prostaglandin as mechanism.

The number of subjects in our study was small. A study using a larger number of subjects is needed. Recently several mechanisms of ASA-induced small bowel complications were treated. But we explained only blood flow one of these several mechanisms. As the future study, we will need to examine relationship among several actions and functions.

In conclusion, low-dose ASA-induced decrease in small bowel blood flow is correlated with small-bowel mucosal injury. Rebamipide does not decrease small bowel blood flow.

COMMENTS

Background

Low-dose aspirin has been widely used for prevention of cardiovascular and cerebrovascular events. Several studies have shown that mucosal breaks caused by taking low-dose aspirin occurred not only in the upper gastrointestinal tract but also in the lower gastrointestinal tract.

Research frontiers

However the cause of small bowel injury is not clear. One of the mechanisms of drug-induced small bowel damage is decrease in blood flow. In this study, the authors investigated the relationship between low-dose aspirin-induced small bowel mucosal damage and blood flow using video capsule endoscopy and contrast-enhanced ultrasonography, and the authors also evaluated the effect of rebamipide on blood flow.

Innovations and breakthroughs

Recent reports have highlighted the mechanisms of aspirin-induced small bowel damage, and the prevention and treatment of aspirin-induced small bowel damage. This is the first study to report that short-term administration of low-dose aspirin is associated with small bowel injuries and blood flow. And rebamipide does not decrease small bowel blood flow.

Applications

This study may represent a future strategy for therapeutic intervention in the treatment of patients with low-dose aspirin-induced small bowel mucosal damage.

Terminology

Rebamipide is an anti-ulcer drug. Its actions include for increasing endogenous prostaglandin, scavenging free radicals, suppressing permeability, and elevating blood flow in the stomach. In this study contrast-enhanced ultrasonography were performed with a 7.5 MHz center frequency linear transducer using an ultrasound unit SSA-790A. The imaging mode was pulse subtraction.

Peer review

This study focuses on the evaluation of small bowel blood flow in healthy subjects with low-dose aspirin (a randomized placebo-controlled double-blinded cross-over study using video capsule endoscopy and contrast-enhanced ultrasonography). Low-dose acetylsalicylic acid-induced decrease in small bowel blood flow is correlated with small-bowel mucosal injury. The collected experiences are interesting

and may represent a future strategy for treatment of patients with drug-induced small bowel mucosal damage.

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¹⁸F-fluorodeoxyglucose positron emission tomography in the diagnosis of small pancreatic cancer

Keiichi Okano, Keitaro Kakinoki, Shintaro Akamoto, Masanobu Hagiike, Hisashi Usuki, Yuka Yamamoto, Yoshihiro Nishiyama, Yasuyuki Suzuki

Keiichi Okano, Keitaro Kakinoki, Shintaro Akamoto, Masanobu Hagiike, Hisashi Usuki, Yasuyuki Suzuki, Department of Gastroenterological Surgery, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan

Yuka Yamamoto, Yoshihiro Nishiyama, Department of Radiology, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan

Author contributions: Okano K designed the study; Okano K and Nishiyama Y analyzed the data and wrote the manuscript; Okano K, Kakinoki K, Akamoto S, Hagiike M, Usuki H and Yamamoto Y acquired, analyzed and interpreted the data; Suzuki Y critically revised the manuscript.

Correspondence to: Keiichi Okano, MD, Department of Gastroenterological Surgery, Faculty of Medicine, Kagawa University, 1750-1, Ikenobe, Kida-gun, Miki-cho, Kagawa 761-0793, Japan. kokano@kms.ac.jp

Telephone: +81-87-8985111 Fax: +81-87-8912186

Received: April 6, 2010 Revised: May 24, 2010

Accepted: May 31, 2010

Published online: January 14, 2011

Abstract

AIM: To investigate the role of ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG-PET) in the diagnosis of small pancreatic cancer.

METHODS: This study involved 31 patients with proven invasive ductal cancer of the pancreas. The patients were divided into 3 groups according to the maximum diameter of the tumor: TS1 (maximum tumor size ≤ 2.0 cm), TS2 (> 2.0 cm and ≤ 4.0 cm) or TS3-4 (> 4.0 cm). The relationships between the TS and various diagnostic tools, including FDG-PET with dual time point evaluation, were analyzed.

RESULTS: The tumors ranged from 1.3 to 11.0 cm in diameter. Thirty of the 31 patients (97%) had a positive FDG-PET study. There were 5 patients classified as TS1, 15 as TS2 and 11 as TS3-4. The sensitivity of FDG-PET, computed tomography (CT) and magnetic resonance

imaging (MRI) were 100%, 40%, 0% in TS1, 93%, 93%, 89% in TS2 and 100%, 100%, 100% in TS3-4. The sensitivity of FDG-PET was significantly higher in comparison to CT and MRI in patients with TS1 ($P < 0.032$). The mean standardized uptake values (SUVs) did not show a significant difference in relation to the TS (TS1: 5.8 ± 4.5 , TS2: 5.7 ± 2.2 , TS3-4: 8.2 ± 3.9), respectively. All the TS1 tumors (from 13 to 20 mm) showed higher SUVs in FDG-PET with dual time point evaluation in the delayed phase compared with the early phase, which suggested the lesions were malignant.

CONCLUSION: These results indicate that FDG-PET with dual time point evaluation is a useful modality for the detection of small pancreatic cancers with a diameter of less than 20 mm.

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Key words: Ductal carcinoma; Pancreas; ¹⁸F-fluorodeoxyglucose; Positron emission tomography; Pancreatic cancer; Dual time point evaluation

Peer reviewer: Itaru Endo, Professor, Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, Yokohama 236-0004, Japan

Okano K, Kakinoki K, Akamoto S, Hagiike M, Usuki H, Yamamoto Y, Nishiyama Y, Suzuki Y. ¹⁸F-fluorodeoxyglucose positron emission tomography in the diagnosis of small pancreatic cancer. *World J Gastroenterol* 2011; 17(2): 231-235 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/231.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.231>

INTRODUCTION

Pancreatic cancer is the 5th leading cause of cancer-related mortality in Japan, with an estimated 20 000 deaths attributable to the disease^[1,2]. The annual mortality rate closely

approximates the annual incidence, thereby reflecting a generally short survival time associated with pancreatic cancer, which is generally less than 1 year. Cancer of the pancreas has the shortest median survival time out of all cancer types in a stage for stage basis. Early diagnosis is the most important factor for improving the overall survival and quality of life in patients with pancreatic cancer.

Recently, positron emission tomography (PET) has demonstrated superiority to computed tomography (CT), ultrasonography (US), and endoscopic US (EUS) in its sensitivity and specificity in diagnosing pancreatic cancer^[3-6]. Furthermore, the metabolic activity of the tumor may be of prognostic significance. We have been reported the efficacy of delayed additional ¹⁸F-fluorodeoxyglucose PET (FDG-PET) imaging in the differential diagnosis of malignant from benign lesions in patients who are suspected of having pancreatic cancer^[7]. Furthermore, the detection rate of liver metastases smaller than 1 cm in diameter from pancreatic cancer was only 33% on early image and 58% on delayed image^[7]. However, the role of dual time point FDG-PET in the diagnosis of small pancreatic cancers has yet to be established.

Therefore, the present study investigated whether small cancers of the pancreas could be accurately diagnosed by FDG-PET with dual time point evaluation.

MATERIALS AND METHODS

Patients

Thirty-one patients with pancreatic carcinoma suspected on the basis of conventional radiological studies (22 males and 9 females; mean age, 65 years; age range, 44-82 years) and who underwent FDG-PET between 2003 and 2007 were retrospectively selected. Patients were excluded from this study if they had poorly controlled diabetes mellitus (presenting with blood glucose level > 200 mg/dL prior to PET imaging). Conventional radiological staging was performed by means of CT or magnetic resonance imaging (MRI). The location of the cancer was in the head of the pancreas in 17 patients and in the body and tail in 14 patients. Twelve of the 31 cancers were diagnosed to be unresectable, and 19 patients eventually underwent surgery with a curative intention, although the cancer turned out to be unresectable in 7 because of intraoperative findings.

Methods

The patients were divided into 3 groups according to the maximum diameter of the tumor: TS1 (maximum size ≤ 2.0 cm), TS2 (> 2.0 cm and ≤ 4.0 cm) or TS3-4 (> 4.0 cm) as indicated by the classification system of the Japan Pancreas Society. FDG-PET was analyzed semi-quantitatively using the standardized uptake values (SUVs). The sensitivity of diagnosing pancreatic cancer was examined for FDG-PET, CT, MRI and the serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) with regard to the size of the tumor. The details of SUVs, the histological findings and correlation of CT findings were evaluated in patients with TS1 pan-

creatic cancer. This study was performed retrospectively by collecting and analyzing data from the patient records.

FDG-PET

The FDG-PET images were acquired with a PET machine (Siemens EXACT HR+, CTI, Knoxville, TN, USA). The patients were required to fast for at least 4 h before PET imaging. The emission images were acquired (early image) 1 h after the intravenous administration of 5 mCi of FDG. Delayed PET emission images of the upper abdomen were acquired at 2 h after administration of ¹⁸F-FDG, using 2 or 3 bed positions with a 3-min acquisition at each^[7]. This acquisition was immediately followed by a transmission scan of the same transverse planes, using a 2-min acquisition at each bed position. The early and delayed PET images were reviewed independently and consecutively by 2 radiologists with extensive experience in FDG-PET imaging. PET images were compared with the corresponding CT and/or MRI images for accurate anatomical identification of the tumor. The findings were considered to be positive when both radiologists strongly suspected malignant disease. In addition, the images were analyzed semi-quantitatively using the SUV, as reported elsewhere. Briefly, for semi-quantitative analysis, a region of interest was placed over the entire FDG-avid lesion including the largest amount of radioactivity using the transverse PET image. The SUV was calculated as: $SUV = (\text{activity in region of interest in mCi}) / (\text{injected dose in mCi} / \text{weight in kg})$.

CT

CT studies were performed with a multidetector row CT scanner (Aquilion, Toshiba, Tokyo, Japan). Helical images of the abdomen were routinely obtained and reconstructed with 5 mm thickness. After pre-contrast CT scans, arterial dominant phase images of dynamic CT were obtained starting 40 s after the beginning of the intravenous bolus injection (3 mL/s) of 100 mL of iodized contrast medium at 350 mg/mL. The pancreatic phase and the late phase (near equilibrium phase) were also obtained, starting at 60 and 180 s after injection, respectively. The CT images were interpreted independently and consecutively by 2 radiologists with extensive experience of more than 10 years in CT scanning. The findings of the CT scans were considered positive when both radiologists strongly suspected malignant disease due to a discrete low-attenuation mass within the pancreas.

MRI

Two 1.5 T superconducting units, Signa Advantage (General Electric, Milwaukee, WI, USA, USA) and Visart (Toshiba, Tokyo, Japan), were used for MRI. T1-weighted gradient-echo imaging; FS-T2-weighted turbo SE imaging and heavily T2-weighted turbo SE images were acquired in the order of scan after initial T1-weighted localizing images were obtained in the coronal and trans-axial directions.

Statistical analysis

The χ^2 test was employed for a statistical comparison of

Table 1 Clinicopathological profiles of the 31 patients

	mean \pm SD (range) or <i>n</i> (%)
Age (yr)	65 \pm 9 (44-82)
Gender (M:F)	22:9
Tumor location	
Head	17 (55)
Body	11 (35)
Tail	3 (10)
Maximum tumor diameter (cm)	3.8 \pm 2.0 (1.3-11.0)
SUV	6.5 \pm 3.3 (2.5-15.8)

SUV: Standardized uptake value.

Table 2 Correlations between tumor size and sensitivity of positron emission tomography, computed tomography, magnetic resonance imaging or tumor markers

TS (cm)	<i>n</i>	PET (%)	CT (%)	MRI (%)	CEA (%)	CA19-9 (%)
TS1 (≤ 2)	5	100 ^a	40	0	0	40
TS2 ($> 2, \leq 4$)	15	93	93	89	20	73
TS3-4 (> 4)	11	100	100	100	73	91

TS: Tumor size; PET: Positron emission tomography; CT: Computed tomography; MRI: Magnetic resonance imaging; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9. ^a*P* = 0.002 *vs* MRI or CEA, *P* = 0.038 *vs* CT or CA19-9.

the sensitivity of FDG-PET, CT, MRI, CEA and CA19-9. The Student *t* test was used to compare the values of the SUV between the groups. All statistical analyses were performed using SPSS software (SPSS, Chicago, USA). A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Table 1 shows the clinicopathological profiles of the 31 patients. The sensitivity of FDG-PET, CT, MRI, the serum levels of CEA and CA19-9 were 100%, 40%, 0%, 0%, 40% in TS1, 93%, 93%, 89%, 20%, 73% in TS2 and 100%, 100%, 100%, 73%, 91% in TS3-4 (Table 2). The sensitivity of PET for detecting TS1, TS2, and TS3 tumors was 100%, 93%, and 100%, respectively. The sensitivity of FDG-PET was significantly higher in comparison to CT, MRI and the serum levels of CEA and CA19-9 in the patients with TS1 (*P* = 0.002 *vs* MRI or CEA, *P* = 0.038 *vs* CT or CA19-9).

Although the sensitivity was higher for larger tumors, the SUV was not significantly associated with the TS factor. The mean SUV did not show a significant difference in relation to the TS (TS1: 5.8 ± 4.5 , TS2: 5.7 ± 2.2 , TS3-4: 8.2 ± 3.9), respectively. The diagnosis of pancreatic adenocarcinoma was histologically confirmed in all patients with TS1 cancer (Table 3). The tumor was well differentiated in 4 patients and poorly differentiated in one patient. The tumor diameter ranged from 13 to 20 mm. All the TS1 tumors showed higher SUVs in the delayed phase compared with that in the early phase. The SUV pattern suggested the small lesions were malignant tumors.

Table 3 Characteristics of TS1 pancreas cancer

	Age (yr)	Gender	Size (mm)	Tumor differentiation	SUV early	SUV delayed
1	77	F	13	Poor	3.59	4.16
2	77	M	20	Well	5.53	7.10
3	82	F	20	Well	2.74	3.14
4	68	M	18	Well	2.87	3.06
5	81	M	20	Well	12.79	13.78

Poor: Poorly differentiated adenocarcinoma; Well: Well differentiated adenocarcinoma; SUV: Standardized uptake value; SUV early: Value at 1 h after iv ¹⁸F-fluorodeoxyglucose; SUV delayed: Value at 2 h.

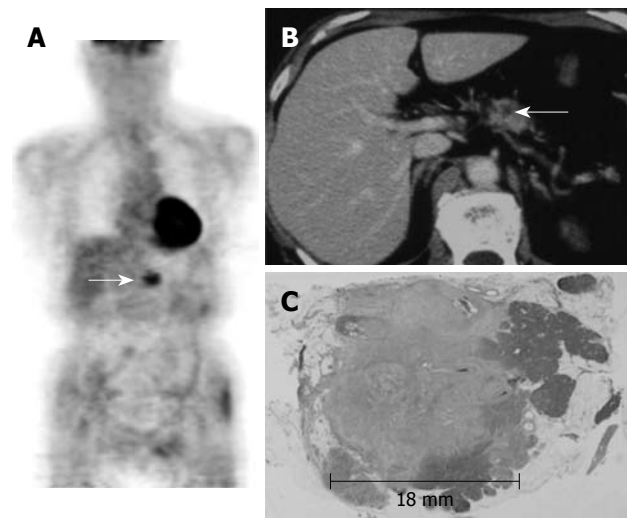


Figure 1 Positron emission tomography images of a 68-year-old male with TS1 pancreatic cancer. A: Whole body positron emission tomography image shows apparent increased uptake of ¹⁸F-fluorodeoxyglucose in the tumor (arrow, delayed point standardized uptake value, 3.06); B: Axial computed tomography image with contrast enhancement shows small low-density mass in the pancreas body (arrow); C: The histological findings (HE staining) of the pancreas revealed invasive ductal cancer in the body of the pancreas with a diameter of 18 mm.

Representative images of one patient (case 4 in Table 3) with TS1 pancreas cancer are shown in Figure 1. A 68-year-old male was transferred to our hospital for evaluation and further management of diabetes mellitus. A whole body FDG-PET image shows apparent increased uptake in the tumor (delayed point SUV, 3.06) (Figure 1A). An axial CT image with contrast enhancement shows a small low-density mass in the pancreas body (Figure 1B). The histological findings (HE staining) of the pancreas revealed invasive ductal cancer in the body of pancreas with a diameter of 18 mm (Figure 1C).

DISCUSSION

The usefulness of FDG-PET in diagnosing distant disease from advanced pancreatic cancer has been previously reported, although the poor spatial resolution of FDG-PET is known to limit the local staging of pancreatic cancer^[3]. CT is better suited to demonstrate the relationship of the tumor, adjacent organs, and vascular structure in advanced pancreatic cancer, but it is rela-

tively insensitive for detecting pancreatic cancers < 2 cm in size^[8-11]. Although the sensitivity of contrast-enhanced helical CT in the detection of pancreatic carcinoma is reported to vary from 76% to 92%, the sensitivity declines to 58% to 67% for tumors smaller than 2 cm^[8-10,12]. The sensitivity of EUS or MRI has been reported to be the same or slightly better in comparison to that of CT^[13,14].

Patients with small pancreatic carcinoma have no typical symptoms, which make it very difficult to detect. In contrast to the inherent limitations of this anatomic imaging modality, functional imaging using FDG PET appears to represent a significant advance in the detection of small pancreatic cancers < 2 cm in size. Seo *et al.*^[15] reported the effectiveness of FDG-PET for the detection of small pancreatic cancers with a sensitivity of 81% for tumors smaller than 2 cm. Although there have been a few reports indicating the value of FDG-PET in the diagnosis of small pancreatic cancer, the efficacy of dual phase FDG-PET in small pancreatic cancer has not been fully evaluated.

Dual time point FDG-PET is a more reliable method than single time point FDG-PET for differentiating pancreatic cancer from a mass identified to be chronic pancreatitis. In addition, delayed PET imaging is also helpful for identifying more lesions in patients with pancreatic cancer^[7]. Dual time point evaluation is routinely performed in our institution for patients with pancreatic cancer. There were 5 tumors smaller than 2 cm in the current series, and the sensitivity of FDG-PET for the detection of these tumors was 100%, although there was no tumor smaller than 1 cm. A dual time point evaluation may help to increase the sensitivity in the diagnosis of small pancreatic cancer.

The increased uptake of FDG due to the enhanced glucose metabolism of cancer cells is a sensitive marker of tumor viability or biological behavior. The SUV is an independent prognostic factor in various malignant tumors. Sperti *et al.*^[16] demonstrated that a high SUV (> 4.0) was associated with shorter survival. Maemura *et al.*^[17] reported that pancreatic tumors with distant metastases showed significantly higher SUV levels than tumors without metastases. The present study showed the SUVs of pancreatic cancer did not differ significantly in relation to tumor size. The results indicate that FDG-PET may, therefore be useful even in patients with small pancreatic cancers that can not be visualized by either CT or other modalities. The present study did not provide data on the specificity because there were no benign lesions. In our previous study^[7], the specificity of FDG-PET for detection of pancreatic cancer was 65%. Benign lesions such as chronic pancreatitis and autoimmune-related pancreatitis can also accumulate FDG and result in false-positive interpretations of PET studies. Further studies including benign lesions are required to clarify the diagnostic accuracy of FDG-PET.

The routine use of PET is not believed to be cost-effective and thus has not been accepted as a standard screening examination for small pancreatic cancer. Although the etiology of pancreatic cancer has not yet been completely elucidated, several factors are thought to be associated with cancer^[18-21]. Smoking is a consistently iden-

tified environmental risk factor which doubles the risk of pancreatic cancer^[19,20]. Dietary factors, such as high energy intake, cholesterol, and high meat consumption are known to increase the risk. Long-standing diabetes, chronic pancreatitis and certain hereditary conditions can affect the risk of developing pancreatic cancer. FDG-PET screening is therefore recommended if the patients are elderly and have been identified to be at risk for pancreatic cancer. FDG-PET screening for the detection of pancreatic cancers should therefore be considered for patients with chronic pancreatitis, because such patients are 16 times more likely to develop pancreatic cancer than healthy controls. Dual time point FDG-PET is a reliable method for differentiating pancreatic cancer from a mass identified to be chronic pancreatitis^[22]. However, there is a limitation in our study. This study was performed by a PET scanner. The coregistration of CT and PET images or integrated PET/CT devices may help to improve some diagnostic problems. Further evolution of PET scanner technology, including the PET/CT hybrid scanner, should provide superior diagnostic performance.

These results indicate that FDG-PET is a useful modality for the detection of small pancreatic cancers with a diameter of less than 20 mm. However, this study was limited due to the small population of patients. As a result, further prospective studies with PET/CT involving a larger population of patients should therefore be conducted to substantiate the results of this study.

COMMENTS

Background

Early diagnosis is the most important factor for improving the overall survival and quality of life in patients with pancreatic cancer. Positron emission tomography (PET) has demonstrated superiority to computed tomography (CT), ultrasonography (US), and endoscopic US (EUS) in its sensitivity and specificity in diagnosing pancreas cancer.

Research frontiers

Delayed additional ¹⁸F-fluorodeoxyglucose PET (FDG-PET) imaging is a useful method in differential diagnosis of malignant from benign lesions. However, the role of dual time point FDG-PET in the diagnosis of small pancreatic cancers has yet to be established.

Innovations and breakthroughs

The usefulness of FDG-PET in diagnosing distant disease from advanced pancreatic cancer has previously been reported, although the poor spatial resolution of FDG-PET is known to limit the local staging of pancreatic cancer. This is the first study to describe the usefulness of dual time point FDG-PET in detection of small pancreatic cancers with a diameter of less than 20 mm.

Applications

The ability to diagnose the early stage of pancreas cancer can be improved by using the dual time point FDG-PET in combination with CT, US and EUS. Early diagnosis is the most important factor for improving the overall survival and quality of life in patients with pancreatic cancer.

Terminology

Dual time point FDG-PET: FDG, a glucose analog, is taken up by high-glucose-using cells such as brain, kidney, and cancer cells, where phosphorylation prevents the glucose from being released intact. FDG-PET can be used for diagnosis, staging, and monitoring treatment of cancers. PET scans detect the areas with increased glucose uptake. The standardized uptake value of FDG is measured from two sequential time points.

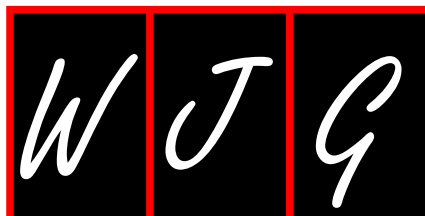
Peer review

This article is a retrospective analysis concerning a diagnostic value of PET for small pancreatic cancer. It is well-written but there are several issues to be resolved.

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S- Editor Wang YR L- Editor Cant MR E- Editor Lin YP



Seroprevalence of anti-HAV among patients with chronic viral liver disease

Hyun Chin Cho, Seung Woon Paik, Yu Jin Kim, Moon Seok Choi, Joon Hyeok Lee, Kwang Cheol Koh, Byung Chul Yoo, Hee Jung Son, Seon Woo Kim

Hyun Chin Cho, Seung Woon Paik, Yu Jin Kim, Moon Seok Choi, Joon Hyeok Lee, Kwang Cheol Koh, Byung Chul Yoo, Division of Gastroenterology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea

Hee Jung Son, Division of Gastroenterology, Center for Health Promotion, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea

Seon Woo Kim, Biostatistics Team, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea

Author contributions: Paik SW provided substantial contributions to conception and design, and approved the final version of the manuscript; Cho HC contributed to drafting the article, acquisition of data, analysis and interpretation of data; Kim YJ, Choi MS, Lee JH, Koh KC, Yoo BC and Son HJ contributed to revising the manuscript and providing data; Kim SW provided contributions to statistical analysis and interpretation of data.

Correspondence to: Seung Woon Paik, MD, Professor, Division of Gastroenterology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea. sw.paik@samsung.com
Telephone: +82-2-34103409 Fax: +82-2-34106983

Received: August 14, 2010 Revised: October 15, 2010

Accepted: October 22, 2010

Published online: January 14, 2011

RESULTS: The overall prevalence of IgG anti-HAV was 86.61% (854/986) in patients with chronic liver disease and was 88.13% (869/986) in age- and gender-matched patients from the Center for Health Promotion. The anti-HAV prevalence was 80.04% (405/506) in patients with chronic hepatitis B, 86.96% (20/23) in patients with chronic hepatitis C, 93.78% (422/450) in patients with HBV related liver cirrhosis, and 100% (7/7) in patients with HCV related liver cirrhosis. The anti-HAV prevalence according to the decade of age was as follows: 20s (6.67%), 30s (50.86%), 40s (92.29%), 50s (97.77%), and 60s (100%). The anti-HAV prevalence was significantly higher in patients older than 40 years compared with that in patients younger than 40 years of age. Multivariable analysis showed that age \geq 40 years, female gender and metropolitan cities as the place of residence were independent risk factors for IgG anti-HAV seropositivity.

CONCLUSION: Most Korean patients with chronic liver disease and who are above 40 years of age have already been exposed to hepatitis A virus.

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Key words: Chronic hepatitis B; Chronic hepatitis C; Hepatitis A virus; Korea; Seroprevalence

Peer reviewers: Fernando Fornari, MD, Department of Gastroenterology, Universidade de Passo Fundo, Rua Teixeira Soares, 817, 99010080 Passo Fundo, Brazil; Fen Liu, MD, University of Minnesota, 6155 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, United States

Cho HC, Paik SW, Kim YJ, Choi MS, Lee JH, Koh KC, Yoo BC, Son HJ, Kim SW. Seroprevalence of anti-HAV among patients with chronic viral liver disease. *World J Gastroenterol* 2011; 17(2): 236-241 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/236.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.236>

Abstract

AIM: To investigate the current seroprevalence of hepatitis A virus (HAV) antibodies in patients with chronic viral liver disease in Korea. We also tried to identify the factors affecting the prevalence of HAV antibodies.

METHODS: We performed an analysis of the clinical records of 986 patients (mean age: 49 ± 9 years, 714 males/272 females) with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection who had undergone HAV antibody testing between January 2008 and December 2009.

INTRODUCTION

Hepatitis A virus (HAV) is an epidemiologically important virus with a worldwide distribution and causes acute hepatitis in humans. This virus has been responsible for numerous disease outbreaks resulting from close personal contact or sexual contact^[1-3], contaminated food or water^[3-7], injection drug use^[8,9] and other modes of transmission^[8,10].

The pattern of this disease includes infection during early childhood followed by life-long immunity^[11]. When acquired in childhood, HAV is a very benign disease, over 70% of patients are asymptomatic and fulminant liver failure is extremely rare^[12,13]. When the infection occurs in adulthood, a much more prolonged course is seen and the rate of jaundice and fulminant liver failure is much higher^[12-14].

During recent decades, due to improvements in sanitation and hygiene, the age of infection by this virus has shifted from early childhood to adolescence or even later^[11,15,16]. Although the overall case-fatality rate of acute HAV among persons of all ages is only 0.01%-0.3%^[17-19], it is higher (1.8%) among adults who are 50 years of age or older^[19]. More importantly, acute HAV superinfection causes severe liver disease, acute liver failure and even higher mortality rates in patients with underlying chronic liver disease (CLD). Numerous studies have identified CLD as a risk factor for fulminant hepatitis and death from acute HAV infection^[7,20-29].

The aim of this study was to investigate the current seroprevalence of HAV antibodies (anti-HAV) in patients with chronic viral liver disease in South Korea. We also tried to determine the age-specific seroprevalence in these patients to assess whether vaccination against HAV is necessary in all patients who have underlying viral liver diseases, and to determine the factors that affect IgG anti-HAV seropositivity.

MATERIALS AND METHODS

Study design, population, and collection of data

We identified a total of 986 patients with chronic viral liver disease who had undergone HAV antibody testing between June 2008 and December 2009 at the Samsung Medical Center, Seoul, South Korea. The inclusion criteria consisted of hepatitis B virus (HBV) surface antigen (HBsAg) positivity or hepatitis C virus (HCV) antibodies (anti-HCV) and HCV RNA positivity in more than two tests for at least 6 mo. Patients with human immunodeficiency virus infection and a past medical history of HAV vaccination were excluded from this study.

The status of underlying liver disease was classified into chronic hepatitis and liver cirrhosis (LC). The diagnosis of LC was made if any one of the following findings was met: (1) compatible intraoperative gross findings or histologically compatible findings; (2) evidence of portal hypertension in patients with liver disease; and (3) compatible radiologic findings and platelet counts less than $100 \times 10^9/L$.

During the same period, 986 age- and gender-matched patients from the Center for Health Promotion were selected as the control group by one-to-one matching, and the study was statistically powered at 89%. There was no loss of subjects in the case group. Patients from the Center for Health Promotion who tested positive for HBsAg or anti-HCV and had a medical history of liver disease were excluded.

Laboratory procedures

Commercially available immunoassays (Anti-HAV IgG IRMA kit, North Institute of Biological Technology, Beijing, China; ARCHITECT HBsAg assay, Abbott Laboratories, Sligo, Ireland; ADVIA Centaur HCV assay, Siemens Healthcare Diagnostics, Los Angeles, CA, USA) were used to detect IgG anti-HAV, HBsAg and anti-HCV, respectively. The HCV RNA was amplified by RNA PCR and hybridization methods (COBAS® AmpliCor HCV test version 2.0, Roche Molecular Systems, Branchburg, NJ, USA, lower limit of detection 50 IU/mL).

Statistical analysis

The Cochran-Armitage trend test was used to assess the association between age and seropositivity rate for anti-HAV. McNemar's test was used to compare the seropositivity rate for anti-HAV between patients with CLD and patients from the Center for Health Promotion. Categorical variables were compared with the χ^2 test. Binary logistic regression analysis was used to determine the relationship between the variables and seropositivity for anti-HAV.

P values < 0.05 were considered statistically significant and Bonferroni's method was used to correct for inflated type I error due to multiple testing. All the statistical analyses were run on SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Ethical considerations

The institutional review board of Samsung Medical Center approved this retrospective study.

RESULTS

Patient demographics

The patient characteristics are detailed in Table 1. The mean age of the patients was 49 years (range: 20-80 years) and the vast majority of patients were over 40 years old (84%). A male preponderance (72.41%) was observed and the vast majority of patients had chronic viral hepatitis B (51.32%) and HBV related LC (45.64%). A relatively large proportion of the patients were from Seoul, the capital of South Korea (39.45%). The overall prevalence of IgG anti-HAV in patients with CLD was 86.61% (854/986).

The prevalence of IgG anti-HAV according to age

When the study participants were classified by decade of age into five groups, from 20s to more than 60 years old, the anti-HAV seroprevalence was 6.67% and 50.86% in

Table 1 Patient characteristics

Variable	n (%)
Mean age (yr, range)	49 ± 9 (20-80)
Gender	
Female	272 (27.59)
Male	714 (72.41)
Chronic liver disease	
Chronic viral hepatitis B	506 (51.32)
Chronic viral hepatitis C	23 (2.33)
HBV related liver cirrhosis	450 (45.64)
HCV related liver cirrhosis	7 (0.71)
Place of residence	
Seoul	389 (39.45)
Gyeonggi-do	274 (27.79)
Metropolitan cities	101 (10.24)
Other provinces	223 (22.62)
Prevalence of IgG anti-HAV	854 (86.61)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HAV: Hepatitis A virus.

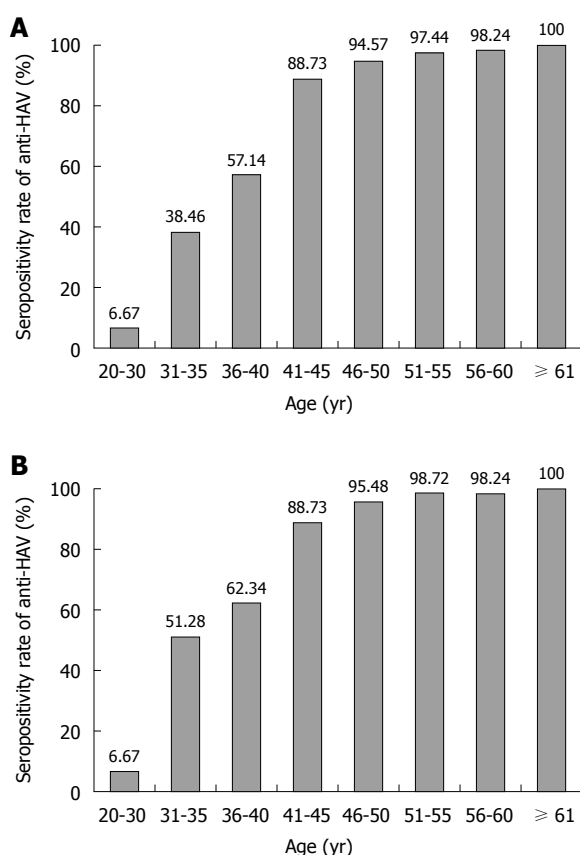


Figure 1 Prevalence of IgG anti-hepatitis A virus according to age in patients with chronic viral liver disease (A) and in age- and gender-matched patients from the Center for Health Promotion (B). HAV: Hepatitis A virus.

the patients in their 20s and 30s, respectively. The positivity rate for anti-HAV in the patients in their 40s, 50s and 60s was 92.29%, 97.77% and 100%, respectively. The prevalence of IgG anti-HAV in patients with CLD, and as divided by 5-year age intervals, is shown in Figure 1A. The seropositivity rate for anti-HAV increased gradually as age increased ($P < 0.001$). The anti-HAV prevalence was significantly higher in patients older than 40 years compared

Table 2 Prevalence of IgG anti-hepatitis A virus in patients with hepatitis B virus *vs* those with hepatitis C virus infection n (%)

Age (yr)	Anti-HAV/HBV	Anti-HAV/HCV
20-30	3/43 (6.98)	0
31-40	58/112 (51.79)	0/2 (0)
41-50	325/351 (92.59)	6/7 (85.71)
51-60	378/387 (97.67)	12/12 (100)
≥ 61	63/63 (100)	9/9 (100)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HAV: Hepatitis A virus.

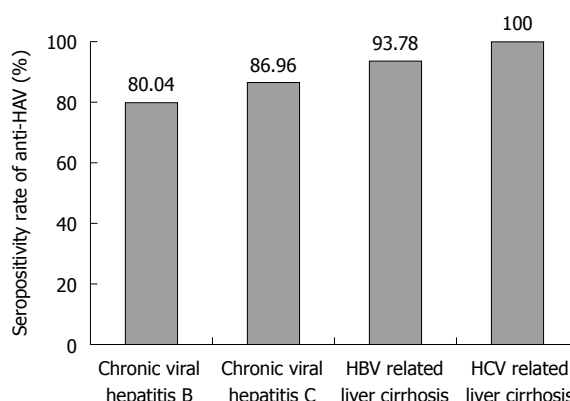


Figure 2 Prevalence of IgG anti-hepatitis A virus according to the status of chronic viral liver disease. HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

with those patients younger than 40 years of age (94.95% *vs* 33.58%, respectively, $P < 0.001$).

The prevalence of IgG anti-HAV according to age in the age- and gender-matched patients from the Center for Health Promotion is shown in Figure 1B. The overall prevalence of anti-HAV was 88.13% (869/986) and the seropositivity rate for anti-HAV increased gradually as age increased ($P < 0.001$). There was no significant difference in the anti-HAV seroprevalence between patients with CLD and those from the Center for Health Promotion ($P = 0.141$).

The prevalence of IgG anti-HAV according to the etiology and status of liver disease

The overall prevalence of anti-HAV was 86.51% in the 956 patients with chronic HBV infection, and it was 90% in the 30 patients with chronic HCV infection. There was no statistically significant difference in seropositivity for anti-HAV between the patients with HBV infection and those with HCV infection ($P = 0.582$). For the HBsAg-positive patients, the anti-HAV prevalence in each group divided by the decade of age increased gradually as age increased, which was similar for all the patients (Table 2).

The prevalence of IgG anti-HAV according to the status of CLD is shown in Figure 2. The anti-HAV seroprevalence was 80.04% (405/506) in patients with chronic hepatitis B, 86.96% (20/23) in patients with chronic hepatitis C, 93.78% (422/450) in patients with HBV related LC and 100% (7/7) in patients with HCV related LC.

Table 3 Prevalence of IgG anti-hepatitis A virus according to gender, the status of liver disease and the place of residence

Characteristics	Anti-HAV positivity, <i>n</i> (%)	<i>P</i> value
Sex		0.049
Male	609/714 (85.29)	
Female	245/272 (90.07)	
Status of liver disease		< 0.001
Chronic viral hepatitis	425/529 (80.34)	
Liver cirrhosis	429/457 (93.87)	
Place of residence		< 0.001
Seoul	311/389 (79.95)	
Gyeonggi-do	241/274 (87.96)	
Metropolitan cities	96/101 (95.05)	
Other provinces	206/223 (92.38)	

HAV: Hepatitis A virus.

Table 4 Factors affecting seropositivity for IgG anti-hepatitis A virus on the multivariable analysis

Characteristics	Anti-HAV positivity, <i>n</i> (%)	OR (95% CI)	<i>P</i> value
Age (≥ 40 yr)	809/852 (94.95)	33.44 (20.14-55.52)	< 0.001
Female	245/272 (90.07)	2.07 (1.16-3.71)	0.014
Etiology			0.487
HBV	827/956 (86.51)	1	
HCV	27/30 (90)	0.61 (0.15-2.47)	
Status of liver disease			0.075
Chronic viral hepatitis	425/529 (80.34)	1	
Liver cirrhosis	429/457 (93.87)	1.64 (0.95-2.82)	
Place of residence			0.035
Seoul	311/389 (79.95)	1	
Gyeonggi-do	241/274 (87.96)	1.51 (0.86-2.66)	0.153
Metropolitan cities	96/101 (95.05)	4.11 (1.37-12.35)	0.012
Other provinces	206/223 (92.38)	1.84 (0.93-3.65)	0.080

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HAV: Hepatitis A virus; OR: Odds ratio; CI: Confidence interval.

Factors affecting the seropositivity of IgG anti-HAV

The anti-HAV prevalence according to gender, the status of liver disease and place of residence is shown in Table 3. Anti-HAV was more frequently detected in female patients (90.07%) than in male patients (85.29%, $P = 0.049$). As for the status of liver disease, anti-HAV antibody was more frequently detected in patients with LC (93.87%) than in those with chronic hepatitis (80.34%, $P < 0.001$). As for the place of residence, anti-HAV antibody was less frequently detected among patients who lived in Seoul or Gyeonggi-do (79.95%-87.96%) than among those living in metropolitan cities or other provinces (92.38%-95.05%, $P < 0.001$).

Multivariable analysis of the factors for anti-HAV seropositivity is shown in Table 4. Age ≥ 40 years ($P < 0.001$), female gender ($P = 0.014$) and metropolitan cities as the place of residence ($P = 0.012$) were independent risk factors for IgG anti-HAV seropositivity.

DISCUSSION

The epidemiological pattern of HAV infection is cur-

rently changing in many developing countries. An improved socioeconomic status, more sanitary conditions and better hygiene practices have reduced the incidence of HAV infection, and the age-specific HAV seroprevalence in the general population has steadily decreased. The decrease in HAV infection in young adults has resulted in a reduction in individuals with protective antibody and increased hepatitis A in the adult population. In Korea, symptomatic hepatitis A has been gradually increasing since the mid-1990s, with a tendency toward an increase in the mean age and disease severity^[30-35].

A number of studies have suggested that the clinical course of HAV infection is more severe in patients with CLD^[7,20-29]. Mortality in patients with HBsAg was found to be significantly higher than that in patients without HBsAg in an outbreak of HAV infection in Shanghai^[23], and an analysis of HAV associated deaths in the United States revealed a higher rate of fatality in HBV carriers than in patients without HBV^[24]. Moreover, patients with HCV infection were reported to experience HAV associated fulminant hepatic failure more often than those patients without CLD. In a prospective cohort study of adults with HCV infection, Vento *et al*^[31] reported that 41.2% of patients with acute HAV superinfection developed acute liver failure and 35.3% died.

In our study, the overall seroprevalence of IgG anti-HAV in the 986 Korean patients with chronic viral liver disease was 86.61%. When the study participants were classified by the decade of age, the anti-HAV seroprevalence was 6.67%, 50.86%, 92.29%, 97.77% and 100% in patients in their 20s, 30s, 40s, 50s and 60s, respectively. These results are consistent with recent Korean studies^[28,29]. The anti-HAV prevalence was significantly higher in patients older than 40 years compared with those younger than 40 years of age (94.95% *vs* 33.58%, respectively). These data indicate that most patients with chronic viral liver diseases and who are above 40 years of age have already been exposed to HAV infection, and have naturally acquired immunity against HAV. Hence, vaccination against HAV should be considered in young anti-HAV-negative patients.

In the present study, we expected the seroprevalence of anti-HAV to be higher in patients with CLD than in those from the Center for Health Promotion, considering the relatively low socioeconomic status of the CLD patients. However, there was no statistically significant difference in anti-HAV seroprevalence between the two groups (86.61% *vs* 88.13%, respectively, $P = 0.141$). This finding may indicate that the immune response to HAV infection is not altered by chronic infection with either HBV or HCV. This result is also consistent with the results of the multivariable analysis in our study.

As shown in the multivariable analysis and Figure 1A, patient age was the most important factor for determining the seropositivity rate of IgG anti-HAV. Although anti-HAV was more frequently detected in patients with LC than in those with chronic viral hepatitis (93.87% *vs* 80.34%, respectively), this was probably attributable to age when considering the results of the multivariable analysis.

Female patients had a relatively higher rate of HAV seropositivity. This finding might be explained by the fact that female subjects in Korea have a larger number of social and household contacts and so probably have more exposure to HAV. We also observed significant differences among the places of residence. The lowest seroprevalence was observed in Seoul, the largest and most urbanized city in Korea, and the highest was in the provinces, with a more rural way of life. Such differences in seroprevalence might well be attributed to the vast variations in living conditions.

The current study has a couple of limitations. First, this is a retrospective study and the available epidemiological data on HAV infection is limited. Socioeconomic characteristics, including the educational level, salary, the number of siblings, the type of residence, the water supply, *etc.*, were not included in this study. Although multivariable analysis of the seropositivity of anti-HAV was carried out with relatively limited variables, the results in the present study may not be applicable to all patients with CLD. Second, although the current study included a large number of CLD patients, it was performed at a single medical center. Therefore, the patients may not be representative of the whole population of Korea. We also used data from the Center for Health Promotion and these patients may not be representative of the general Korean population without CLD when considering the fact that patients with a relatively higher level of socioeconomic status visit the Center for Health Promotion.

In conclusion, the overall prevalence of IgG anti-HAV in Korean patients with chronic viral liver disease was 86.61%, and most patients who are above 40 years of age have already been exposed to HAV. Therefore, vaccination against HAV should be considered, particularly for young anti-HAV-negative patients with chronic liver disease.

COMMENTS

Background

An improved socioeconomic status, more sanitary conditions and better hygiene practices have reduced the incidence of hepatitis A virus (HAV) infection. In Korea, symptomatic hepatitis A has been gradually increasing since the mid-1990s, with a tendency toward an increase in the mean age and disease severity. Acute HAV superinfection causes severe liver disease, acute liver failure and even higher mortality rates in patients with underlying chronic liver disease.

Research frontiers

Identifying the current seroprevalence of HAV antibodies in patients with chronic viral liver disease would be valuable for establishing appropriate vaccination guidelines for patients with chronic liver disease.

Innovations and breakthroughs

Recent studies reported a rapid epidemiological shift in HAV infection. In this study, the authors evaluated seroprevalence of IgG anti-HAV in patients with chronic liver disease using large population based data, and investigated the age-specific seroprevalence and the factors that affect IgG anti-HAV seropositivity.

Applications

There has been an apparent epidemiological shift in HAV seroprevalence in patients with chronic liver disease. Most patients who are above 40 years of age have already been exposed to HAV. Therefore, vaccination against HAV should be considered, particularly for young anti-HAV-negative patients with chronic liver disease.

Peer review

This manuscript is of interest.

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S- Editor Sun H L- Editor Webster JR E- Editor Lin YP

Magnesium citrate with a single dose of sodium phosphate for colonoscopy bowel preparation

Yong Sung Choi, Jung Pil Suh, Jong Kyu Kim, In Taek Lee, Eui Gon Youk, Doo Seok Lee, Do Sun Kim, Doo Han Lee

Yong Sung Choi, Jung Pil Suh, Jong Kyu Kim, Department of Gastroenterology, Daehang Hospital, Seoul 137-820, South Korea
 In Taek Lee, Eui Gon Youk, Doo Seok Lee, Do Sun Kim, Doo Han Lee, Department of Surgery, Daehang Hospital, Seoul 137-820, South Korea

Author contributions: Choi YS performed data analysis and edited the manuscript; Suh JP and Kim JK were involved in editing the manuscript; Lee IT, Youk EG and Lee DS were involved in data collection and analysis; Kim DS and Lee DH provided financial support for publishing this work.

Correspondence to: Jung Pil Suh, MD, Department of Gastroenterology, Daehang Hospital, Seoul 137-820, South Korea. yschoi71@dreamwiz.com

Telephone: +82-2-63888114 Fax: +82-2-63888115

Received: July 20, 2010 Revised: August 17, 2010

Accepted: August 24, 2010

Published online: January 14, 2011

Abstract

AIM: To evaluate the efficacy and acceptability of magnesium citrate and a single dose of oral sodium phosphate (45 mL) solution for morning colonoscopy bowel preparation.

METHODS: A total of 159 patients were randomly assigned to receive two split doses of 90 mg of sodium phosphate (Group I, $n = 79$) or magnesium citrate (250 mL, the day before the procedure) followed by 45 mL of sodium phosphate (the day of procedure, Group II, $n = 80$). The quality of bowel cleansing and the acceptability of each regimen were compared, including the satisfaction, taste, willing to repeat and adverse effects of each regimen.

RESULTS: The quality of bowel cleansing of Group II was as good as that of Group I (An Aronchick scale score of good or excellent: 70.9% vs 81.0%, respectively, $P = 0.34$; the Ottawa system score: 4.4 ± 2.6 vs 3.8 ± 3.0 , respectively, $P = 0.76$). There was no statistically

significant difference between both groups with regard to acceptability, including the satisfaction, taste and willingness to repeat the regimen. A significantly greater number of older patients (over 65 years old) in Group II graded the overall satisfaction as satisfactory (48.1% vs 78.1%, respectively; Group I vs Group II, $P = 0.01$). There were no significant adverse reactions.

CONCLUSION: Magnesium citrate and a single dose of sodium phosphate was as effective and tolerable as the conventional sodium phosphate regimen and is a satisfactory option.

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Key words: Colonoscopy; Bowel preparation; Efficacy; Acceptability; Magnesium citrate; Sodium phosphate

Peer reviewer: Josep M Bordas, MD, Department of Gastroenterology IMD, Hospital Clinic, Llusanes 11-13 at, Barcelona 08022, Spain

Choi YS, Suh JP, Kim JK, Lee IT, Youk EG, Lee DS, Kim DS, Lee DH. Magnesium citrate with a single dose of sodium phosphate for colonoscopy bowel preparation. *World J Gastroenterol* 2011; 17(2): 242-248 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i2/242.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.242>

INTRODUCTION

The worldwide introduction of screening colonoscopy programs has led to a dramatic increase in the number of colonoscopies. Despite the development of techniques and instruments, the difficulties in bowel preparation for a complete colonoscopic examination still remain a challenge to the physician and the examinee. These difficulties may result in significant noncompliance and thus subop-

timal preparation, which subsequently may lead to missed lesions^[1], an increased procedure time and the need for repeat colonoscopy^[2], as well as reluctance to undergo follow-up examinations.

Sodium phosphate is a low-volume laxative, and it is known to be superior to 4l polyethylene glycol (PEG) solution, both for acceptability by the patient and for the quality of bowel cleansing^[3-6]. However, a significant proportion of patients complain that its bad taste is unacceptable and there are adverse reactions such as nausea, vomiting and headache. Moreover, more potential adverse reactions associated with hyperphosphatemia may theoretically develop under any conditions that increase absorption or decreased elimination of phosphate^[7]. Unfortunately, poor compliance with the instructions about the dosage or interval of oral sodium phosphate and undetected chronic illness, particularly in the elderly, may result in a higher possibility of unexpected adverse events. Recent reports of renal failure associated with sodium phosphate have raised increasing concerns^[8-12] in spite of some reports demonstrating its safety in a series of clinical trials^[13,14].

In this study, we wanted to find a better and easier preparation regimen for a complete colonoscopic examination. We have experienced that the usual bowel preparation for sigmoidoscopy in our hospital using magnesium citrate the day before the procedure scheduled in the next morning was able to debulk the colon of solid fecal material without much patient discomfort. Therefore, we hypothesized that taking magnesium citrate the day before the procedure and then taking a single dose of sodium phosphate (45 mL) on the day of procedure would be as effective a regimen as the conventional two doses of sodium phosphate and more tolerable for morning colonoscopy.

MATERIALS AND METHODS

Study design, patient enrollment and the end outcomes

This was a prospective, randomized, endoscopist-blinded, controlled study. All consecutive patients referred to the Outpatient Department of Gastroenterology and Surgery at Daehang Hospital (Seoul, Korea) between September 2009 and December 2009 for colonoscopy in the morning were enrolled in this clinical study. The exclusion criteria were as follows: ileus or suspected bowel obstruction, significant gastroparesis or gastric outlet obstruction, the presence of serious medical conditions such as severe cardiac, renal, hepatic or metabolic diseases, pregnancy, lactation and a history of prior colon or rectal surgery. Written informed consent was obtained from each patient. No patient refused to participate. The primary end outcomes in this study were the efficacy of bowel preparation according to the Aronchick scale and the Ottawa Bowel Preparation Scale (OBPS), the patients' tolerance, the overall satisfaction, the taste and the willingness to repeat the same preparation regimen, if necessary. The sec-

ondary end outcomes included assessment of the OBPS components (i.e. the right vs middle vs left colon, and the fluid score) and a subgroup assessment according to age, gender, a prior bowel cleansing regimen and so forth.

Colon bowel preparation

A total of 159 patients were randomly assigned to receive two split doses of 90 mg of oral sodium phosphate (Group I, $n = 79$) or 250 mL of magnesium citrate (magnesium carbonate 4.3 g and anhydrous citric acid 7.8 g/100 mL) (the day before the procedure) and this was followed by 45 mL of sodium phosphate (the day of the procedure, Group II, $n = 80$). Group I ingested a single dose (45 mL) of sodium phosphate starting at 8:00 PM on the day before the procedure and then they ingested the remaining 45 mL of sodium phosphate at 5:00 AM on the day of colonoscopy. Group II ingested magnesium citrate (one bottle; 250 mL) at 8:00 PM on the preceding day and a single dose (45 mL) of sodium phosphate at 5:00 AM on the procedure day. Both groups took one tablet of bisacodyl as pretreatment 30 min before each laxative on the day before the procedure. All the patients who had taken oral sodium phosphate were instructed to drink an additional 1-1.5 L of water. Both groups had a thick liquid diet for dinner on the day before the procedure, and they took nothing further by mouth after 6:00 PM. All the colonoscopy procedures were performed between 8:30 AM and 11:30 AM. The quality of bowel cleansing, the tolerability, the satisfaction, the willing to repeat the procedure and the adverse effects of each regimen were compared.

Evaluation of bowel preparation

Efficacy of bowel cleansing: The endoscopists, who were blinded to the form of preparation, performed the colonoscopy exams. To standardize and minimize interobserver variation, we had a meeting before the start of this study and an investigator gave a standardized explanation for assessing the quality of bowel preparation during the meeting. A poster with endoscopic photos that demonstrated the scoring system was placed in each endoscopy room as a reminder. The endoscopists were also requested to take pictures of each colon segment before cleansing the segments with saline during the procedure.

Bowel cleansing was assessed at the end of the colonoscopy using the OBPS and the Aronchick scale. The OBPS has a potential score ranging from 0 (excellent preparation, no fluid) to 14 (inadequate in all segments with a large amount of fluid) (Table 1). The Aronchick scale was as follows: excellent (a small volume of clear liquid or greater than 95% of the surface was seen); good (a large volume of clear liquid covering 5% to 25% of the surface but greater than 90% of the surface was seen); fair (some semisolid stool that could be suctioned or washed away but greater than 90% of the surface was seen); and poor (semisolid stool that could not be suctioned or washed away and less than 90% of the surface was seen).

There were ten colonoscopists who performed colonos-

Table 1 Summary of the scoring system for the Ottawa Bowel Preparation Scale

Cleanliness
Excellent (0): mucosal detail clearly visible. If fluid present, it is clear. Almost no stool residue
Good (1): some turbid fluid or stool residue but mucosal detail still visible. Washing and suctioning not necessary
Fair (2): turbid fluid or stool residue obscuring mucosal detail and contour. However, mucosal detail becomes visible with suctioning. Washing not necessary
Poor (3): presence of stool obscuring mucosal detail and contour. However, with suctioning and washing, a reasonable view is obtained
Inadequate (4): solid stool obscuring mucosal detail and contour despite aggressive washing and suctioning
Fluid: small (0), moderate (1), large (2)

Table 2 Baseline characteristics (mean \pm SD) *n* (%)

Variables	Group I (<i>n</i> = 79)	Group II (<i>n</i> = 80)	<i>P</i> value
Age (yr), median (range)	59 (46-77)	60 (48-78)	NS
Male	40 (50.6)	50 (62.5)	NS
Cecal intubation time (s)	300.6 \pm 171.9	314 \pm 190.5	NS
Weight (kg)	61.5 \pm 15.6	64.1 \pm 15.3	NS
Prior colonoscopy	45 (56.9)	44 (55.0)	NS
With PEG	11 (13.9)	14 (17.5)	
With Sodium phosphate	34 (43.0)	30 (37.5)	
Hypertension	12 (15.2)	15 (18.8)	NS
Diabetes	8 (10.1)	5 (6.3)	NS
Constipation (< 3/wk)	12 (15.2)	14 (17.6)	NS

PEG: Polyethylene glycol; NS: Not significant.

copies in this study. All the colonoscopists were experts who had experienced at least 1000 cases of colonoscopy after their gastrointestinal or coloproctologic fellowship. To check the interobserver variation for rating the efficacy of bowel preparation, all the colonoscopy pictures were reviewed by two experienced endoscopists (Choi YS and Suh JP) who were blinded to the preparation methods.

Patient acceptability

All the patients completed a questionnaire before colonoscopy. It was used to assess the past medical history, compliance, discomfort symptoms during preparation and the overall satisfaction. The investigated symptoms were nausea or vomiting (present = 1, absent = 0), abdominal pain or discomfort (present = 1, none = 0) and abdominal distension (present = 1, none = 0). The overall satisfaction with the preparation was rated on a 3 scale (satisfactory, fair and unsatisfactory). The willingness to repeat the same preparation method in the future, if necessary, was also evaluated with a “yes” or “no” question.

Statistical analysis

All the continuous variables are presented as means and standard deviations or as medians and ranges, as appropriate. The χ^2 test was used for comparisons between categorical variables and Student's *t* test was used for com-

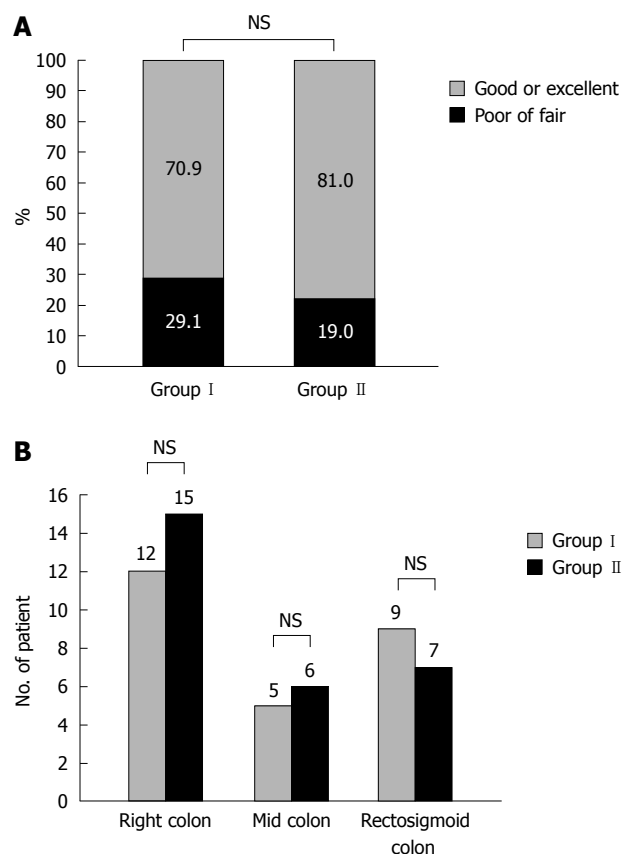


Figure 1 Quality of bowel cleansing. A: Quality of bowel cleansing. The patients are graded as good or excellent quality vs fair or poor quality with the conventional sodium phosphate regimen (Group I) or the magnesium citrate and a single dose sodium phosphate regimen (Group II); B: Segmental quality of bowel cleansing. The patients are scored with colon segment grades of poor or inadequate with the conventional sodium phosphate regimen (Group I) or the magnesium citrate and a single dose sodium phosphate regimen (Group II). NS: Not significant.

parisons between continuous variables. *P* values less than 0.05 were regarded as statistically significant.

RESULTS

Baseline characteristics

A total of 180 consecutive patients were enrolled and randomized into two groups (90 patients in each group). Twenty-one patients were excluded because the procedure was postponed or canceled. Finally, 159 patients were included in the analysis. There was no significant difference in the baseline characteristics between both groups (Table 2). In all cases, cecal intubation was successful. There were no serious complications or adverse events immediately after the procedure.

Assessment of the quality of bowel cleansing

There was no significant difference in bowel cleansing quality as evaluated by the Aronchick scale between both bowel preparation regimens. Group I had 56 satisfactory preparations (defined as excellent or good) of 79 (70.9%) compared with 62 of 80 (81.0%) for Group II (*P* = 0.34) (Figure 1A).

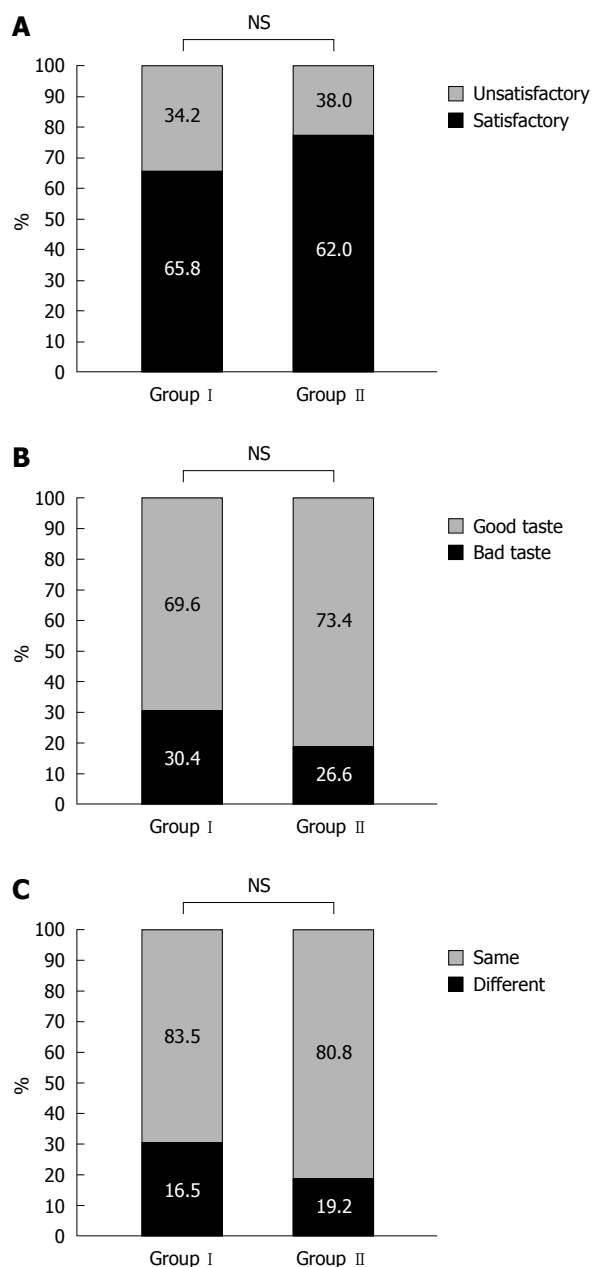


Figure 2 Patient acceptability. A: For overall satisfaction, 65.8% in Group I and 82.0% in Group II replied that they were “satisfied” or it was “fair”; B: For assessing taste, the percentage of patients who responded “good or tolerable” was not significantly different between Group I (69.6%) and Group II (73.4%); C: For the willingness to repeat the regimen, the percentage of patients who responded “yes” was not significantly different between Group I (83.5%) and Group II (80.8%). NS: Not significant.

When bowel preparation was assessed by the Ottawa scale, the preparation quality was not significantly different as well (Table 3). The mean score was not significantly different between both groups for the right colon ($P = 0.54$), the mid colon ($P = 0.47$), the rectosigmoid colon ($P = 0.97$) or the total amount of fluid ($P = 0.48$). In both groups, the patients with colon segment grades of poor or inadequate were 12 (15.2%) *vs* 15 (18.8%), 5 (6.3%) *vs* 6 (8.0%) and 9 (11.4%) *vs* 7 (9.0%) for the right colon, the mid colon and the rectosigmoid colon, respectively (Figure 1B).

Table 3 Efficacy of bowel cleansing by Ottawa scoring system (mean \pm SD)

Variables	Group I (n = 79)	Group II (n = 73)	P value
Right colon	1.4 \pm 1.1	1.3 \pm 1.1	NS
Mid colon	1.0 \pm 0.9	0.8 \pm 0.9	NS
Rectosigmoid colon	1.3 \pm 0.9	1.2 \pm 1.0	NS
Fluid present	0.7 \pm 0.6	0.4 \pm 0.6	NS
Overall score	4.4 \pm 2.6	3.8 \pm 3.0	NS

NS: Not significant.

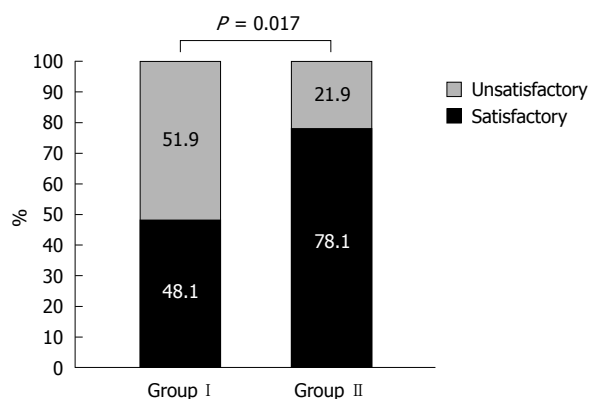


Figure 3 Overall satisfaction with bowel preparation in the elderly (> 65 years). 48.1% in Group I and 78.1% in Group II replied that they were “satisfied” or it was “fair” ($P = 0.017$).

There was no significant difference of the segmental bowel preparation quality either.

Assessment of patient acceptability (overall satisfaction, taste and willing to repeat)

The percentage of patients with symptoms such as abdominal distension with the two preparation regimens was 23% *vs* 34%, respectively, nausea or vomiting 45% *vs* 41%, respectively, and abdominal pain 15% *vs* 13%, respectively.

When patients were asked whether they were satisfied with the bowel preparation regimen, 65.8% (52 of 79) in Group I and 82.0% (62 of 80) in Group II replied that they were “satisfied” or it was “fair” (Figure 2A). When patients were asked how the laxatives tasted, the patients who responded “good or tolerable” were not significantly different between Group I (69.6%) *vs* Group II (73.4%) (Figure 2B). When patients were asked whether they were willing to repeat the same preparation regimen if necessary, the percentage of patients who responded “yes” was not significantly different between Group I (83.5%) *vs* Group II (80.8%) (Figure 2C).

Subgroup assessment of the bowel cleansing quality and patient acceptability

When the patients over 65 years old were asked whether they were satisfied with the bowel preparation regimen, 48.1% (13 of 27) in group I and 78.1% (25 of 32) in group II replied that they were “satisfied” or it was “fair” ($P = 0.017$) (Figure 3). The question about taste and will-

ingness to repeat revealed no significant differences between both groups.

When only the patients who had previously undergone colonoscopy and had taken PEG or oral sodium phosphate were asked about patient acceptability, the prior sodium phosphate users generally had a preference for this new regimen rather than the new PEG users concerning overall satisfaction (80.0% *vs* 64.3%, respectively, $P = 0.26$), taste tolerance (80% *vs* 71.4%, respectively, $P = 0.52$) and willing to repeat (90.0% *vs* 61.3%, respectively, $P = 0.04$).

DISCUSSION

The early detection of colonic neoplasm *via* colonoscopy and its removal by polypectomy is popular and increasing, yet many people avoid screening colonoscopy because they fear the preparation required for colonoscopy. The two most commonly-used regimens for colonoscopic bowel preparation are currently a large volume of osmotically balanced PEG solution and a small volume of an osmotically active agent, oral sodium phosphate. Four liters of PEG solution is known to be safe and effective, but poorly tolerated. In contrast, sodium phosphate is known to be well-tolerated and as effective as PEG solution. Oral sodium phosphate has been shown to be very safe in a large series of clinical trials^[13,14]. Its administration is known to be associated with only an asymptomatic, mild increase in serum phosphate (mean increase: 3 mg/dL) and a minimal decrease in serum calcium (mean decrease: 0.3 mg/L); these changes are readily reversible^[10]. Clinically significant adverse reactions, including severe hyperphosphatemia, hypocalcemia, seizure, tetany, hypovolaemia and acute renal failure are thought to be extremely rare, with a reported incidence of 1.44 per million patients^[10,15]. Under normal conditions, phosphate is absorbed in the small intestine and eliminated by the kidney as calcium phosphate^[15]. The conventional dosage of oral phosphate for bowel cleansing is one bottle (45 mL) the evening before the procedure and another bottle the next morning. Any condition that can increase absorption or decrease elimination of phosphate such as repeated dosing, dehydration, the use of diuretics and ACE inhibitor, or a preexisting illness that may impair phosphate homeostasis, for example, hyperparathyroidism, renal insufficiency or delayed intestinal transit can be a risk factor. Indeed, fatal cases have been observed among the patients with a history of renal dysfunction^[16-18], ischemic colitis^[17], cirrhosis^[18] and in elderly patients with normal renal function^[19,20]. The maximum safe dose of sodium phosphate is 90 mL^[21]. Several studies on the adverse effects of high doses of sodium phosphate have suggested that these high doses should be avoided^[13-15]. If the recommended dose of 60 g (90 mL) is surpassed, or if the interval between doses is < 5 h, severe hyperphosphatemia could develop^[10,15,18,22,23]. Fine *et al*^[24] have found that the mortality rate was 33%, and that the risk of death was high if the serum phosphate level increased beyond 32.69 mg/dL (10.56 mmol/L). Most of the deaths reported in the lit-

erature have been caused by arrhythmia or heart attack associated with electrolyte changes and dehydration^[25]. Therefore, adequate fluid intake is necessary. Markowitz *et al*^[10] has suggested that patients must be encouraged to drink eight cups of fluids (1920 mL) and Rex *et al*^[2] have promoted taking 3.6 L of clear fluids. However, it was reported that the standard dose of sodium phosphate could induce the development of hyperphosphatemia even in low-risk and well-hydrated patients^[7].

In this study, we attempted to debulk the colon of solid fecal material with magnesium citrate at first and thereby decrease the volume of oral sodium phosphate required for bowel cleansing without compromising the quality of the bowel preparation. Rather, we achieved superior overall satisfaction in the elderly patients who were pretreated with magnesium citrate. It is practically significant to find that this new regimen is better tolerated by elderly patients since the potential for complications associated with bowel preparation may be relatively increased in elderly patients due to poor compliance with the recommended fluid intake. In addition, elderly patients have a greater possibility of having an underlying chronic illness that may be undetected. Therefore, we think a lower volume of sodium phosphate can be safer and better suited for everybody. The patients who had previously taken two doses of sodium phosphate for bowel preparation had a tendency to prefer this new regimen rather than the PEG solution, suggesting that this regimen can be an alternative option for the intolerant patients.

We found that overall rates of adequate bowel cleansing in both groups were relatively high (Group I, 70.9% and Group II, 81%), which suggested that bisacodyl as pretreatment and procedure time scheduled for the morning had influenced the overall quality of bowel cleansing. Both regimens were scheduled for morning because afternoon colonoscopies have higher failure rates than morning colonoscopies, and the suggested scheduling of all outpatient colonoscopies is preferentially in the morning to avoid suboptimal procedures^[26]. The dosing time of magnesium citrate was determined as 9 h before taking the oral sodium phosphate. In a previous study that used magnesium citrate as pretreatment to decrease the volume of PEG solution, magnesium citrate was taken at 1-2 h before ingesting 2 L of PEG solution^[27,28]. However, we expect that a 9 h time interval would be optimal, with reference to the usual interval time for the split-dosed PEG solution regimen or the oral sodium phosphate regimen.

Our study has several limitations. First, the safety profiles, including electrolytes, hemoglobin and the BUN/creatinine change, were not evaluated to prove the safety advantages of this new regimen compared to the conventional sodium phosphate regimen, although the volume of sodium phosphate was reduced by half. Second, we excluded the patients with medical conditions that are currently contraindicated for conventional sodium phosphate use such as heart failure, cirrhosis, renal disease, very old age patients or those taking diuretics or ACE inhibitors and so forth. Thus, the findings in our study need to be

confirmed in a large study with various groups of patients. Third, there were ten colonoscopists involved in this study. Although all the endoscopists were trained in using the scale with photographs illustrating each point on the OBPS, and the degree of agreement was acceptable according to a κ coefficient of greater than 0.4, the study could have been more powerful if all the colonoscopies had been performed by a single colonoscopist. Fourth, there was a discrepancy between “better” overall satisfaction and a “similar” propensity of willingness to repeat the bowel preparation regimen among the elderly patients. Further tailored evaluation of a large group of elderly patients is needed to verify this.

As mentioned above, the use of the conventional dose of sodium phosphate is theoretically limited for patients with any condition that can increase the absorption or decrease the elimination of phosphate. If we think a lower volume of sodium phosphate might be safer, then this must be verified in individual cases since the mechanism of an adverse reaction to sodium phosphate and its risk factors has not been fully discovered. Further evaluation of the safety profile is mandatory.

In conclusion, a regimen of magnesium citrate and a single dose of oral sodium phosphate was as effective as the conventional two doses of sodium phosphate, and our new regimen was well tolerated. Therefore, this regimen could be a good option to routinely prepare for morning colonoscopy.

COMMENTS

Background

Oral sodium phosphate is a hyperosmolar, low-volume laxative for colonoscopy bowel preparation, and it is known to be as effective as polyethylene glycol solution. It is generally better tolerated, but the bad taste and uncomfortable abdominal symptoms such as nausea and vomiting frequently lead to poor compliance with the oral sodium phosphate regimen, and all this can subsequently cause incomplete bowel cleansing. Moreover, potential adverse reactions associated with hyperphosphatemia may develop.

Innovations and breakthroughs

This is the first clinical study to evaluate the efficacy and tolerability of magnesium citrate and a single dose of oral sodium phosphate for bowel preparation prior to morning colonoscopy. The findings demonstrated this modified regimen, in which the volume of sodium phosphate was reduced by half was as effective and tolerable as the conventional sodium phosphate regimen.

Applications

The study results suggest that magnesium citrate and a single dose of oral sodium phosphate can be an effective and satisfactory option to routinely prepare for morning colonoscopy.

Terminology

Magnesium citrate acts as an osmotic laxative which is often used for bowel preparation

Peer review

In this well designed paper the author has proposed a combination of magnesium citrate the day before the procedure and sodium phosphate early in the morning of the day of the procedure, associated with one tablet of bisacodyl as compared to standard cleansing using two doses of sodium phosphate, with one tablet of bisacodyl. The paper is interesting and the design is adequate, as are the results and comments.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zheng XM

Risk factors for hilar cholangiocarcinoma: A case-control study in China

Wen-Ke Cai, Hui Sima, Ben-Dong Chen, Guang-Shun Yang

Wen-Ke Cai, Hui Sima, Ben-Dong Chen, Guang-Shun Yang, Department of Biliary Surgery II, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Cai WK and Yang GS designed the research; Cai WK, Sima H and Chen BD performed the research and analyzed the data; Cai WK and Yang GS wrote the paper.

Correspondence to: Guang-Shun Yang, Professor, Department of Biliary Surgery II, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China. caiwenke002@126.com

Telephone: +86-21-81875292 Fax: +86-21-81875291

Received: August 24, 2010 Revised: September 26, 2010

Accepted: October 3, 2010

Published online: January 14, 2011

lithiasis, hepatolithiasis, cholecystolithiasis) and parasitic liver disease (biliary ascariasis, liver fluke, liver schistosomiasis) are the risk factors for HC in Chinese population.

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Key words: Hilar cholangiocarcinoma; Choledocholithiasis; Hepatitis B virus; Hepatitis C virus; Liver fluke

Peer reviewer: Ching-Chung Lin, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 111, Taiwan, China

Cai WK, Sima H, Chen BD, Yang GS. Risk factors for hilar cholangiocarcinoma: A case-control study in China. *World J Gastroenterol* 2011; 17(2): 249-253 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/249.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.249>

Abstract

AIM: To study the association between hilar cholangiocarcinoma (HC) and pre-existing medical conditions.

METHODS: Three hundred and thirteen HC patients admitted to the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) in 2000-2005 and 608 healthy controls were enrolled in this study. Association between HC and pre-existing medical conditions was studied with their adjusted odds ratio (OR) calculated by logistic regression analysis.

RESULTS: The prevalence of choledocholithiasis (adjusted OR = 2.704, $P = 0.039$), hepatolithiasis (adjusted OR = 3.278, $P = 0.018$), cholecystolithiasis (adjusted OR = 4.499, $P < 0.0001$), cholecystectomy (adjusted OR = 7.012, $P = 0.004$), biliary ascariasis (adjusted OR = 7.188, $P = 0.001$), liver fluke (adjusted OR = 10.088, $P = 0.042$) and liver schistosomiasis (adjusted OR = 9.913, $P = 0.001$) was higher in HC patients than in healthy controls.

CONCLUSION: Biliary tract stone disease (choledocholithiasis, hepatolithiasis, cholecystolithiasis) and parasitic

INTRODUCTION

Hilar cholangiocarcinoma (HC) is a rare and highly malignant cancer of the bile duct, accounting for less than 2% of all human malignancies. Although the entire biliary tree is a potential risk, tumors involving the biliary confluence or the right or left hepatic ducts are most common, accounting for 40%-60% of all tumors. Its etiology remains poorly understood and its incidence has been increasing in China. The long-term prognosis of HC is poor due to its early metastasis. Complete resection has been recognized as the most effective therapy for HC. However, surgical management still remains a major challenge because of its location close to the portal vein, hepatic artery, and liver parenchyma. So far, most studies have been focused on the risk factors for intrahepatic cholangiocarcinoma (ICC)^[1-3], while few studies on the risk factors for HC are available^[4]. Due to the increasing incidence and poor long-term prognosis of HC, it is extremely important to evaluate the risk factors for HC in order to decrease its incidence.

MATERIALS AND METHODS

Study population

Three hundred and thirteen HC patients who received surgical dissection at the Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University (Shanghai, China) from January 2000 to December 2005 were included in this study. HC was diagnosed by pathological examination of samples taken from HC patients. Those who were diagnosed as HC but did not undergo surgery were excluded from the study.

Controls

Six hundred and eight healthy individuals who visited the Eastern hepatobiliary Surgery Hospital of the Second Military Medical University for a routine checkup served as controls and were matched to the HC patients for sex and age (± 4 years). Moreover, the years of search were matched in HC patients and controls for risk factors to minimize their difference.

Several potential risk factors for HC were studied and divided into 3 broad categories: biliary tract condition and operation, infectious diseases, and miscellaneous potential risk factors. Biliary tract condition and operation included choledocholithiasis, hepatolithiasis, cholecystolithiasis, primary sclerosing cholangitis (PSC), parasitic liver disease and cholecystectomy. Infectious diseases included hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Miscellaneous potential risk factors included alcoholic liver disease, type II diabetes mellitus, smoking and ulcerative colitis.

Statistical analysis

Prevalence of HC and potential risk factors for HC were compared in HC patients and controls. χ^2 test or Fisher's exact test was used for categorical variables, and *t* test was used for discrete variables.

Variables with a *P* value < 0.05 were considered statistically significant. Odds ratio (OR) and 95% CI of each risk factor for HC were computed as estimate of the relative risk by unconditional logistic regression analysis, using the maximum-likelihood estimate.

RESULTS

Demographic and baseline parameters of patients and controls

A total of 313 patients (194 men and 119 women with male-to-female ratio of 1.63:1) diagnosed as HC were enrolled in the present study. Their mean age was 56.64 ± 10.585 years (range 32-80 years). Most HC developed during the fourth to sixth decade, and reached the peak at the age of 55 years (Figure 1).

The distribution of HC in controls and HC patients according to their age and gender is shown in Table 1. No significant difference was found in gender and mean age between HC patients and controls, suggesting that the pairing is effective.

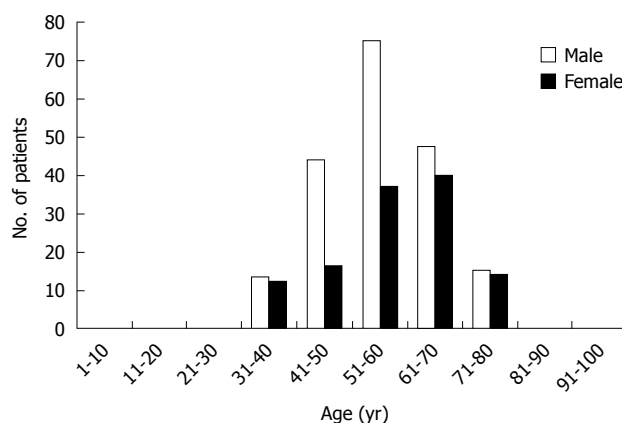


Figure 1 Distribution of age and sex in patients with hilar cholangiocarcinoma admitted to the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) during 2000-2005.

Table 1 Demographic characteristics of patients with hilar cholangiocarcinoma and control subjects

Variable	HC patients (<i>n</i> = 313)	Controls (<i>n</i> = 608)	<i>P</i>
Mean age	56.64 ± 10.585	55.58 ± 10.792	0.895
Gender			
Male	194	380	0.878
Female	119	228	

HC: Hilar cholangiocarcinoma.

Risk factors

The risk factors were divided into 3 categories: biliary tract condition and operation, infectious diseases and other risk factors. In biliary tract condition and operation, the prevalence of choledocholithiasis, hepatolithiasis cholecystolithiasis, cholecystectomy, biliary ascariasis, liver schistosomiasis and liver fluke was higher in HC patients than in controls (*P* < 0.05), while no significant difference was found in the prevalence of PSC between HC patients and controls. In infectious diseases, the prevalence of HBV infection was 7.3% in HC patients and 6.3% in controls (*P* = 0.526), the prevalence of HCV infection was 1.0% in HC patients and 2.1% in controls (*P* = 0.151). In other risk factors, the prevalence of ulcerative colitis (UC), alcoholic liver disease, type II diabetes mellitus and smoking was 0.3%, 0.6%, 5.4% and 22%, respectively, in HC patients, and 0.0%, 0.3%, 6.1% and 20.0%, respectively, in controls.

The distribution of risk factors in each category is summarized in Table 2.

Multiple logistic regression analysis

Logistic regression model was used to adjust the demographics (age, sex), and the adjusted OR and 95% CI for the different risk factors were calculated. Multivariate analysis showed that choledocholithiasis, hepatolithiasis, cholecystolithiasis, cholecystectomy, biliary ascariasis, liver fluke and liver schistosomiasis were the risk factors for HC, while HBV infection, HCV infection, PSC, UC, alco-

Table 2 Distribution of risk factors in hilar cholangiocarcinoma patients and controls *n* (%)

Risk factors	Controls (<i>n</i> = 608)	HC patients (<i>n</i> = 313)	<i>P</i>
Biliary tract condition and operation			
Cholelithiasis	8 (1.3)	28 (8.9)	< 0.0001
Hepatolithiasis	7 (1.2)	25 (8.0)	< 0.0001
Cholecystolithiasis	45 (7.4)	98 (31.3)	< 0.0001
Cholecystectomy	3 (0.5)	23 (7.3)	< 0.0001
PSC	0 (0.0)	2 (0.6)	0.115
Biliary ascariasis	4 (0.7)	15 (4.8)	< 0.0001
Liver fluke	1 (0.2)	4 (1.3)	0.048
Liver schistosomiasis	3 (0.5)	12 (3.8)	< 0.0001
Infectious etiologies			
HBV infection	38 (6.3)	23 (7.3)	0.526
HCV infection	13 (2.1)	3 (1.0)	0.151
Other risk factors			
UC	0 (0.0)	1 (0.3)	0.340
Alcoholic liver disease	2 (0.3)	2 (0.6)	0.498
Diabetes mellitus type II	37 (6.1)	17 (5.4)	0.689
Smoking	134 (22.0)	63 (20.0)	0.503

HC: Hilar cholangiocarcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis.

holic liver disease, type II diabetes mellitus and smoking were not the significant risk factors for HC (Table 3).

DISCUSSION

In this case-control study, the risk factors for HC were examined in Chinese population, including choledocholithiasis, hepatolithiasis, cholecystolithiasis, cholecystectomy, biliary ascariasis, liver fluke and liver schistosomiasis. Although HBV and HCV infection have been reported to be intimately associated with intrahepatic cholangiocarcinoma (ICC)^[1,2], they were not demonstrated to be significantly associated with HC in our study. In addition, PSC, UC, alcoholic liver disease, type II diabetes mellitus and smoking were not significantly related to HC.

The prevalence of biliary tract stone disease (hepatolithiasis, cholecystolithiasis and choledocholithiasis) and cholecystectomy was higher in HC patients than in controls. Hepatolithiasis is more frequently seen in East Asian countries than in Western countries, and represents a high-risk factor for ICC due to inflammation and epithelial proliferation^[3]. In parallel, extrahepatic lithiasis may promote chronic inflammatory changes in extrahepatic bile ducts, thus increasing the risk of developing extrahepatic cholangiocarcinoma (ECC), which can explain why choledocholithiasis increases the risk of developing HC. However, the reasons why hepatolithiasis increases the risk of developing HC are less clear. It was reported that cholecystolithiasis is a risk factor for ECC according to its ecological and epidemiological evidence, and the incidence of HC is decreased 10 or more years after cholecystectomy for cholecystolithiasis^[5,6]. In our study, the prevalence of both cholecystolithiasis and cholecystectomy was higher in HC patients than in controls. Cholecystectomy was

Table 3 Risk factors for hilar cholangiocarcinoma and their *P* value, odds ratio and 95% CI

Risk factors	<i>P</i>	OR	95% CI	
			Lower	Upper
Choledocholithiasis	0.039	2.704	1.054	6.941
Hepatolithiasis	0.018	3.278	1.226	8.766
Cholecystolithiasis	< 0.0001	4.499	2.990	6.769
Cholecystectomy	0.004	7.012	1.895	25.954
Biliary ascariasis	0.001	7.188	2.245	23.015
Liver fluke	0.042	10.088	1.085	93.775
Liver schistosomiasis	0.001	9.913	2.702	36.369

OR: Odds ratio.

not a risk factor for HC because the majority of surgical procedures (76%) were performed within a year prior to tumor diagnosis, which is consistent with the findings in a recent US case-control study^[7].

Chronic infectious liver diseases, such as HBV and HCV infection, were not significantly associated with HC in our study. The prevalence of HBV and HCV infection was 7.3% and 1.0%, respectively, in HC patients, and 6.3% and 2.1%, respectively, in controls. The prevalence of HBV is rather high in China, and HBV is strongly associated with hepatocellular carcinoma (HCC). Moreover, the HBV genome has been detected not only in infected hepatocytes but also in bile duct epithelial, endothelial, and smooth muscle cells^[8]. It has been shown that HBV is also an independent risk factor for ICC in Chinese population^[9]. In the present study, HBV was not associated with HC, which is, however, not consistent with the reported findings in China^[10], suggesting that HBV is related to ECC. It was reported that HCV can also damage the bile duct epithelial cells, thus leading to a range of proliferative, inflammatory, and generative changes^[11]. Recent studies from Korea, Japan and Italy showed that HCV is associated with cholangiocarcinoma^[12-14]. A population-based case-control study in US^[6] showed that HCV is associated with ICC, but not with ECC. This study also showed that HCV was not a risk factor for HC. Although both HBV and HCV can be detected in hepatic bile ducts, most studies demonstrated that they are related only to intrahepatic bile duct carcinoma^[15,16]. The detailed mechanism still needs further investigation.

It has been shown that parasitic liver disease is related with cholangiocarcinoma^[17-19]. In the present study, biliary ascariasis, liver fluke (*Clonorchis sinensis*) and liver schistosomiasis were significantly associated with HC in Chinese population. Ascariasis is endemic in China and 4.8% cases had a history of biliary ascariasis in our study. *Ascaris* invasion into the bile duct may cause biliary colic, pyogenic cholangitis, pancreatitis and septicemia. A residual dead worm may destroy the biliary epithelial cells, resulting in fibrosis or nidi that may form stones. It was reported that ova and fragments of *ascaris* can serve as for stone formation^[20] and choledocholithiasis is a risk factor for HC. The bile duct damage induced by *ascaris*,

worm and stone formation may play an important role in genesis and development of cholangiocarcinoma. *Clonorchis sinensis* infestation is common in East Asia countries, including China, Korea, and Far East Russia, where the local inhabitants are used to eat raw fish or shrimp^[21]. In our study, 4 patients were infected with *Clonorchis sinensis* and had a history of eating fresh fish and shrimp. An epidemiological study from Thailand suggested that liver fluke (*opisthorchis viverrini*) is associated with cholangiocarcinoma^[22]. A hospital-based case control study in Korea also revealed that *Clonorchis sinensis* is strongly associated with cholangiocarcinoma^[23]. Schistosomiasis is the second most common parasitic infection worldwide after malaria. *Schistosomiasis Japonica*, occurring in Japan and China, may be a risk factor and an independent adverse prognostic factor for HCC^[24,25]. Besides, it has been shown that schistosomiasis *Japonica* is a risk factor for HC in Egyptian^[4]. Our study also confirmed that schistosomiasis *Japonica* was associated with HC in Chinese population. Carcinogenesis occurs in patients with parasitic infection, because the existence of parasites within the host induces chronic inflammation^[26,27]. Phagocytes release reactive oxygen and nitrogen species in patients with chronic inflammation are potential to damage DNA, proteins and cell membranes, modulate enzyme activities and gene expression, thus promoting carcinogenesis^[28-30].

In the Western world, the most common risk factor for HC is PSC^[31,32], an inflammatory disease of the bile duct, which is in turn closely associated with UC. The prevalence of UC in patients with PSC is 70%-100%^[33-35]. It was reported that the lifetime risk of developing cholangiocarcinoma in patients with PSC is 10%-15%^[36]. Parker *et al*^[37] described the association between UC and cholangiocarcinoma in 1954. Converse *et al*^[38] in 1971 found that the incidence of HC is higher in patients with PSC than in those with UC. The prevalence of PSC and UC is higher in Western world than in Asia^[39]. No epidemiological investigation of PSC and UC is available in China. In our study, PSC was diagnosed in 2 patients and UC in 1 patient. However, no PSC or UC was observed in controls.

It was reported that alcoholic liver disease and type II diabetes mellitus are related to extrahepatic cholangiocarcinoma in US population^[40]. However, our study demonstrated that they were not significantly associated with HC in Chinese population.

Our study had two potential limitations. One is that clinical records were used as the source for risk factor information, and the other is that most patients and controls came from certain geographic regions, which may omit some risk factors prevalent in other regions of China.

In summary, cholecystolithiasis, hepatolithiasis, choledocholithiasis, biliary ascariasis, liver fluke and liver schistosomiasis are the risk factors for HC, while PSC and UC are the suspected risk factors for HC in China. Further study is needed to explore the role of these risk factors in the development of HC.

COMMENTS

Background

Although several risk factors are associated with the development of hilar cholangiocarcinoma (HC), such as primary sclerosing cholangitis (PSC), liver fluke or biliary tract stone disease, the risk factors for HC in Chinese patients have not been fully studied.

Research frontiers

The etiology of cholangiocarcinoma has been well studied, and several risk factors for HC have been documented. Chronic infectious liver diseases, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, have been fully investigated in recent studies and are considered as the important risk factors for cholangiocarcinoma.

Innovations and breakthroughs

In this study, neither HBV infection nor HCV infection was associated with HC in Chinese population, which is not consistent with previous studies. PSC and ulcerative colitis were not the risk factors for HC possibly due to their low incidence in Chinese population. In addition to liver fluke, biliary ascariasis and liver schistosomiasis are also the risk factors for HC, which have not been reported in previous studies.

Applications

HC is a rare malignant disease with a poor prognosis. The findings in this study may contribute to its control.

Peer review

The authors investigated the potential risk factors associated with HC, and demonstrated that biliary tract stone and parasitic liver disease are the risk factor for HC, which may contribute to its control.

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S- Editor Sun H L- Editor Wang XL E- Editor Zheng XM

Effects of penehyclidine hydrochloride in small intestinal damage caused by limb ischemia-reperfusion

Yan Zhang, Yu-Fang Leng, Xing Xue, Yue Zhang, Tao Wang, Yu-Qing Kang

Yan Zhang, Yu-Fang Leng, Xing Xue, Yue Zhang, Tao Wang, Yu-Qing Kang, Department of Anesthesiology, First Hospital, Lanzhou University, Lanzhou 730000, Gansu Province, China

Author contributions: Leng YF contributed to the conception and design of the study and edited the manuscript; Zhang Y carried out experiment and wrote the manuscript; Xue X, Zhang Y, Wang T and Kang YQ conducted the experiment.

Supported by Lanzhou City Development Plan of Science and Technology, No. 2009-1-52

Correspondence to: Yu-Fang Leng, Professor, Department of Anesthesiology, First Hospital, Lanzhou University, Lanzhou 730000, Gansu Province, China. lengyf@lzu.edu.cn

Telephone: +86-931-8625200 Fax: +86-931-8619797

Received: August 20, 2010 Revised: September 26, 2010

Accepted: October 3, 2010

Published online: January 14, 2011

Abstract

AIM: To investigate the protective effect of penehyclidine hydrochloride post-conditioning in the damage to the barrier function of the small intestinal mucosa caused by limb ischemia-reperfusion (LIR) injury.

METHODS: Male Wistar rats were randomly divided into three groups (36 rats each): the sham-operation group (group S), lower limb ischemia-reperfusion group (group LIR), and penehyclidine hydrochloride post-conditioning group (group PHC). Each group was divided into subgroups ($n = 6$ in each group) according to ischemic-reperfusion time, i.e. immediately 0 h (T_1), 1 h (T_2), 3 h (T_3), 6 h (T_4), 12 h (T_5), and 24 h (T_6). Bilateral hind-limb ischemia was induced by rubber band application proximal to the level of the greater trochanter for 3 h. In group PHC, 0.15 mg/kg of penehyclidine hydrochloride was injected into the tail vein immediately after 3 h of bilateral hind-limb ischemia. The designated rats were sacrificed at different time-points of reperfusion; diamine oxidase (DAO), superoxide dismutase (SOD) activity, myeloperoxidase (MPO) of small intestinal tissue, plasma endotoxin, DAO, tumor necrosis factor- α

(TNF- α), and interleukin (IL)-10 in serum were detected in the rats.

RESULTS: The pathological changes in the small intestine were observed under light microscope. The levels of MPO, endotoxin, serum DAO, and IL-10 at T_1 - T_6 , and TNF- α level at T_1 - T_4 increased in groups LIR and PHC ($P < 0.05$) compared with those in group S, but tissue DAO and SOD activity at T_1 - T_6 decreased ($P < 0.05$). In group PHC, the tissue DAO and SOD activity at T_2 - T_6 , and IL-10 at T_2 - T_5 increased to higher levels than those in group LIR ($P < 0.05$); however, the levels of MPO, endotoxin, and DAO in the blood at T_2 - T_6 , and TNF- α at T_2 and T_4 decreased ($P < 0.05$).

CONCLUSION: Penehyclidine hydrochloride post-conditioning may reduce the permeability of the small intestines after LIR. Its protection mechanisms may be related to inhibiting oxygen free radicals and inflammatory cytokines for organ damage.

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Key words: Penehyclidine hydrochloride; Post-conditioning; Limb ischemia-reperfusion injury; Small intestine; Protection

Peer reviewer: Adriana M Torres, Professor of Pharmacology, Suipacha 531, Rosario 2000, Argentina

Zhang Y, Leng YF, Xue X, Zhang Y, Wang T, Kang YQ. Effects of penehyclidine hydrochloride in small intestinal damage caused by limb ischemia-reperfusion. *World J Gastroenterol* 2011; 17(2): 254-259 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/254.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.254>

INTRODUCTION

Limb ischemia-reperfusion (LIR) injury has been observed

in atherosclerosis, thrombosis or injury to the great vessels in the extremities, severe crushing injury and surgery^[1-3]. It can lead to limb edema, skeletal muscle dysfunction, and necrosis, and may further result in the dysfunction and structural damage to other organs such as the heart, lungs, brain, small intestines, and so on^[4,5]. Therefore, protection against ischemia-reperfusion injury has become an important focus of research in clinical work.

In recent years, many narcotics have been used to reduce the damage caused by ischemia-reperfusion^[6-8]. The new anti-cholinergic drug penehyclidine hydrochloride (PHC), reported to have protective effects against organ injury^[9-11], was developed by the Institute of Pharmacology and Toxicology in China (Academy of Military Medical Sciences) to minimise side effects harmful to the cardiovascular system^[12,13]. To date, no reports on the protective effects of PHC against LIR in the small intestines have been published. In this study, the serum and small intestinal tissue diamine oxidase (DAO), plasma endotoxin (reflecting the barrier function of the small intestinal mucosa), superoxide dismutase (SOD), and myeloperoxidase (MPO) activity of the small intestinal tissue, as well as serum tumor necrosis factor- α (TNF- α) and interleukin (IL)-10 levels (reflecting damage to the small intestine) were examined in rats.

MATERIALS AND METHODS

Experimental animals

One hundred and eight healthy 6-mo-old male Wistar rats weighing 220-250 g were provided by the Medical Experimental Animal Center of the Gansu College of Traditional Chinese Medicine, China. The rats were randomly divided into 3 groups: the sham-operation group (group S), limb ischemia-reperfusion group (group LIR), and penehyclidine hydrochloride post-conditioning group (group PHC). Each group was divided into sub-groups ($n = 6$ in each group) according to ischemic-reperfusion time, i.e. immediately (T_1), 1 h (T_2), 3 h (T_3), 6 h (T_4), 12 h (T_5), and 24 h (T_6).

Animal model

The LIR model was established as follows: the rats were fasted 12 h preoperatively with unlimited drinking water; and exposed to 2% isoflurane until the loss of righting reflex, and fixed onto a sit-board on the operating table. The posterior limbs of the rats were ligated with elastic rubber bands above the greater trochanter to completely block the blood flow. Group S was anesthetized, but did not undergo ligation. Group LIR was ligated until complete ischemia of the lower limbs, and released after 3 h to restore blood flow to the posterior limbs; reperfusion was conducted for 1, 3, 6, 12, and 24 h. In group PHC, the rubber band was released after 3 h of ischemia, and then 0.15 mg/kg PHC was injected into the tail veins, followed by reperfusion for 1, 3, 6, 12 and 24 h. After the reperfusion at pre-set time points (T_1 - T_6), the rats were sacrificed under deep isoflurane anaesthesia. All experiments were conducted according to the protocols approved by the Lanzhou University Animal Care and Use Committee.

About 5 mL of blood was drawn from the inferior vena cava of the rats. The blood was centrifuged at 3000 r/min for 15 min to separate the serum, which was stored at -20°C for further target detection. The small intestines of the rats were quickly removed up to 5 cm from the ileocecal valve and washed three times with normal saline. Then, 0.5 g of the small intestines were ground into tissue homogenate in a glass homogeniser; after centrifugation at 3500 r/min for 20 min, the resultant supernatant was diluted into a 10% solution with normal saline and stored at -20°C for further target detection. The remaining small intestines were fixed in 10% formalin, embedded in paraffin, stained with hematoxylin and eosin, sectioned, and observed for pathological changes under optical microscope.

Evaluation of changes in the barrier function of the small intestinal mucosa

The rat small intestinal tissue and serum DAO were detected using a spectrophotometer with an automatic biochemical analyser (OLYMPUS-AU5400; kit provided by the Nanjing Jiancheng Bioengineering Institute, China). Plasma endotoxin was measured using a quantitative chromogenic substrate assay (Xiamen TAL Experimental Plant Co., Ltd.).

Evaluation of damage mechanism in the small intestine

SOD and MPO activities in the rat small intestinal tissue were measured by colorimetry (Nanjing Jiancheng Bioengineering Institute). Serum TNF- α and IL-10 were measured by enzyme-linked immunosorbent assay (Wuhan Boster Biological Technology, Ltd., China).

Statistical analysis

All data were reported as mean \pm SD. Statistical significance was determined by one-way analysis of variance using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Rat small intestinal pathological changes

The small intestinal microstructure of the group S rats were normal: the villi were lined with normal epithelial cells, the interstitium was congestion-free, and gland morphology was normal. The small intestinal tissue of the group LIR rats had obvious pathological changes: the villi were malpositioned, atrophied, and shorter and thicker; loose interstitial edema with lymphocytic infiltration was observed, and lymph node follicles and submucosal lymphatic vessels were filled with lymphocytes. The small intestinal microstructure of the group PHC had certain pathologic changes that were less pronounced than in the group LIR.

Changes in the barrier function of the small intestinal mucosa

DAO is an enzyme synthesised primarily in gastrointestinal mucosal cells. The intestinal tissue and serum levels of DAO have been used as an indicator of the integrity and functional mass of the intestinal mucosa^[14,15]. When intes-

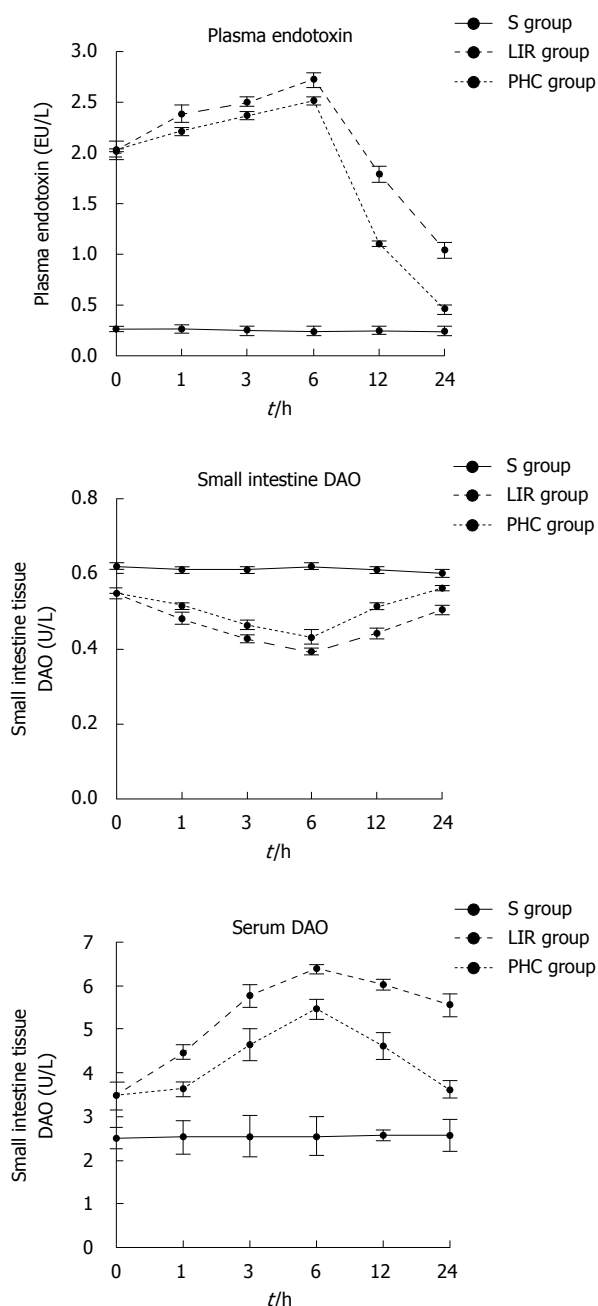


Figure 1 Changes of intestinal tissue diamine oxidase, serum diamine oxidase and plasma endotoxin in rats. S group: Sham-operation group; LIR group: Lower limb ischemia-reperfusion group; PHC group: Penheyclidine hydrochloride post-conditioning group. DAO: Diamine oxidase.

tinal mucosal integrity was disrupted during shock, burns, abdominal aortic surgery, liver disease, ischemia-reperfusion injury, and so on, an increase in intestinal permeability and translocation of bacteria endotoxin, and plasma endotoxin as well, was observed^[16,17]. In the groups LIR and PHC, serum DAO and plasma endotoxin levels at all preset time-points of reperfusion (T₁-T₆) increased compared with the group S ($P < 0.05$), whereas the tissue DAO content decreased ($P < 0.05$). Compared with the T₂-T₆ of group LIR, the tissue DAO activity of group PHC increased ($P < 0.05$), whereas the serum DAO activity and plasma endotoxin concentration were decreased ($P < 0.05$).

The tissue and serum DAO activity and plasma endotoxin concentration reached its peak ($P < 0.05$) (Figure 1) compared with other preset time-points of reperfusion at T₄ in the groups LIR and PHC.

Changes of certain factors in damage mechanism in the small intestine

In the groups LIR and PHC, the MPO activity of the small intestinal tissue and serum IL-10 at T₁-T₆, and serum TNF- α at T₁-T₄ were higher than those in the group S ($P < 0.05$), whereas the tissue SOD activity at T₁-T₆ was lower ($P < 0.05$). In group PHC, the tissue SOD activity at T₂-T₆ and serum IL-10 at T₂-T₅ were significantly higher than those in group LIR ($P < 0.05$); however, the tissue MPO activity at T₂-T₆ and serum TNF- α at T₂ and T₄ decreased ($P < 0.05$). In the groups LIR and PHC, the tissue SOD and MPO activities reached their peak at T₄ ($P < 0.05$) while TNF- α reached its peak at T₂ ($P < 0.05$). Thereafter, TNF- α gradually decreased, and even decreased at T₅ and T₆ in group S ($P \geq 0.05$); IL-10 at T₃ exhibited the highest concentration ($P < 0.05$) even at T₄-T₅, and the serum IL-10 also maintained a relatively high concentration (Figure 2).

DISCUSSION

The gastrointestinal tract in ischemia-reperfusion injury is of interest, not only because its functions are damaged, but also it is a potential factor of multiple organ dysfunction syndrome (MODS) associated with reperfusion injury^[18,19]. When the intestinal barrier is injured, the gut endotoxins may enter into the extraintestinal tissues and produce free radicals and cytokines that potentiate the development of MODS^[20-22]. In the group LIR, at different reperfusion time points, the small intestinal mucosa had varying degrees of injury, including neutrophil and lymphocyte infiltration, as well as small intestinal epithelial cell degeneration, necrosis, or even sloughing off. This result shows that LIR injury induces injury to the small intestinal mucosa. Meanwhile, the small intestinal tissue DAO activity in the group LIR was lower than in the group S; however, the activity of serum DAO and the plasma endotoxin were significantly higher than in the group S, indicating that the mucous membrane barrier of the rat small intestines was destroyed and gut permeability changed, leading to the absorption of gut cavity endotoxin and small intestinal tissue DAO into the blood stream.

LIR injury leading to permeability changes in intestinal mucosal reperfusion injury may be closely related to the production of excessive oxygen free radicals and inflammatory cytokines^[19,20,23,24]. Oxygenated free radicals appear to play a prominent role in mediating the damage associated with gastrointestinal diseases. The production of reactive oxygen metabolites in ischemia-reperfusion involves oxidases found in resident phagocytic cells, as well as microvascular and mucosal epithelial cells^[25]. SOD is a key enzyme that eliminates free radicals by converting superoxide anions into hydrogen peroxide, which is

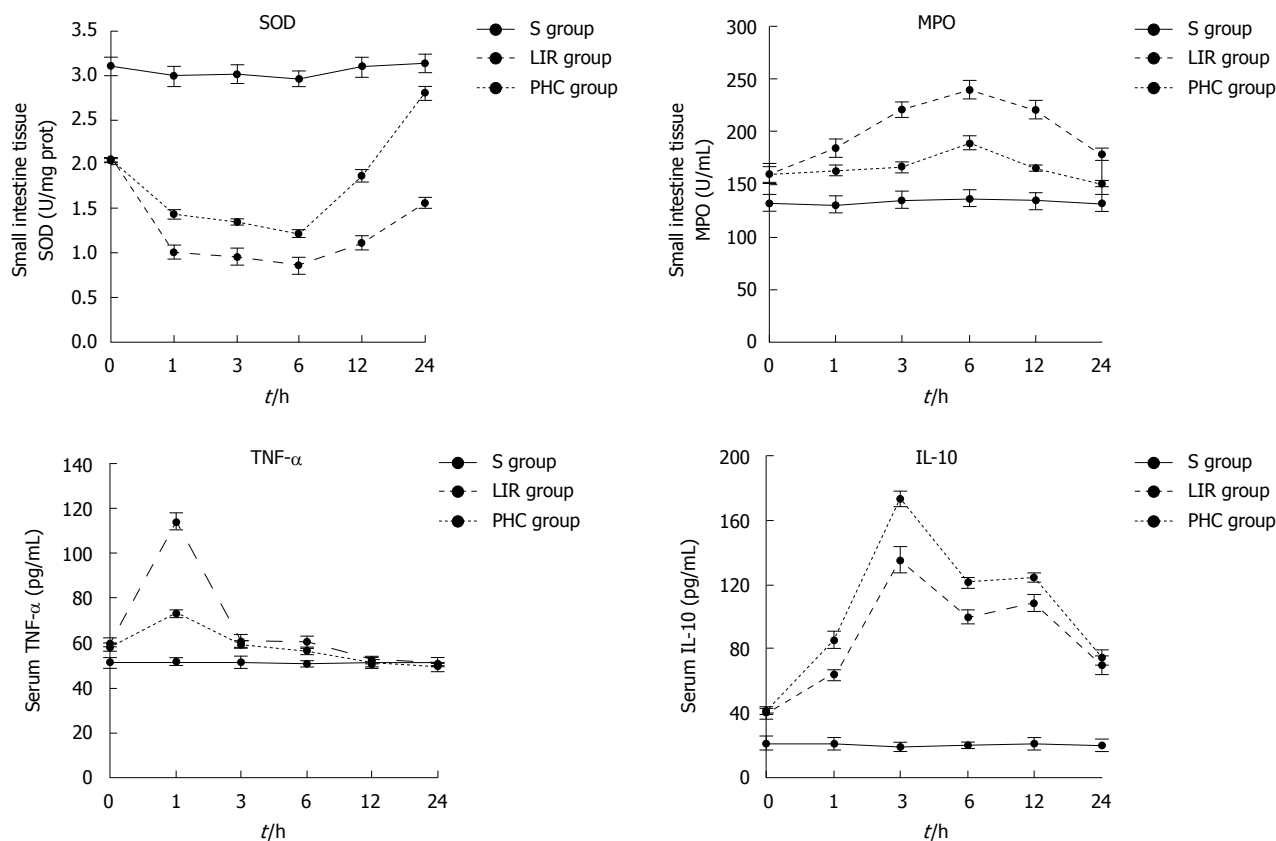


Figure 2 Changes of intestinal tissue superoxide dismutase, intestinal tissue myeloperoxidase, serum tumor necrosis factor- α and interleukin-10 in rats. S group: Sham-operation group; LIR group: Lower limb ischemia-reperfusion group; PHC group: Penehyclidine hydrochloride post-conditioning group. SOD: Superoxide dismutase; MPO: Myeloperoxidase; TNF- α : Tumor necrosis factor- α ; IL-10: Interleukin-10.

then removed by glutathione peroxidase and catalase. A high amount of oxygen free radicals is generated during ischemia followed by reperfusion, which leads to excessive consumption of SOD^[26]. MPO, on the other hand, is released upon activation to catalyse the formation of oxidants, which can lead to tissue damage during chronic inflammation, and serves as a major enzymatic catalyst of lipid peroxidation at inflammation sites^[27]. In this paper, the small intestinal tissue SOD activity of the group LIR during reperfusion was lower than that of the group S, but higher than that of MPO. This shows that an increase in oxygen free radicals and lipid peroxidation occurs, resulting in changes in the pathophysiology of the small intestinal mucosa, causing mucosal epithelial damage, edema, and activation of inflammatory immune cells.

Ischemia and reperfusion injury are associated with the coordinated activation of a series of cytokines and adhesion molecules^[27-29]. When the intestinal damage releases a large amount of inflammatory cytokines, including rapidly produced TNF- α , inflammatory cells accumulate and intestinal inflammatory damage occurs. IL-10 modulates pro-inflammatory cytokine production and tissue injury following ischemia-reperfusion injury^[30]. A study showed that the exogenous administration of IL-10 reduced the systemic inflammatory response in a rodent model of intestinal reperfusion injury, an effect associated with the inhibition of cytokine production and neutrophil accumulation^[31]. Being anti-inflammatory, the release of IL-10

can modulate pro-inflammatory cytokine production and reperfusion-associated tissue injuries^[32]. This experiment also suggested that IL-10 may inhibit the role of TNF- α .

PHC mainly blocks muscarinic acetylcholine receptors, which shows a wide range of biological activities, including antioxidation, cytoprotective activity, and so on. PHC can inhibit lung vascular leak, inflammation and p38MAPK activation, signalling a potential role in lipopolysaccharide and alleviation of lung injuries by inhibiting apoptosis in lung tissue cells^[9]. Wang *et al.*^[10] found that PHC attenuated the acute lung injury induced by endotoxin involving the nuclear factor- κ B (NF- κ B) pathway. The inhibition of NF- κ B activation in intestinal epithelial cells prevented the increase in systemic TNF- α concentrations after intestinal ischemia and reperfusion^[33]. In this study, PHC post-conditioning significantly reduced the pathological damage to the small intestine with lower limb ischemia-reperfusion injury. Although this damage was inevitable, the small intestinal injury in the group PHC was less severe than that in the group LIR. PHC post-conditioning increased SOD activity and reduced MPO activity, thereby reducing oxygen free radicals to diminish tissue damage. PHC post-conditioning can effectively lower the blood levels of DAO, endotoxin and TNF- α , which disrupt the effects of the organisation. PHC post-conditioning can also promote the production of anti-inflammatory factor IL-10, which inhibits TNF- α and reduces inflammatory cell accumulation in the local organization.

In conclusion, PHC post-conditioning can improve small intestinal mucosal injury induced by lower limb ischemia-reperfusion. It increases SOD activity to scavenge oxygen free radicals, reduces the production of inflammatory cytokine TNF- α , and increases the production of anti-inflammatory factor IL-10. The protective action of PHC post-conditioning on ischemia-reperfusion injury may be controlled by various mechanisms, which should be explored by further investigations.

ACKNOWLEDGMENTS

We would like to thank Dr. Gen Chen from Lanzhou University for providing the laboratory facilities.

COMMENTS

Background

Limb ischemia-reperfusion (LIR) injury can not only lead to damage of limb itself and other organs such as the heart, lungs, brain, small intestines and so on, but also may further trigger a systemic inflammatory response and multiple organ dysfunction mainly induced by dysfunction and structural damage of intestines. Penahyclidine hydrochloride (PHC) is a new anticholinergic drug with antimuscarinic, antinicotinic activities, retained potent central and peripheral anticholinergic activities. Although PHC has protective effects against septic shock and organ injury, the effects of PHC post-conditioning against LIR in the damage of the small intestines remains unknown.

Research frontiers

Damage of the gastrointestinal tract in ischaemia-reperfusion injury is of interest because it functions not only as a target organ but also as a potential effector of the multiple organ dysfunction associated with reperfusion injury. The authors used PHC post-conditioning to protect the damage to the barrier function of the small intestinal mucosa caused by LIR injury, because PHC post-conditioning can significantly inhibit the production of tumor necrosis factor- α (TNF- α), suppress excessive expression of inducible nitric oxide synthase, counteract lipid peroxidation and increase superoxide dismutase (SOD) level.

Innovations and breakthroughs

The authors aimed to investigate the protective effect of PHC post-conditioning in the damage to the barrier dysfunction of the small intestine caused by LIR injury. The results indicate that PHC post-conditioning may reduce the permeability of the small intestines after LIR. Its protection mechanisms may be related to inhibiting oxygen free radicals and inflammatory cytokines for organ damage.

Applications

Although additional studies are necessary to confirm this effect in humans, the authors found the protective effect of PHC in the clinical management of patients with limb ischemia-reperfusion. PHC used in surgical operation might reduce the damage of small intestine from limb ischemia-reperfusion injury.

Terminology

PHC is a new anti-cholinergic drug, selectively blocking M1, M3 receptors and N receptor. Compared with other anticholinergics, the notable advantage of PHC is that it does not accelerate heart rate, and it can improve microcirculation, inhibit lipid peroxidation, attenuate the release of lysosome, and depress microvascular permeability.

Peer review

This manuscript demonstrated that PHC post-conditioning can improve small intestinal mucosal injury induced by lower limb ischemia-reperfusion. It increases SOD activity to scavenge oxygen free radicals, reduces the production of inflammatory cytokine TNF- α , and increases the production of anti-inflammatory factor interleukin-10.

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S- Editor Sun H L- Editor Ma JY E- Editor Lin YP

CYP1A1 Ile462Val polymorphism contributes to colorectal cancer risk: A meta-analysis

Jian-Qiang Jin, Yuan-Yuan Hu, Yu-Ming Niu, Gong-Li Yang, Yu-Yu Wu, Wei-Dong Leng, Ling-Yun Xia

Jian-Qiang Jin, Yu-Yu Wu, Department of Pathology, Affiliated No. 4 Hospital of Soochow University, Wuxi 214062, Jiangsu Province, China

Yuan-Yuan Hu, Yu-Ming Niu, Wei-Dong Leng, Ling-Yun Xia, Department of Stomatology, Affiliated Taihe Hospital of Hubei University of Medicine, Shiyan 442000, Hubei Province, China

Gong-Li Yang, Department of Gastroenterology, Affiliated Taihe Hospital of Hubei University of Medicine, Shiyan 442000, Hubei Province, China

Author contributions: Jin JQ and Hu YY contributed equally to this work; Jin JQ and Hu YY searched and analyzed the literature; Niu YM, Yang GL and Xia LY revised the manuscript; Wu YY and Leng WD provided the idea of this study.

Correspondence to: Yu-Yu Wu, Associate Professor, Department of Pathology, Affiliated No.4 Hospital of Soochow University, 200 Huihe Road, Wuxi 214062, Jiangsu Province, China. jinqianqiang2010@yeah.net

Telephone: +86-510-88683076 Fax: +86-510-85808820

Received: September 4, 2010 Revised: October 16, 2010

Accepted: October 23, 2010

Published online: January 14, 2011

95% CI: 1.16-1.86, $P = 0.002$; dominant model: OR = 1.33, 95% CI: 1.01-1.75, $P = 0.04$; recessive model: OR = 1.49, 95% CI: 1.18-1.88, $P = 0.0009$). Subgroup ethnicity analysis showed that CYP1A1 Ile462Val polymorphism was also significantly related with colorectal cancer risk in Europeans (Ile/Val vs Ile/Ile: OR = 1.22, 95% CI: 1.05-1.42, $P = 0.008$; dominant model: OR = 1.24, 95% CI: 1.07-1.43, $P = 0.004$) and Asians (Val/Val vs Ile/Ile: OR = 1.40, 95% CI: 1.07-1.82, $P = 0.01$; recessive model: OR = 1.46, 95% CI: 1.12-1.89, $P = 0.005$).

CONCLUSION: CYP1A1 Ile462Val may be an increased risk factor for colorectal cancer.

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Key words: CYP1A1; Polymorphism; Colorectal cancer; Meta-analysis

Peer reviewer: Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Jin JQ, Hu YY, Niu YM, Yang GL, Wu YY, Leng WD, Xia LY. CYP1A1 Ile462Val polymorphism contributes to colorectal cancer risk: A meta-analysis. *World J Gastroenterol* 2011; 17(2): 260-266 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/260.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.260>

Abstract

AIM: To study the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk by meta-analysis.

METHODS: A meta-analysis was performed to investigate the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk by reviewing the related studies until September 2010. Data were extracted and analyzed. Crude odds ratio (OR) with 95% confidence interval (CI) was used to assess the strength of relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk.

RESULTS: Thirteen published case-control studies including 5336 cases and 6226 controls were acquired. The pooled OR with 95% CI indicated that CYP1A1 Ile462Val polymorphism was significantly related with colorectal cancer risk (Val/Val vs Ile/Ile: OR = 1.47,

INTRODUCTION

Colorectal cancer, one of the most prevalent cancers worldwide, ranks fourth in frequency in men and third in women^[1]. In recent years, the incidence of colorectal cancer has increased in most countries but its prognosis is still poor. A number of researches have shown that colorectal cancer is possibly related with tobacco and alcohol con-

sumption as well as other environmental sources^[2-4]. It has been shown that inter-individual differences including single nucleotide polymorphism (SNP) may influence human susceptibility to colorectal cancer^[5,6].

Metabolic enzymes including phase I and phase II enzymes are involved in activation and detoxification of xenobiotics, which play an important role in the pathogenesis of colorectal cancer^[7]. Cytochrome P450, including family 1, subfamily A, polypeptide 1 (CYP1A1), is one of the phase I enzymes, metabolizing a large number of endogenous and exogenous substances, such as polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines, and N-nitrosamines^[8,9]. Thus, CYP1A1 plays an important role in human susceptibility to colorectal cancer due to various exogenous factors.

Non-synonymous SNP (rs1048943) leads to amino acid change in exon 7 of CYP1A1 from Ile to Val (nucleotides A-G) at codon 462, which may alter the protein activity and the human susceptibility to colorectal cancer. Since the first study on the relation between colorectal cancer and CYP1A1 Ile462Val polymorphism conducted by Sivaraman *et al*^[10] in 1994, a large number of epidemiological studies on the relation between colorectal cancer and CYP1A1 Ile462Val polymorphism have been conducted, but their conclusions are different or even contradictory. In this study, a meta-analysis of the published case-control studies was performed to assess the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk.

MATERIALS AND METHODS

Search strategy

Studies on the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk were search from PubMed from 1994 to September 2010 using the key words "CYP1A1", "colorectal cancer", "colon cancer", "rectum cancer", and "polymorphism". Related studies were also searched from the references of original papers or reviews. All studies were selected according to the following criteria: only case-control studies on the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk, sufficient published data for estimating odds ratio (OR) with 95% confidence interval (CI), and selection of the largest or most recent studies when several publications reporting the same or overlapping data^[11]. Only the data published in 2007 were selected from two studies by Kiss *et al*^[12,13] who reported overlapping data in Hungarians. Finally, 13 case-control studies including 5336 patients with colorectal cancer and 6226 controls were enrolled in our meta-analysis.

Data extraction

Two investigators independently extracted the following data from the included publications, including name of the first author, publication data, country origin, source of control, racial descent of the study population, genotyping method, number of different genotypes, and Hardy-Weinberg equilibrium (HWE) in controls.

Statistical analysis

Crude OR with 95% CI was computed to assess the strength of relation between CYP1A1 Ile462Val polymorphisms and colorectal cancer risk. Codominant model (Val/Val *vs* Ile/Ile, Ile/Val *vs* Ile/Ile), dominant model [(Val/Val + Ile/Val) *vs* Ile/Ile] and recessive model [Val/Val *vs* (Ile/Val + Ile/Ile)] were evaluated. Subgroup statistical analysis of the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk in Asians and Europeans was performed. Heterogeneity assumption was checked by chi-square based Q-test^[14]. Pooled OR estimation of each study was calculated with the random-effect model (DerSimonian and Laird method) when $P < 0.10$ ^[15]. Otherwise, the fixed-effect model (Mantel-Haenszel method) was selected^[16]. The publication bias was evaluated with the funnel plot and linear regression asymmetry test as previously described^[17]. Statistical analysis was performed using the STATA version 9.2 (Stata Corporation, College Station, TX) and Review Manager (version 4.2, Oxford, England), using two-sided *P*-values.

RESULTS

Study characteristics

Thirteen published case-control studies including 5336 patients with colorectal cancer and 6226 controls met the inclusion criteria for the meta-analysis^[10,13,18-27]. The distribution of studies in different populations is listed in Table 1. The minor allele frequency of Val in controls ranged from 0.030 in Europeans^[22] to 0.255 in Asians^[23]. Genotyping methods included polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR, TaqMan, MassARRAY system, microarray system, and APEX. The distribution of genotypes in controls of all studies was in agreement with HWE except for two studies^[19,20].

Meta-analysis

The results of meta-analysis and heterogeneity test are shown in Table 2. The colorectal cancer risk was significantly higher in individuals carrying the Val/Val genotype than in those carrying the Ile/Ile genotype (OR = 1.47, 95% CI: 1.16-1.86, $P = 0.002$, $P_{\text{heterogeneity}} = 0.44$, Figure 1A). The dominant and recessive models also showed that colorectal cancer risk was significantly related with the CYP1A1 Ile462Val polymorphism [(Val/Val + Ile/Val) *vs* Ile/Ile: OR = 1.33, 95% CI: 1.01-1.75, $P = 0.04$, $P_{\text{heterogeneity}} < 0.01$, Figure 1B; Val/Val *vs* (Ile/Val + Ile/Ile): OR = 1.49, 95% CI: 1.18-1.88, $P = 0.0009$, $P_{\text{heterogeneity}} = 0.77$, Figure 1C] in the total population. Subgroup race analysis showed that the CYP1A1 Ile462Val polymorphism was significantly related with colorectal cancer risk in Europeans [Ile/Val *vs* Ile/Ile: OR = 1.22, 95% CI: 1.05-1.42, $P = 0.008$, $P_{\text{heterogeneity}} = 0.25$; (Val/Val + Ile/Val) *vs* Ile/Ile: OR = 1.24, 95% CI: 1.07-1.43, $P = 0.004$, $P_{\text{heterogeneity}} = 0.24$] and in Asians [Val/Val *vs* Ile/Ile: OR = 1.40, 95% CI: 1.07-1.82, $P = 0.01$, $P_{\text{heterogeneity}} = 0.23$; Val/Val *vs* (Ile/Val + Ile/Ile): OR = 1.46, 95% CI: 1.12-1.89, $P = 0.005$, $P_{\text{heterogeneity}} = 0.24$].

Table 1 Characteristics of case-control studies included in meta-analysis

Author	Country /region	Racial descent	Source of controls	Case (n)	Control (n)	Genotype distribution						Genotyping type	HWE
						Case (n)			Control (n)				
						Ile/Ile	Ile/Val	Val/Val	Ile/Ile	Ile/Val	Val/Val		
Sivaraman <i>et al</i> ^[10] , 1994	USA	Mixed	Population control	43	47	32	9	2	33	14	0	Allele-specific PCR	0.230
Ishibe <i>et al</i> ^[18] , 2000	USA	European	Population control	212	221	176	31	5	186	31	4	PCR-RFLP	0.057
Sachse <i>et al</i> ^[19] , 2002	UK	European	Population control	490	592	415	68	7	539	48	5	TaqMan	< 0.01
Slattery <i>et al</i> ^[20] , 2004	USA	European	Population control	997	1170	910	82	5	1077	86	7	Allele-specific PCR	< 0.01
Slattery <i>et al</i> ^[20] , 2004	USA	European	Population control	794	1010	722	66	6	920	85	5	Allele-specific PCR	0.052
Landi <i>et al</i> ^[21] , 2005	Italy	European	Hospital control	362	323	333	28	1	298	25	0	Microarray and APEX	0.469
Little <i>et al</i> ^[22] , 2006	UK	European	Population control	251	396	235	16	0	372	24	0	PCR-RFLP	0.534
Kiss <i>et al</i> ^[13] , 2007	Hungary	European	Hospital control	500	500	386	110	4	415	83	2	Allele-specific PCR	0.315
Yeh <i>et al</i> ^[23] , 2007	China	Asian	Hospital control	717	729	400	228	89	410	266	53	PCR-RFLP	0.280
Yoshida <i>et al</i> ^[24] , 2007	Japan	Asian	Not report	66	121	34	27	5	79	37	5	PCR-RFLP	0.800
Pereira Serafim <i>et al</i> ^[25] , 2008	Brazil	Mixed	Population control	114	114	14	97	3	81	33	0	PCR-RFLP	0.071
Kobayashi <i>et al</i> ^[26] , 2009	Japan	Asian	Hospital control	105	225	65	32	8	125	87	13	MassARRAY system	0.674
Nisa <i>et al</i> ^[27] , 2010	Japan	Asian	Population control	685	778	418	231	36	461	276	41	PCR-RFLP	0.970

HWE: Hardy-Weinberg equilibrium in control; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; APEX: Arrayed primer extension.

Table 2 Odds ratio and 95% confidence interval of CYP1A1 Ile462Val polymorphism and colorectal cancer risk

Contrast	Racial descent	OR	95% CI	P _h
Val/Val vs Ile/Ile	Total	1.47	1.16-1.86	0.44
	European	1.43	0.83-2.48	0.93
	Asian	1.40	1.07-1.82	0.23
Ile/Val vs Ile/Ile	Total	1.28	0.96-1.72	< 0.01 ¹
	European	1.22	1.05-1.42	0.25
	Asian	0.91	0.79-1.05	0.19
(Val/Val + Ile/Val) vs Ile/Ile	Total	1.33	1.01-1.75	< 0.01 ¹
	European	1.24	1.07-1.43	0.24
	Asian	0.98	0.96-1.13	0.17
Val/Val vs (Ile/Val + Ile/Ile)	Total	1.49	1.18-1.88	0.77
	European	1.39	0.80-2.41	0.94
	Asian	1.46	1.12-1.89	0.24

¹Estimates for random effects. P_h: Test for heterogeneity; CYP1A1: Cytochrome P450, including family 1, subfamily A, polypeptide 1; OR: Odds ratio; CI: Confidence interval.

Publication bias

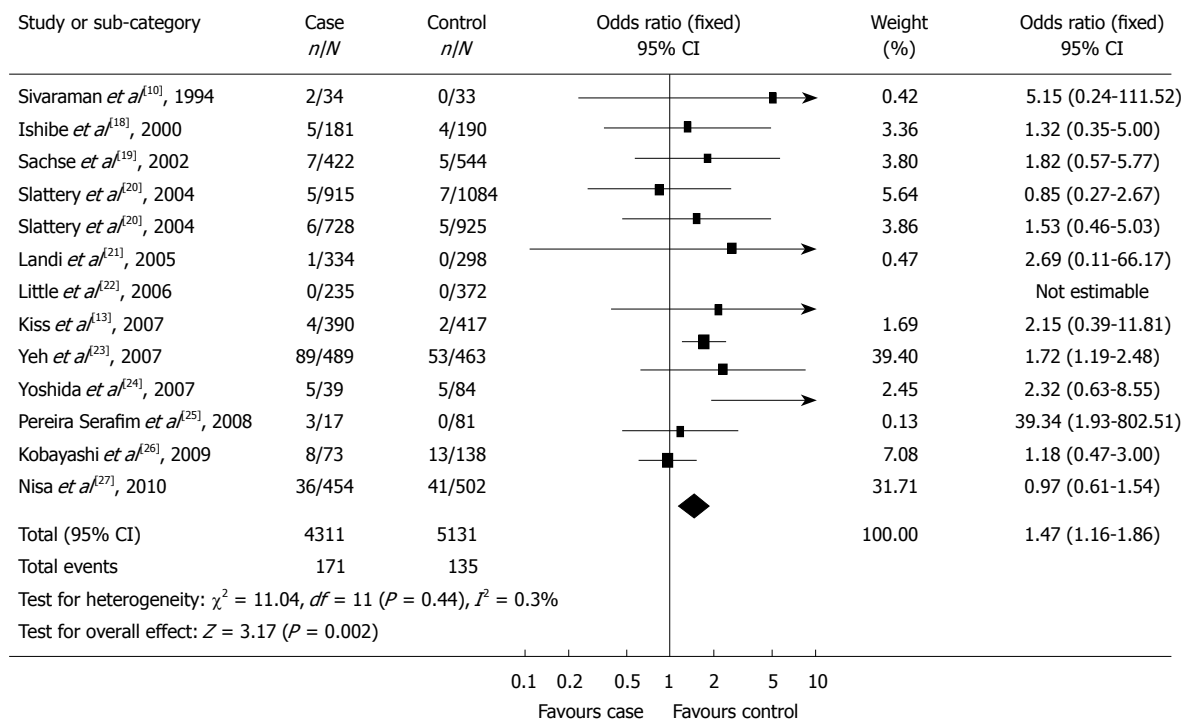
Funnel plot and Egger's test were used to estimate the publication bias of studies. The funnel plots seemed symmetrical in all models (Val/Val vs Ile/Ile: $P = 0.17$, (Val/Val + Ile/Val) vs Ile/Ile: $P = 0.17$, Val/Val vs (Ile/Val + Ile/Ile): $P = 0.39$, Figure 2). No publication bias concerning the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk was detected.

DISCUSSION

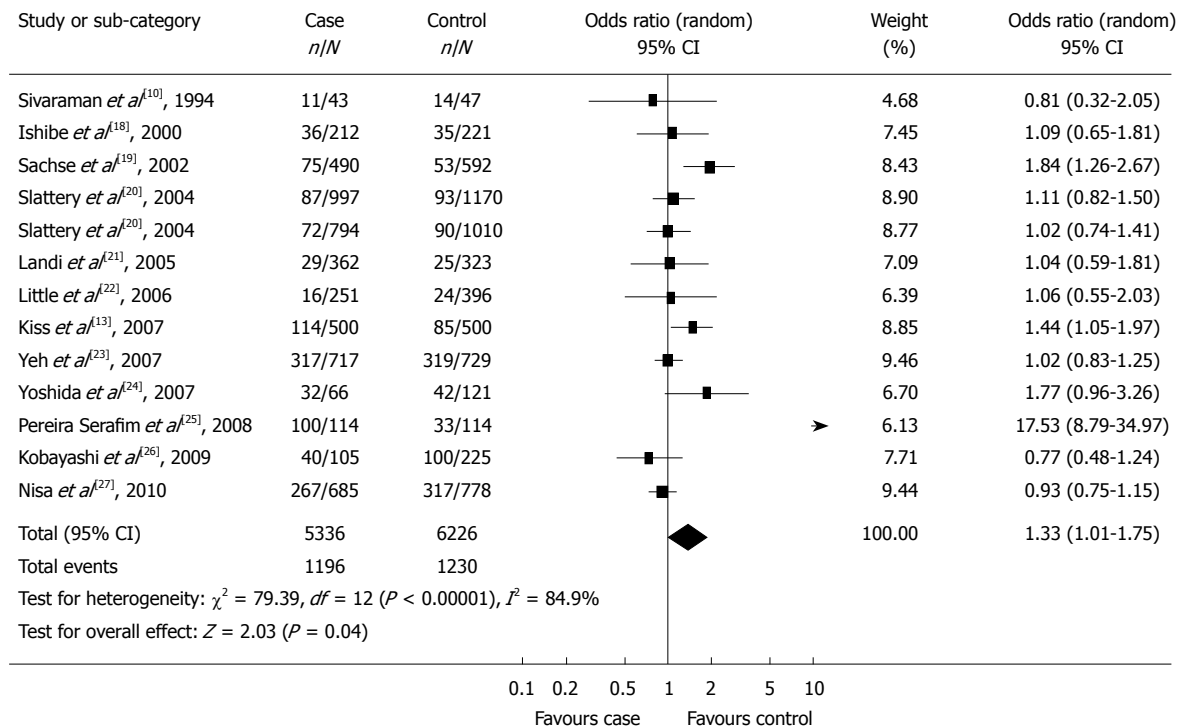
CYP1A1, a phase I enzyme encoded by the CYP1A1 gene, has been mapped to chromosome 15q24.1. The CYP group of enzymes is involved in metabolic activation and detoxification of tobacco-derived carcinogen and other xenobiotics. It has been shown that alcohol intake and cigarette smoking are two important risk factors for colorectal cancer^[20,25,27]. Reactive intermediates can bind to DNA when they are activated, resulting in adducts that cause mutations if not repaired, thereby initiating carcinogenesis^[28]. Meanwhile, valine for isoleucine transition at codon 462 can lead to genetic disequilibrium from adenine to guanine mutation. It has been shown that CYP1A1 Ile462Val polymorphism can increase the activity of enzymes and activation of carcinogens may increase the risk of colorectal cancer^[29,30]. At the same time, CYP1A1 Ile462Val polymorphisms in genotypes show considerable variations in their activities in different diseases and ethnics, as the variant Val, exhibiting an elevated breast cancer risk in Caucasian^[31], is a risk factor for esophageal cancer in Asians but not in Caucasians^[32]. However, Ile/Val polymorphism is not related with the increased risk of prostate cancer^[33].

The first study, published in 1994^[10], did not reveal the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer. To date, no consensus conclusion is

A Review: CYP1A1 Ile462Val polymorphisms and colorectal cancer
 Comparison: Val/Val vs Ile/Ile
 Outcome: Total



B Review: CYP1A1 Ile462Val polymorphisms and colorectal cancer
 Comparison: (Val/Val + Ile/Val) vs Ile/Ile
 Outcome: Total



C

Review: CYP1A1 Ile462Val polymorphisms and colorectal cancer

Comparison: Val/Val vs (Ile/Val + Ile/Ile)

Outcome: Total

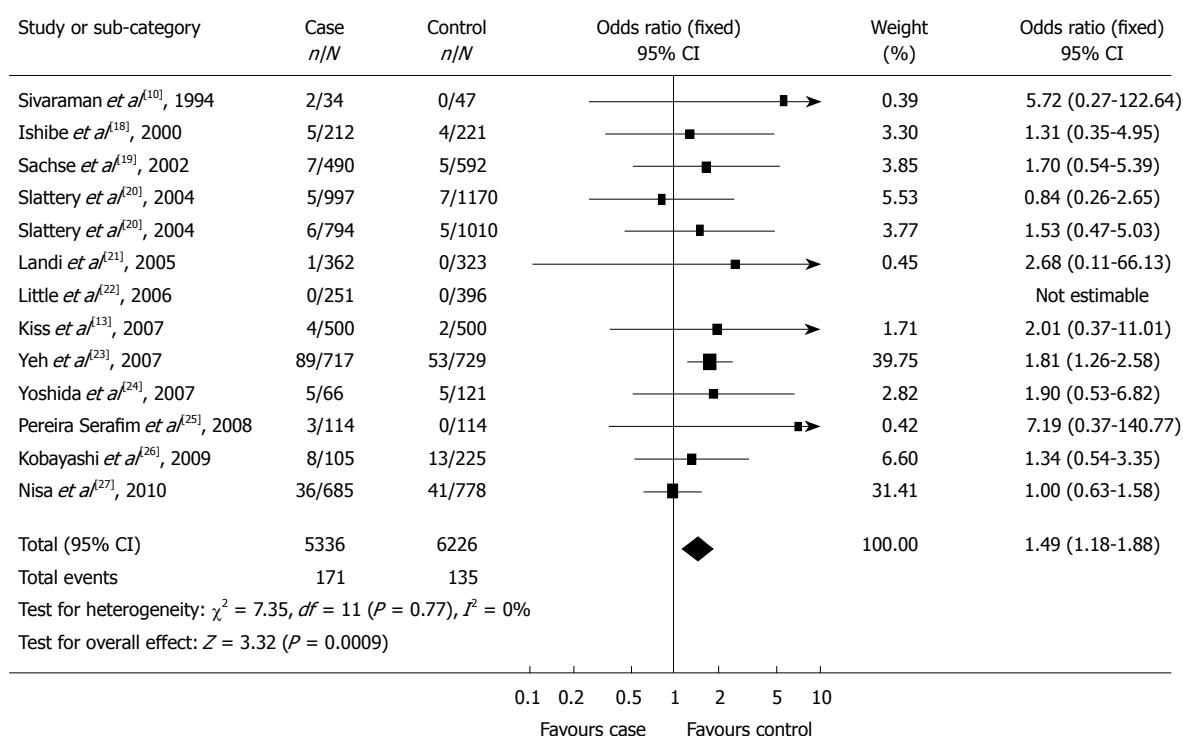


Figure 1 Odds ratio of colorectal cancer associated with CYP1A1 Ile462Val for Val/Val vs Ile/Ile genotypes (A), Val/Val + Ile/Val vs Ile/Ile genotypes (B), and Val/Val vs Ile/Val + Ile/Ile genotypes (C).

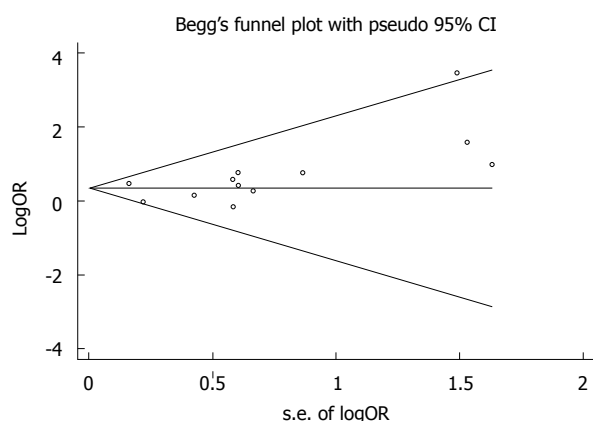


Figure 2 Funnel plot analysis showing publication bias for Val/Val vs Ile/Ile genotypes. Each point represents a separate study for the indicated association.

available on the relation of CYP1A1 Ile462Val polymorphism and colorectal cancer. Pereira Serafim *et al*^[25] demonstrated that the risk of colorectal cancer is 5-fold higher in Brazilians with the Val genotype (OR = 5.14, 95% CI: 3.15-10.80). Sachse *et al*^[19] reported that the risk of colorectal cancer is about 2-fold higher in Europeans with the homozygous Val allele (OR = 2.15, 95% CI: 1.36-3.41). Kiss *et al*^[13] and Yeh *et al*^[23] also reported that the risk of colorectal cancer is similar to those reported by Pereira Serafim *et al*^[25] and Sachse *et al*^[19] in Hungarians and Asians with

the Val genotype. However, other studies from USA and Europe showed that colorectal cancer risk is not significantly related with CYP1A1 Ile462Val polymorphism^[10,18,20-22,24,26,27], but positively related with Val allele and smoking (OR = 2.5, 95% CI: 1.3-4.8) in Europeans^[20]. The present meta-analysis of 13 eligible case-control studies including 5336 cases and 6226 controls showed that CYP1A1 Ile462Val polymorphism could contribute to colorectal cancer risk. The stratified analysis according to the ethnicity revealed that CYP1A1 Ile462Val polymorphism was positively related with colorectal cancer risk both in Asians and in Europeans. However, no report is available on the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk in Africans. On the other hand, gender factor may change the risk of colorectal cancer sometimes. It was reported that the colorectal cancer risk is 3.1-fold higher in Chinese women with CYP1A1 Val/Val and XRCC3 Thr/Thr genotypes than in those with CYP1A1 Ile and XRCC3 Met alleles^[23], suggesting that CYP1A1 Ile462Val polymorphism may be an important risk factor for colorectal cancer.

Heterogeneity is another problem found in our meta-analysis. A significant heterogeneity was observed in Ile/Val vs Ile/Ile and (Val/Val + Ile/Val) vs Ile/Ile. However, subgroup ethnicity analysis showed that the heterogeneity was removed apparently, indicating that the genetic background and environment are different in different ethnicities.

Several limitations in our meta-analysis need to be addressed. First, the results were obtained based on the unadjusted estimates and lacked of original data about the eligible studies, thus limiting the evaluation of effects of gene-gene and gene-environment interactions on the pathogenesis of colorectal cancer. Second, other single nucleotide polymorphisms of CYP1A1 were identified, but no linkage disequilibrium and haplotype analysis of these polymorphisms was performed. Third, the real relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk might have been influenced since the sample size was relatively small in this analysis, thus a further analysis of the relation between CYP1A1 polymorphism and colorectal cancer should be performed.

In conclusion, CYP1A1 Ile462Val polymorphism may contribute to colorectal cancer risk. Further study is needed with a large-scale case-control sample to validate the identified risk in our current meta-analysis, and potential gene-gene and gene-environment interactions should be taken into account when the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk is further studied.

COMMENTS

Background

Colorectal cancer is one of the most prevalent malignances worldwide. CYP1A1 is one of the phase I enzymes. Ile to Val transition has been supposed as a risk factor for colorectal cancer. A large number of studies on the association between CYP1A1 and colorectal cancer risk have been conducted, but their conclusions are different or even contradictory.

Research frontiers

Many studies indicate that CYP1A1 Ile462Val polymorphism plays an important role in pathogenesis of esophageal cancer in Asians and breast cancer in Caucasians. However, the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk remains controversial and no meta-analysis has been conducted.

Innovations and breakthroughs

This meta-analysis systemically assessed the relation between CYP1A1 Ile462Val polymorphism and colorectal cancer risk, showing that the Val allele may be a risk factor for colorectal cancer in both Europeans and Asians.

Applications

The results of meta-analysis in this study show that the CYP1A1 Ile462Val polymorphism contributes the human susceptibility to colorectal cancer in both Europeans and Asians, which may help us to make early prevention and treatment of colorectal cancer.

Peer review

This is an interesting meta-analysis of the association between CYP1A1 Ile462Val polymorphism and colorectal cancer risk. The authors carefully reviewed the literature and collected the original data. The methods they used in meta-analysis are proper.

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S- Editor Sun H L- Editor Wang XL E- Editor Lin YP

Splenic infarction associated with sorafenib use in a hepatocellular carcinoma patient

Sang Ock Kim, Sang Young Han, Yang Hyun Baek, Sung Wook Lee, Ji Sun Han, Byung Geun Kim, Jin Han Cho, Kyung Jin Nam

Sang Ock Kim, Sang Young Han, Yang Hyun Baek, Sung Wook Lee, Ji Sun Han, Byung Geun Kim, Department of Gastroenterology and Hepatology, Dong-A University Hospital, Dongdaesin-dong, Seo-gu, Busan 602-103, South Korea
 Sang Young Han, Liver Center, Dong-A University College of Medicine, 3-ga, Dongdaesing-dong, Seo-gu, Busan 602-103, South Korea

Jin Han Cho, Kyung Jin Nam, Department of Radiology, Dong-A University Hospital, Dongdaesin-dong, Seo-gu, Busan 602-103, South Korea

Author contributions: Kim SO and Han SY contributed equally to this work; Kim SO, Han SY, Baek YH and Lee SW contributed to drafting the report; Cho JH and Nam KJ interpreted CT images; Kim SO, Han SY and Han JS wrote and revised the paper; all authors contributed to analyzing the patient's data.

Supported by Dong-A University fund

Correspondence to: Sang Young Han, MD, PhD, Professor, Liver Center, Dong-A University College of Medicine, 3-ga, Dongdaesing-dong, Seo-gu, Busan 602-103, South Korea. syhan@dau.ac.kr

Telephone: +82-51-2402861 Fax: +82-51-2402087

Received: August 15, 2010 Revised: September 26, 2010

Accepted: October 3, 2010

Published online: January 14, 2011

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Key words: Hepatocellular carcinoma; Sorafenib; Tyrosine kinase inhibitor; Adverse effects; Splenic infarction

Peer reviewer: Yuichiro Eguchi, MD, Department of Internal Medicine, Saga Medical School, 5-1-1 Nabeshima, Saga, 849-8501, Japan

Kim SO, Han SY, Baek YH, Lee SW, Han JS, Kim BG, Cho JH, Nam KJ. Splenic infarction associated with sorafenib use in a hepatocellular carcinoma patient. *World J Gastroenterol* 2011; 17(2): 267-270 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/267.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.267>

INTRODUCTION

Sorafenib (Nexavar[®], Bayer) is an oral vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI) that has been approved by the United States Food and Drug Administration for the treatment of hepatocellular carcinoma (HCC). Many studies regarding the adverse effects of this drug have been reported, which include hand-foot skin reaction, diarrhea, anorexia, asthenia, alopecia, weight loss, hypertension and arterial thromboembolism. The rate of arterial thromboembolic events following the use of sorafenib has been reported to be about 3.8%^[1]. However, most cases have been cardiac or cerebrovascular events. There have been no clinical reports of splenic infarction associated with sorafenib, to date. We report a patient who developed a spontaneous infarction of the spleen after treatment with sorafenib used for the treatment of HCC.

CASE REPORT

A 69-year-old female with a history of HCC presented to

Abstract

Sorafenib, a multitargeted tyrosine kinase inhibitor, has been shown to improve survival in patients with advanced hepatocellular carcinoma (HCC). As the clinical use of sorafenib increases, many adverse effects have been reported, such as hand-foot skin reaction, diarrhea, anorexia, asthenia, alopecia, weight loss, hypertension and arterial thromboembolism. However, there are no prior reports of splenic infarction as an adverse effect of sorafenib. Here, a case of splenic infarction in a patient with HCC who was treated with sorafenib is reported. The patient had no other predisposing factors to explain the splenic infarction except for the administration of sorafenib. The splenic infarction improved after sorafenib was discontinued; however, the HCC progressed.



Figure 1 Contrast-enhanced abdominal computed tomography. A wedge-shaped hypodense lesion in the spleen is consistent with splenic infarction (arrow). Hepatic artery catheter is seen in hepatic artery as artifact.

the gastrointestinal department with left upper quadrant (LUQ) discomfort. The patient was previously treated with percutaneous ethanol injection therapy (PEIT) for primary HCC in 1999, and subsequently transarterial chemoembolization (TACE) for local recurrent HCC in 2008 and 2009. Recently, two cycles of hepatic arterial infusion (HAI) chemotherapy with FUDR (0.3 mg/kg) and sorafenib (400 mg *po bid*) were provided for intrahepatic metastases. Initially, this combination chemotherapy was started the day after an HAI catheter was inserted. During these treatments, intermittent abdominal pain and itching of both palms were observed. At that time, endoscopic findings showed acute gastromucosal lesions; therefore, treatment with sucralfate and a proton pump inhibitor was started. The abdominal pain improved. However, after several days other characteristics of abdominal discomfort returned. The patient reported a sudden onset of dull, left-sided upper quadrant abdominal pain. The patient stopped her medications including sorafenib and the symptoms gradually improved.

A physical examination was unremarkable except for mild LUQ tenderness. The laboratory investigations including complete blood counts, electrolytes, liver and kidney function tests were all within normal limits. EKG, chest X-ray and cardiac enzymes were also within normal range. An abdominal computed tomography (CT) scan showed a wedge-shaped opacity in the spleen suggestive of an acute infarction (Figure 1). Further evaluations were performed to determine the possible cause of the spontaneous splenic infarction such as infectious endocarditis, atrial fibrillation, hematologic disorders, autoimmune disease or other possible infectious diseases. Additional laboratory tests of the peripheral blood smear, i.e. protein C and S, antithrombin III, lipid profile, homocysteine and autoantibodies including antinuclear antibody, antineutrophil cytoplasmic antibodies (ANCA), rheumatoid factor, antiphospholipid antibodies, and complement levels, were not specific. Tumor markers of α -fetoprotein (316.18 ng/mL) and PIVKA-II (393 mAU/mL) were elevated. Although she had underlying liver cirrhosis, the absence of encephalopathy and ascites, and the normal prothrombin



Figure 2 Follow up abdominal computed tomography (1 mo). New hypodense lesion has developed in upper level of the spleen (arrow).

time and albumin resulted in a Child-Pugh A functional class. The 2D echocardiogram and venous Doppler were unremarkable.

During the evaluation, the patient continued HAI chemotherapy with FUDR according to schedule without sorafenib. At discharge, sorafenib was administered again, because the symptoms had gradually improved; however, a few days later, the sorafenib was discontinued due to a second attack of LUQ pain. One month later, the follow up CT scan showed another wedge-shaped lesion in the spleen without aggravation of the HCC (Figure 2). The follow up laboratory data showed no significant changes, including for platelet count. Intra-arterial chemotherapy without sorafenib was provided. One month later, a CT scan showed diminished infarction size in the spleen; however, the HCC progressed (Figure 3).

DISCUSSION

The vascular endothelial growth factors (VEGFs) play a critical role in angiogenesis and stimulate endothelial cell proliferation, migration and tube formation^[2]. Sorafenib was developed because of its anti-angiogenic action in inhibiting tyrosine kinase of the VEGF receptor; it has been shown to improve the clinical outcome of patients with HCC in a large phase III trial^[3]. The side effects associated with this agent are mostly mild to moderate. Rash, exfoliative dermatitis, hand-foot skin reaction, diarrhea and fatigue are the most common adverse events, occurring in 33%-38% of patients^[4]. However, unexpected toxicities have also been reported, including thrombotic events^[5]. Most of the adverse events are downstream effects of the suppression of VEGF signaling in endothelial cells of normal organs. The thrombotic events are also associated with anti-VEGF effects. VEGF not only stimulates endothelial cell proliferation, but also promotes endothelial cell survival and helps maintain vascular integrity. Inhibition of VEGF could thereby diminish the regenerative capacity of endothelial cells and cause abnormalities that expose pro-coagulant phospholipids of the luminal plasma membrane or underlying matrix, leading to thrombosis^[6]. In addition, VEGF increases production of NO and

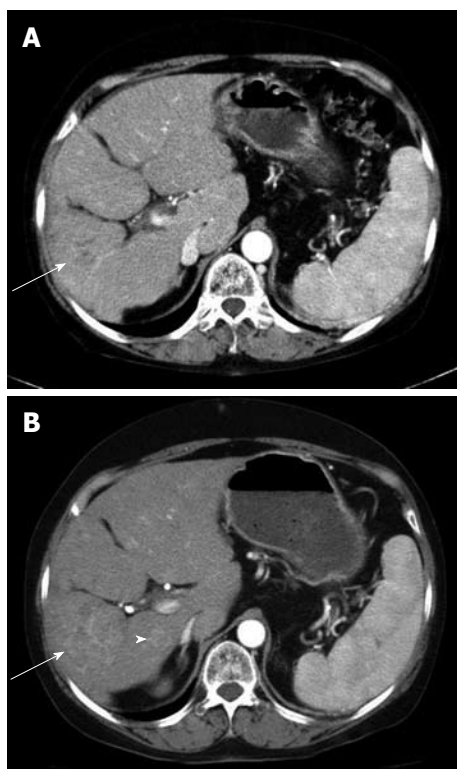


Figure 3 Contrast-enhanced computed tomography scan in arterial phase at the time of diagnosis of initial splenic infarction (A) and 2 mo later (B). A: When splenic infarction was diagnosed, an approximately 4 cm x 4 cm sized hepatocellular carcinoma (HCC) (arrow) was shown in S6; B: Two months later, the size of HCC was enlarged to 5 cm x 5 cm (arrow) and an intrahepatic metastatic nodule was also seen (arrowhead) beside main mass.

prostacyclin, and inhibits proliferation of vascular smooth muscle cells. Reduction in NO and prostacyclin, after inhibition of VEGF signaling, may predispose to thromboembolic events^[7]. Moreover, VEGF inhibition may also increase the risk of thrombosis by increasing the hematocrit and blood viscosity *via* overproduction of erythropoietin^[8]. Other concurrent pathological findings in a patient might also play a central role. There are some reports of anti-VEGF agent-related thromboembolic events such as myocardial infarction and cerebrovascular accidents. To date, thrombotic risks with intravenous bevacizumab have been studied extensively, in contrast to oral VEGFR TKIs where data on arterial events have not yet been evaluated. Recently, Choueiri *et al.*^[9] reported the relative risk of arterial thrombotic events with bevacizumab and a VEGFR TKI (sorafenib or sunitinib); a two-fold and three-fold increase was reported, respectively. This risk did not depend on the type of malignancy.

In a meta-analysis of bevacizumab-treated patients, the major underlying risk factors of arterial thrombotic events were advanced age, hypertension, diabetes, and a prior history of thrombotic events. The treatment duration for the incidence of thrombotic events was within the first 3 mo of therapy; however, the data regarding the occurrence of the events, during the course of the trial, were frequently not reported^[10]. In the case reported here, the patient was of advanced age but had no predisposing factors. The

thromboembolic event was a splenic infarction with the clinical symptom of LUQ pain. The symptoms developed 2 mo after administration of sorafenib. Most prior reports of TKI-associated arterial thrombotic events have shown myocardial infarctions and/or cerebrovascular accidents; a splenic artery infarction has not been previously reported. Possible causes of splenic infarction were investigated. The physical examination, laboratory findings, imaging studies and other drug history did not suggest any other possible causes except for the sorafenib administration. Although the patient had a history of other therapeutic procedures such as PEIT, TACE, and HAI chemotherapy, the last TACE was administered 16 mo previously and the HAI chemotherapy was continued without LUQ pain. The HAI catheter was inserted at the correct hepatic arterial level, and the distal end was connected to the port at the right femoral artery. Anatomically this should not contribute to occlusion of the splenic artery. Furthermore, the patient tolerated HAI chemotherapy without the sorafenib. The time interval from the initial administration of sorafenib to onset of LUQ pain, and the fact that discontinuing sorafenib was associated with resolution of symptoms, suggested that the sorafenib was the cause of the acute splenic infarction. Two months later, the follow up CT scan showed improvement of the splenic lesion; however, the HCC had progressed.

In this case, discontinuing sorafenib resolved the splenic infarction, however, at the expense of the HCC. Perhaps if the sorafenib had been continued with other pain medications and anticoagulants, the outcome would have been better. The use of low-dose aspirin for the prophylaxis of arterial thromboembolic events in high-risk patients is supported by an extensive body of literature^[11] and is recommended as the standard of care^[12]. Therefore, if a patient has a good response to sorafenib, it might be better to continue the sorafenib with the addition of low-dose aspirin to prevent events such as a splenic infarction, which is not life threatening.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

Potential efficacy of ginger as a natural supplement for nonalcoholic fatty liver disease

Amirhossein Sahebkar

Amirhossein Sahebkar, Biotechnology Research Center and School of Pharmacy, Mashhad University of Medical Sciences, PO Box 91775-1365, Mashhad, Iran

Amirhossein Sahebkar, Cardiovascular Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, PO Box 91775-1365, Mashhad, Iran

Amirhossein Sahebkar, Research Center of Chemical Injuries, Baqiyatallah University of Medical Sciences, PO Box 19945-581, Tehran, Iran

Author contributions: Sahebkar A contributed all to the paper. Correspondence to: Amirhossein Sahebkar, PhD, Biotechnology Research Center and School of Pharmacy, Mashhad University of Medical Sciences, PO Box 91775-1365, Mashhad, Iran. sahebkarah811@mums.ac.ir

Telephone: +98-511-8823255 Fax: +98-511-8823251

Received: August 31, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: January 14, 2011

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Key words: Nonalcoholic fatty liver disease; Ginger; Insulin resistance; Oxidative stress; Inflammation

Peer reviewer: Furqaan Ahmed, MD, South City Hospital St-1, Block #, Shahrah-e-Firdousi, Clifton, Karachi 75000, Pakistan

Sahebkar A. Potential efficacy of ginger as a natural supplement for nonalcoholic fatty liver disease. *World J Gastroenterol* 2011; 17(2): 271-272 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i2/271.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i2.271>

Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases and its prevalence is likely to reach epidemic proportions. According to the "two-stage hypothesis" proposed for the pathophysiology of NAFLD, insulin resistance, oxidative stress and pro-inflammatory cytokines are among the key promoters of the disease. Here, ginger has been hypothesized to prevent NAFLD or blunt its progression *via* several mechanisms, such as sensitizing insulin effects, activating peroxisome proliferator-activated receptor γ which induces adiponectin and down-regulates pro-inflammatory cytokines, changing the balance between adiponectin and tumor necrosis factor- α in favor of adiponectin, promoting considerable antioxidant effects and antidyslipidemic properties, and reducing hepatic triglyceride content which can prevent steatosis. The aforementioned mechanisms imply that ginger possesses interesting potentials for serving as a natural supplement for the prevention and treatment of NAFLD. Therefore, conducting trials to explore its benefits in clinical practice is greatly recommended.

TO THE EDITOR

Nonalcoholic fatty liver disease (NAFLD), one of the most common liver diseases, is growing fast into an epidemic problem in Western countries. NAFLD, covering a wide spectrum of hepatic conditions from simple steatosis to nonalcoholic steatohepatitis (NASH), may lead to more severe disorders such as cirrhosis and hepatocellular carcinoma. The pathophysiology of NAFLD has been conceptualized to be a two-stage process, consisting of fat accumulation in hepatocytes and consequent hepatic steatosis in the first stage, and hepatic injury or NASH in the second stage. Insulin resistance plays a central role in both stages of NAFLD pathogenesis while oxidative stress and pro-inflammatory cytokines [in particular tumor necrosis factor (TNF)- α] are among the important promoters of the second stage.

Ginger (underground rhizomes of *Zingiber officinale*) is a famous spice which has been used for centuries as a medicinal plant in different traditional medicine systems. The therapeutic effects of ginger have also been validated by modern research, rendering it as a potential medication for a variety of disorders. Gingerols and shogaols represent the predominant pungent constituents of ginger responsible for many of its medicinal properties. Herewith, it is hypoth-

esized that ginger might be applied as a potential natural medicine that could counteract the biochemical abnormalities involving the pathogenesis of NAFLD as follows.

Insulin resistance is a common feature in patients with NAFLD and NASH. Regarding the key role of insulin resistance and resulting hyperinsulinemia in hepatic triglyceride accumulation, insulin sensitizing is an important therapeutic mechanism against NAFLD. A previous preliminary study reported that the insulin sensitivity to adipocytes could be improved using ginger, with gingerol as its active component for this effect^[1].

It is considered that peroxisome proliferator-activated receptors α (PPAR α) and γ (PPAR γ) can influence hepatic triglyceride accumulation and thereby pathogenesis of NAFLD. It was reported that PPAR γ can improve insulin sensitivity and decrease the flux of fatty acids into liver^[2]. Moreover, PPAR γ activation is associated with other beneficial effects such as induction of adiponectin expression and secretion, and down-regulation of the expression of pro-inflammatory cytokines including TNF- α . Hence, PPAR γ agonists have been hypothesized to be of therapeutic importance in NAFLD^[2]. Noteworthy, ginger's 6-shogaol has been reported to be a significant agonist of PPAR γ in adipocytes^[3].

Previous studies showed that both TNF- α and adiponectin play an important role in the development of hepatic steatosis and in its progression to NASH^[4,5]. These cytokines have conflicting activities and antagonize each other's production and effects. While adiponectin has several protective effects against NAFLD such as improvement of insulin resistance and reduction of fat accumulation in hepatocytes. TNF- α antagonizes these effects and promotes hepatic steatosis. Therefore, down-regulation of TNF- α may be a potential approach to the treatment of NAFLD and amelioration of liver damage. Ginger is deemed to be effective for this purpose as several previous studies have shown that ginger extract and its bioactive constituents can decrease the TNF- α expression^[6,7]. Moreover, it has been found that both 6-gingerol and 6-shogaol can significantly inhibit TNF- α mediated down-regulation of adiponectin expression^[5].

Along with cytokines, oxidative stress also plays an important role in the second stage of NAFLD, mediating the progression of hepatic steatosis to NASH. Therefore, antioxidants such as vitamin E have gained therapeutic application in the treatment of NAFLD. In addition to the other benefits mentioned, ginger possesses considerable antioxidant properties including radical scavenging activity and inhibitory effect on lipid peroxidation, which can be ascribed to the presence of polyphenols such as gingerol and curcumin in this plant^[8]. Besides, ginger protects the liver against hepatotoxic agents by enhancing the hepatic antioxidant activity^[9,10].

Triglyceride accumulation in hepatocytes is the hallmark of NAFLD. There is evidence that ginger can reduce hepatic triglyceride content, induce LDL receptor and down-regulate HMG-COA expression in the liver^[11].

Moreover, ginger extract has been reported to exert its anti-hyperlipidemic effects by decreasing serum levels of total cholesterol, LDL-C and triglycerides and increasing HDL-C^[12]. Thus, supplementation with ginger might be effective in the prevention of steatosis and control of dyslipidemia which is a risk factor for NAFLD^[10].

In spite of the aforementioned interesting potentials of ginger, the efficacy of this wonderful spice has not been sufficiently investigated in relation to NAFLD. Given the long reputation of ginger as a medicinal herb and dietary spice with a good safety, tolerability and low price, future clinical trials are warranted to identify its efficacy as a multifunctional adjunctive therapy for NAFLD.

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S- Editor Tian L L- Editor Wang XL E- Editor Zheng XM



ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Kyoichi Adachi, MD, Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

Matilde Bustos, MD, PhD, Hepatology and Gene Therapy Area, CIMA (Center for Applied Medical Research), Avda Pio XII, 55, 31008 Pamplona, Spain

Jen-Hwey Chiu, MD, PhD, Professor, Division of General Surgery, Department of Surgery, Taipei-Veterans General Hospital, Taipei 112, Taiwan, China

Ana J Coito, Associate Professor of Surgery, Department of Surgery, The Dumont, UCLA Transplant Center, 77-120 CHS, Box 957054, Los Angeles, CA 90095-7054, United States

Giamila Fantuzzi, PhD, Associate Professor, Department of Kinesiology and Nutrition, University of Illinois at Chicago, 1919 W Taylor Street MC 517, Chicago, IL 60613, United States

Roberto de Franchis, Professor of Medicine (Gastroenterology), Department of Medical Sciences, University of Milan, Head, Gastroenterology 3 Unit, IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milano, Italy

Paolo Del Poggio, Dr., Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Toru Hiyama, MD, PhD, Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima 739-8521, Japan

Udayakumar Navaneethan, MD, Department of Internal Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267, United States

William R Parker, Dr., PhD, Assistant Professor, Department of Surgery, Duke University Medical Center, Box 2605, Durham, NC 27710, United States

Giuseppe Sica, MD, PhD, Department of Surgery, University Hospital Tor Vergata, Viale Oxford 81, 00133 Rome, Italy

Parviz M Pour, Professor of Pathology, Member of UNMC/Eppley Cancer Center, 98168 Nebraska Med Center, Omaha, NE 68198-680, United States

Richard A Rippe, Dr., Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Damian Casadesu Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Paul Sharp, Dr., BSc (Hons), PhD, Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London, SE1 9NH, United Kingdom

Jon Arne Søreide, MD, PhD, FACS, Professor, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway

Subbaramiah Sridhar, MB, BS, MPH, FRCP (Edin), FRCP (Glasg), FRCP (Lond), FRSS (Lond), FRSC (Medicine and gastroenterology), FACP, FACC, FASGE, AGAF, Section of Gastroenterology, BBR 2544, Medical College of Georgia, 15th Street, Augusta, GA 30912, United States

Qin Su, Professor, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Shoichiro Sumi, MD, PhD, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan

Yoshihisa Takahashi, MD, Department of Pathology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan

Lucia Ricci Vitiani, Dr., Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, Rome 00161, Italy



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 04-05, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 07-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYs, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

CSSN

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by

Baishideng Publishing Group Co., Limited

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67 , $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

Instructions to authors

uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:....; B:....; C:....; D:....; E:....; F:....; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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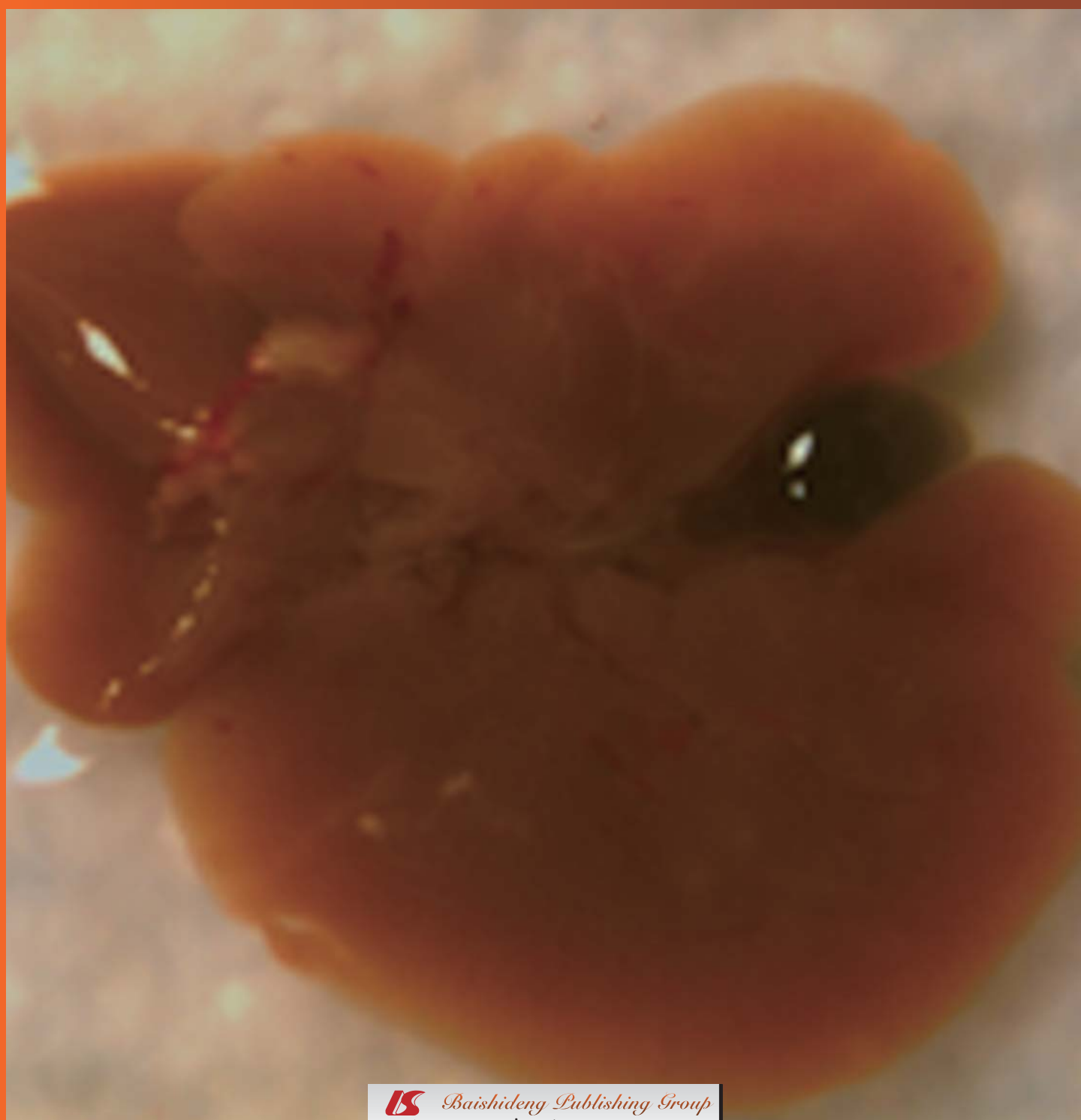
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World J Gastroenterol 2011; 17(3): 300-312
<http://www.wjgnet.com/1007-9327/full/v17/i3/300.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
January 21, 2011

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

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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm. If you do not have web access please contact the editorial office.

ONLINE SUBMISSION
<http://www.wjgnet.com/1007-9327/office>

Gastric electrical stimulation for gastroparesis: A goal greatly pursued, but not yet attained

Mauro Bortolotti

Mauro Bortolotti, Department of Internal Medicine and Gastroenterology, University of Bologna, Via Massarenti 48, Bologna 40138, Italy

Author contributions: Bortolotti M solely contributed to this paper.

Correspondence to: Mauro Bortolotti, MD, Professor, Department of Internal Medicine and Gastroenterology, University of Bologna, Via Massarenti 48, Bologna 40138, Italy. bormau@tin.it

Telephone: +39-51-307901 Fax: +39-51-307901

Received: June 6, 2010 Revised: September 6, 2010

Accepted: September 13, 2010

Published online: January 21, 2011

Abstract

The lack of an effective medical treatment for gastroparesis has pushed the research of new techniques of gastric electrical stimulation (GES) for nearly half a century of experimentation with a large variety of electrical stimuli delivered to the gastric wall of animals and patients with gastroparesis. Three principal methods are currently available: gastric low-frequency/high-energy GES with long pulse stimulation, high-frequency/low-energy GES with short pulse stimulation and neural sequential GES. The first method aims to reset a regular slow wave rhythm, but has variable effects on contractions and requires devices with large and heavy batteries unsuitable for implantation. High-frequency/low-energy GES, although inadequate to restore a normal gastric electro-mechanical activity, improves dyspeptic symptoms, such as nausea and vomiting, giving patients a better quality of life together with a more satisfactory nutritional status and is suitable for implantation. Unfortunately, the numerous clinical studies using this type of GES, with the exception of two, were not controlled and there is a need for definitive verification of the effectiveness of this technique to justify the cost and the risks of this procedure. The last method, which is neural sequential GES, consists of a microprocessor-controlled sequential activation of a series of annular electrodes along the distal two thirds of the stomach and is able to

induce propagated contractions causing forceful emptying of the gastric content. The latter method is the most promising, but has been used only in animals and needs to be tested in patients with gastroparesis before it is regarded as a solution for this disease.

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Key words: Gastric electrical stimulation; Gastric emptying; Gastric motility; Gastric myoelectric activity; Gastroparesis; Prokinetic drugs

Peer reviewer: Dr. Chikashi Shibata, Department of Surgery, Tohoku University, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan

Bortolotti M. Gastric electrical stimulation for gastroparesis: A goal greatly pursued, but not yet attained. *World J Gastroenterol* 2011; 17(3): 273-282 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/273.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.273>

INTRODUCTION

Gastroparesis is a chronic disorder characterized by a severe functional delay in gastric emptying (GE), which not only causes distressing symptoms, such as upper abdominal discomfort or pain, a sense of epigastric fullness after meals, early satiety, nausea, and vomiting, but may also lead to nutritional depletion requiring enteral or parenteral nutrition. The treatment of this condition represents a clinical challenge and is one of the most disappointing areas in medicine. The current available medical therapy is represented, by dietary modifications and administration of prokinetic agents, such as domperidone, metoclopramide and derivatives, cholinomimetics, such as neostigmine, macrolides, such as clarithromycin, erythromycin, and "motilides" and, more recently, the 5HT₄ selective agonists, such as prucalopride^[1], while cisapride has been withdrawn from most markets due to its dangerous side effects which

affect the heart. However, some patients with gastroparesis can not undergo chronic treatment with prokinetic drugs, due to the occurrence of severe side-effects, such as nervous disturbances caused by metoclopramide, hyperprolactinemia due to domperidone, and antibiotic activity with erythromycin. In addition, tachyphylaxis may occur with some drugs, such as domperidone and erythromycin, and refractoriness to prokinetic agents is observed in a significant number of patients. The intrapyloric endoscopic injection of botulinum toxin seems to relieve the symptoms of gastroparesis^[2,3]. However, a preliminary controlled double-blind study apparently failed to confirm previous results with regard to symptoms, although showed a significant improvement in solid GE^[4]. If all of these treatments are unsuccessful and nutrition “per os” is insufficient, patients must undergo enteral nutrition. Surgical jejunostomy performed by laparoscopy and percutaneous endoscopic jejunostomy are indicated for patients with refractory gastroparesis unable to maintain sufficient nutrition “per os”, in order to provide nutrients, fluids and medications^[5], on condition that there are no motor disturbances of the intestine, such as pseudo-obstruction. If enteral nutrition is not possible, the patient is usually referred to the surgeon for partial or even subtotal gastrectomy with Roux en Y reconstruction^[6]. If gastric resection is risky, or refused by the patient, or does not resolve the nutritional problems, the patient must undergo permanent enteral or parenteral nutrition. No alternative to surgery and chronic artificial nutrition was imaginable until 1963, when investigators hoped that gastric electrical stimulation was a new way to cure ileus^[7]. Following cardiac stimulation with a pacemaker, these authors thought that it would be sufficient to deliver an electrical stimulus to the gut wall to restore an efficient contraction. However, this simple idea turned out to be more difficult than expected and remained a dream for decades, because the electrical activity, that governs the motor function of the stomach, is much more complex than that of the heart.

The present paper presents a critical overview of various methods of gastric electrical stimulation used in animals and humans aiming to restore efficient gastric motor function and improve dyspeptic symptoms in gastroparesis.

GASTRIC MYOELECTRICAL ACTIVITY

To understand the mechanism of gastric electrical stimulation it is necessary to know what gastric myoelectrical activity is. This consists of an uninterrupted sequence of electrical potential variations called “slow waves”, that spring out continuously, at a frequency of about 3/min in man, from a small zone of the proximal gastric corpus near the great curvature (pacemaker area), and propagate distally along the gastric wall toward the pylorus in the form of incomplete depolarization-repolarization annular bands. When the depolarization reaches a determined threshold, the smooth muscle cell membrane depolarizes completely with consequent contraction and another kind of electrical activity, called “spike potentials”, appears superim-

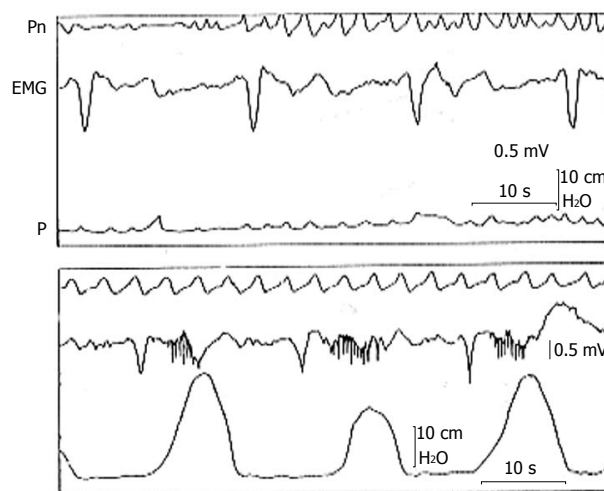


Figure 1 Gastric myoelectric and pressure activities recorded with an intraluminal electromyographic and manometric technique from the gastric antrum of a healthy subject during a period of absence of pressure waves (top tracing) and a period of contractile activity (bottom tracing). Note the slow waves of normal morphology and the frequency of 3 cycles/min that in correspondence with the contractions are followed by bursts of spikes^[6]. EMG: Gastric myoelectric; Pn: Pneumogram; P: Pressure.

posed on the second part of the slow wave^[8,9] (Figure 1). The origin of slow waves lies in the interstitial cells of Cajal type I (ICC), a series of highly ramified cells located between the longitudinal and circular muscle coats, making close contacts with the Auerbach plexus and the smooth muscle cells of both layers mediating the cholinergic excitatory and nitrergic inhibitory inputs^[10]. These cells, also called myoneural, have the property of automatically generating and transmitting to smooth muscle cells, the slow waves with an intrinsic frequency decreasing caudally^[10,11]. The absence of ICC is associated to the absence of coordinated slow waves^[12] and depletion of these cells in pathologic conditions, such as diabetic gastroparesis, may interrupt the propagation of both spontaneous and artificially paced slow waves^[13].

Hence, the orad area generates the most frequent slow wave activity and functions as a pacemaker. Slow waves initiated at proximal areas migrate caudally and “capture” (“entrain”) contiguous distal areas of less frequent intrinsic activity, driving them at their own rate (“coupling”). The slow waves propagate from one cell to another through special contacts in the cell membrane called “nexuses”, which provide a pathway of low electrical resistance regulated by the neuro-humoral control system. From these data one can understand the complexity of gastric myoelectrical activity and the importance of its role in gastric motor function. It represents the end point of the motility control system, on which neurocrine, endocrine and paracrine systems operate, and, as it establishes frequency, direction and propagation velocity of peristaltic waves, it may be considered the indispensable condition (“conditio sine qua non”) of any coordinated motor activity of the stomach^[14].

In gastroparesis, there are more or less severe alterations in gastric myoelectrical activity^[15], which may be record-

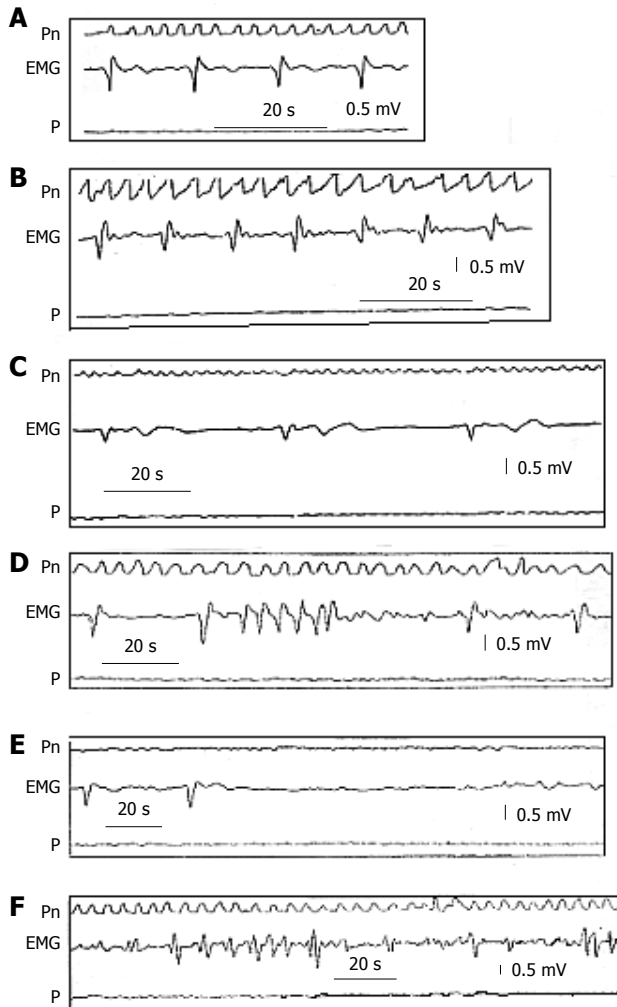


Figure 2 Electrogastrographic alterations. Series of alterations in the gastric myoelectric activity recorded with an electromyographic and manometric technique from the gastric antrum of patients with severe gastroparesis in confront with (A) normal myoelectric activity recorded in a healthy subject showing slow waves of normal morphology and frequency of 3 cycles/min. B: Tachygastric; C: Bradygastric; D: Run of high frequency tachygastric; E: Bradyarrhythmia; F: Complete disorganization of myoelectric activity ("gastric fibrillation"). Note that all these alterations are associated with absence of gastric contractions^[1]. EMG: Gastric myoelectric; Pn: Pneumogram; P: Pressure.

ed with intraluminal, serosal and cutaneous electrodes^[16,17]. The electrogastrographic alterations consist of various kinds of arrhythmias (Figure 2), very similar to those observed on the electrocardiogram in some cardiac diseases, such as tachygastric, tachyarrhythmia, bradyarrhythmia, asystolia (electrical silence), and gastric fibrillation^[16]. The latter is a complete disorganization of gastric electrical activity due to impairment of coupling and propagation of gastric slow waves. All these alterations result in a lack of propagated gastric contractions with a more or less severe delay in GE. However, it is also possible that, despite a regular slow wave rhythm, the gastric wall is unable to contract (electro-mechanical dissociation), because of alterations in the smooth muscle cell contractile system activation and operation.

It is paramount to remember that the motility structures of the gastric wall, such as the smooth muscle cell contractile system, interstitial cells of Cajal pacemaker

network, enteric neurons (motor, sensory, integratory) and afferent and efferent fibres connected with the CNS, work using a depolarization-repolarization mechanism. An electrical stimulus delivered to the gastric wall may influence the electrical activity of these structures with consequent modifications of their function and its effect depends on the characteristics of excitability of the target tissues and on the stimulus parameters.

GASTRIC ELECTRICAL STIMULATION

Gastric electrical stimulation (GES) consists of the delivery of electrical stimuli by means of electrodes implanted in the musculature of the gastric wall which are connected to a stimulator device in order to restore effective gastric contractions with normal GE and improve the symptoms of refractory gastroparesis.

Since the 1960s, many investigators have tried to re-establish normal gastric myoelectrical activity to generate coordinated peristaltic activity in patients with refractory gastroparesis^[18]. They used a large variety of electrical stimuli differing in pulse width, amplitude and frequency with diverse approaches and various results. It is the difference in frequency of the electrical stimuli from 3-4 cycles/min (cpm) to 50 cycles/s (Hz), which is mainly responsible for the different effects on the target structures of the gut wall.

Two principal types of GES are available: (1) Low-frequency/high-energy GES with long pulse stimulation, the frequency of which is just above that of the native slow wave with a pulse duration in the order of some tenths of a second; and (2) High-frequency/low-energy GES with short pulse stimulation, the frequency of which is markedly above that of the native slow wave with a pulse duration less than one thousandth of a second, delivered singly or in bursts of various length.

Low-frequency/high-energy GES with long pulse (gastric electrical pacing)

The electrical stimulus likely activates the interstitial cells of Cajal and/or muscle cells directly without involving intramural cholinergic nerves, because the administration of atropine does not block the appearance of electrical-ly-induced slow waves^[19].

This stimulation is called low-frequency/high-energy GES, because the frequency is slightly above that of the slow wave and its request for energy is high, due to prolonged delivery of a pulse current, for which it is also called "long pulse stimulation". It may be properly defined as "gastric electrical pacing" (GEP), as it refers to electrical stimuli which are aimed to induce propagated slow waves that replace the spontaneous waves.

If a pulse stimulus with a constant current of 2-4 mA lasting 30-500 ms is given to the gastric wall during a non-refractory period, an extra slow wave is induced, which propagates along the gastric wall both in oral and aboral directions, depending on the site of stimulation^[20,21]. When a series of stimuli is given, a series of slow waves is induced (entrainment) only if the stimulus frequency is

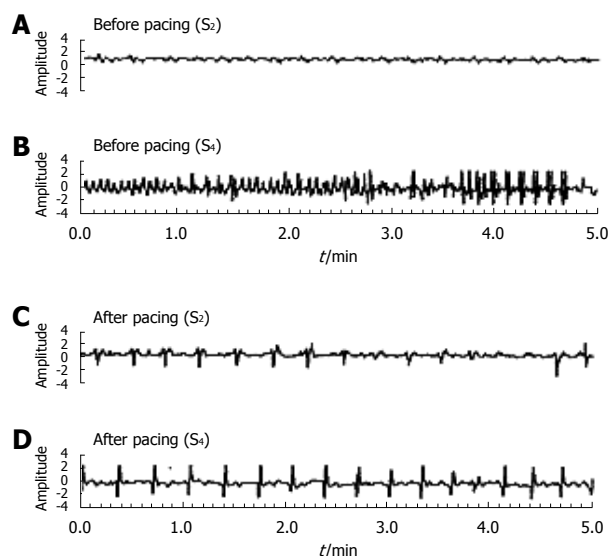


Figure 3 Normalization of ectopic tachygastria with low-frequency/high-energy gastric electrical stimulation [pacing frequency: 3.2 cycles/min (cpm), pulse width: 300 ms, amplitude: 4 mA] in a patient with gastroparesis. The pacing was carried out in the proximal corpus and the recording electrodes were in the mid gastric corpus (S_2) and in the gastric antrum (S_4). Before pacing a slow wave of about 3.5 cpm was recorded at S_2 (A) and tachyarrhythmias at S_4 (B), whereas the pacing with a frequency of 3.2 cpm entrained gastric slow waves at S_2 (C) and S_4 (D)^[44].

slightly above that of the intrinsic frequency, but not more than 4.7 cpm in man^[18,20,22] (Figure 3). The entrainment, however, is not sufficient to re-establish a propagated contraction in all cases and consequently to improve GE, especially if the neuromuscular structures are severely damaged^[20]. In fact, GEP in dogs following stimulation of 100-300 ms duration, 5-7 cpm frequency and 2-4 mA amplitude was able to entrain slow waves, even after an artificial gastroparesis induced by the association of vagotomy and glucagon, but the effect on gastric contraction and GE was obtained only in some cases^[18,23-27]. However, in patients with gastroparesis, GEP performed by means of an external device and transcutaneous electrodes fixed in the proximal corpus, with a frequency of 3-3.3 cpm, an amplitude of 2-4 mA and a pulse duration of 30-300 ms, was able to induce a regular rhythm in most patients, and in some cases restored efficient contractions, accelerated GE and improved symptoms^[28-32].

In conclusion, besides these limitations this kind of extrinsic pacing may re-establish a normal frequency of slow wave activity in gastric dysrhythmias, but does not guarantee the appearance of true contractions and consequent improvement in GE, especially in conditions of severe gastric atony, and has little effect on vomiting. Unfortunately, long duration pulses require high energy which must be provided by batteries too heavy and large to be implanted in a patient for long-term treatment.

High-frequency/low-energy GES with short pulse (gastric neuro-stimulation)

This type of stimulation is called “high-frequency GES” (HF-GES), because the frequency of the stimulation is well above the intrinsic frequency, and is also called

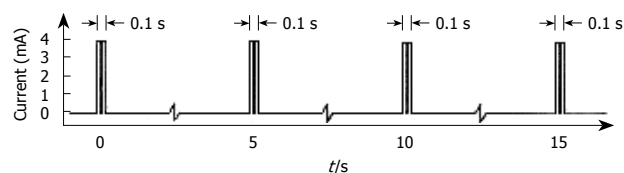


Figure 4 Type of electrical stimulation used by the Enterra system. Short bursts of short duration rectangular pulses (330 μ s each) with amplitude of 4 mA were given at a frequency of 14 Hz in each burst. Bursts in turn lasted 0.1 s and were delivered every 5 s^[39].

“high-frequency/low-energy” GES, because it requires a low quantity of energy, and, being short the length of pulse, is also called “short pulse stimulation”.

If a series of stimuli of 2-5 mA amplitude is delivered to the gastric wall with a frequency higher than 4.8 cpm cycles/min at 2-5 mA, no slow waves are induced, because the frequency stimulus is above the “maximum driven frequency” of the stomach. The native slow wave continues to spread with its own frequency and slight modifications, while the effects on contractions and GE are variable^[27,33-38]. Stimulation may be performed with a single pulse of constant current^[35] of short duration (approximately 300 μ s) or by a couple of pulses of 300 μ s at 70 μ s intervals^[34] or by a burst of pulses of high frequency (up to 50 Hz)^[33] and variable length. As the power consumption is low, this system does not require unwieldy batteries and allows the implantation of a portable device.

The type of HF-GES most used is performed with an implantable stimulator called Enterra (Medtronic, Minneapolis, MN, USA). It delivers electrical stimuli consisting of couples of pulses with a frequency of 14 Hz, amplitude 5 mA, duration 330 μ s, which are delivered for 0.1 s at a frequency of 12 cpm^[39] (Figure 4). The electrodes are positioned *via* laparotomy or laparoscopy in the musculature of the gastric corpus, whereas the pulse generator is inserted in a subcutaneous pocket^[34,40,41]. This method of stimulation is approved by the USA Food and Drug Administration (FDA) within certain limits on humanitarian grounds and in a few selected centres of research with the approval of the Institutional Review Board, to be used in patients with diabetic or idiopathic refractory gastroparesis, but has not been authorized by NICE in the UK. Moreover, some centres in the USA have discontinued the implantation of this device, because of few benefits to patients in terms of cost and risks.

With regard to the effects of HF-GES on gastric electro-mechanical activity, the slow waves remain practically unchanged^[19,24,26,27,35,37,38], whereas the effects on contractions are contradictory^[19,26,27,37]. This may be due to the fact that none of these investigators considered the possible spontaneous occurrence of activity fronts of the migrating motor complex that may increase the motility index casually in correspondence with the period of stimulation. GE was found to be unchanged, worsened or improved, sometimes after months or years of stimulation^[26,34,35,38,42-51]. However, these studies were not controlled and in some cases the patients continued to take

prokinetic drugs during the period of stimulation^[35,38]. Some investigators solved the problem of little effect on GE by adding a pyloroplasty to GES obtaining an obvious improvement in GE^[52]. Other investigators devised a dual stimulation protocol alternating pulses of short duration (0.3 ms) with pulses of long duration (500 ms) every 10 s with the aim of obtaining not only an antiemetic effect, but also to correct dysrhythmias and improve GE^[53]. In conclusion, the effect of HF-GES on slow waves and contractions is absent or at least dubious, while there is a slight and inconstant effect on GE.

With regard to the effects of HF-GES on symptoms of gastroparesis, the first uncontrolled trials from a small number of centres reported significant and prolonged gastric symptoms improvement in both diabetic and idiopathic gastroparesis with about 80% reduction in nausea and vomiting^[34,46,54-59]. One further study^[48] from three regional centres regarding 214 patients carrying the device for an average of 4 years, reported a continued improvement of at least one of the gastroparesis symptoms in 50% to 92% of patients. However, no symptom score and quality of life measurements at baseline and at follow-up were carried out with respect to a control group of 25 non-implanted patients. In addition, no survival benefits were observed in implanted patients with respect to non-implanted patients.

The major fault in all these studies was the absence of a double-blind randomized crossover design, with the exception of one complete study^[60] and one abstract^[61]. In the first study which included the Enterra system, 33 patients (17 diabetic and 16 idiopathic) were randomized to ON and OFF stimulation for 1 mo periods in a double-blind crossover design, followed by a non-blinded ON period of 6-12 mo. With regard to the results there were, however, as noted in a follow-up "Letter to the Editor"^[62], discrepancies between the initial submission to the USA FDA, where a decrease in vomiting frequency was reported without significant differences between the ON and OFF periods, and the subsequent publication in *Gastroenterology*, where a reduction in vomiting frequency was observed in diabetic patients during the ON period and not during the OFF period. The decrease in vomiting frequency continued during the uncontrolled phase of stimulation, confirming the results of other studies^[58], but no significant decreases in postprandial fullness, early satiety, pain and bloating were observed. Due to these limited results, the authors announced in a reply to the previously reported "Letter to the Editor"^[62], that a new controlled double-blind multicentre trial of GES with Enterra was underway.

The results of a new prospective multicentric double-blind randomized controlled crossover study with Enterra was presented at the DDW of 2010^[61] on the effects observed in 32 patients with idiopathic gastroparesis. After 6 wk of stimulation, a double-blind randomized consecutive 3-mo crossover period with the device ON or OFF was followed by an unblinded ON period up to 12 mo after implantation. However, during the crossover period there was a non-significant reduction in weekly vomiting frequency with a median of 9.8 episodes during the OFF

period vs 6.4 during the ON period. At one year after implantation, symptoms and quality of life were significantly improved as well as GE at 2 h, but not at 4 h.

In patients treated with this system the decrease in vomiting was associated with an improvement in some nutritional parameters, such as body weight and serum albumin, and with a decrease in necessity for parenteral or enteral nutrition. In addition, the need for visits and hospitalization and the use of prokinetic and antiemetic drugs were significantly decreased, whereas in diabetic gastroparesis the glycaemic control was improved, as well as the health-related quality of life^[34,46,49,50,56,57,60]. A comparison between medical therapy for gastroparesis and the necessity for health care resources was determined, however, this was carried out only in one study with a very small randomized control group and without a detailed indication of the drugs and doses used^[55].

Other studies on the effect of GES were performed in other types of patients with gastroparesis, such as those who underwent a partial gastric resection with or without Roux en Y gastric bypass and those who underwent esophagectomy or heart-lung and kidney-pancreas transplant procedures^[42,63,64], who reported a decrease in symptoms for long periods of time. However, the energy requirement for successful stimulation was higher in patients with postsurgical gastroparesis with respect to any other type of gastroparesis^[65].

The major problem with this procedure is the scarce responsiveness which may occur in patients with prominent bloating or pain^[58], and in idiopathic with respect to diabetic gastroparesis^[58,60]. Also, patients with interstitial cell of Cajal loss showed little response to HF-GES^[66]. The possible causes of a poor response may lie in the incorrect positioning of electrodes and in opiate use at the time of implantation which may blunt the response to stimulation^[58]. Consequently, some investigators suggest an intraoperative endoscopic ultrasound to confirm the correct positioning of the electrodes within the gastric muscle layer^[67]. To test the response before the implantation of a permanent stimulatory device, other investigators placed percutaneous stimulating electrodes at the time of gastrostomy or used a PEG technique^[68]. Self anchoring percutaneous electrodes have been used^[69,70], which may allow prolonged stimulation up to 2 mo^[71]. Other investigators propose to adjust the stimulation parameters using hand-held programming devices to increase the voltage or pulse frequency.

Another important problem of the Enterra system is the complications that may take place in up to 20% of patients, such as infections, migration and erosion of the stimulating device^[34,60], stomach wall perforation, pain due to adhesive bands from pacing wires to the abdominal wall^[72], dislodgment, breakage and erosion of leads into small bowel^[73], and stomach wall perforation and intestinal obstruction. All these complications require another surgical intervention and are sometimes lethal^[46].

The mechanism of action of this type of HF-GES is unknown. In fact, symptom improvement is not due to an entrainment of slow waves, or to a correction of underlying slow wave dysrhythmias^[56,74], or to an improvement in

GE. The improvement in symptoms was associated with a decrease in gastric retention at 4 h in rare cases^[43], whereas in the majority of cases there was a discrepancy between the improvement in symptoms and the disappointing results on gastric motor function. A decrease in vomiting with the Enterra system was also observed in patients with hereditary intestinal pseudo-obstruction or with simple functional dyspepsia^[70,75], as well as in patients with nausea and vomiting regardless of GE rates^[74,76]. These results indicate the existence of a mechanism independent of GE improvement. The fact that in a couple of controlled studies^[60,61] a similar improvement in symptoms occurred during the crossover double-blind ON and OFF stimulation, suggests that this mechanism could be a placebo effect or that the surgery itself on the stomach and abdominal wall may have given rise to some kind of afferent stimuli that decreased the sensation of nausea and vomiting. In addition, a spontaneous improvement in gastric motility and dyspeptic symptoms cannot be excluded, as this was noted in patients with postviral gastroparesis and in patients with intractable diabetic and idiopathic gastroparesis under tube feeding, who resumed spontaneously with oral feeding^[5,77,78]. No significant effect on the blood levels of gut hormones with gastrokinetic activity, such as motilin, gastrin, neurotensin and pancreatic polypeptide was observed. Other investigators took into consideration modifications in sympathetic-vagal activity, adrenergic and cholinergic functions^[79,80] or modulation of thoracic spinal neurons activity^[81], as well as that of the paraventricular nucleus of the hypothalamus^[82] and that of the thalamus, which were found on PET to be activated in gastroparetic patients by gastric stimulation^[80]. However, the hypothesis that the device acts through vagal pathways^[54,83] was disproved by the fact that HF-GES also works well in patients with vagotomy^[42]. Symptom improvement is possibly due to an action on afferents fibres^[84] which inhibit the vomiting centre or influence symptom perception in the brain or promote fundic relaxation through nonvagal nitrergic pathways^[38,85-87]. In fact, experiments with a gastric barostat showed that HF-GES decreases sensitivity to gastric distension and enhances gastric accommodation to a meal in patients with severe idiopathic gastroparesis^[85]. As to why this kind of GES improves nausea and vomiting remains an enigma.

Neural sequential GES is another type of high-frequency GES

The application of a train of electrical square waves with a duration of a few ms and a frequency > 50 Hz with an amplitude of 8-16 V for 4-16 s invokes a contraction of the gut wall at the site of the electrodes. This type of electrical stimulation induces a release of acetylcholine from the intramural cholinergic fibres, which in turn stimulates muscle cell contraction. In fact, its effect is prevented by the previous administration of atropine^[88] and for this reason is called "neural GES"^[89]. This contraction, however, does not propagate spontaneously, but either circumferentially and aborally^[90]. To have a contraction involving a circular band of gastric musculature it is necessary to use

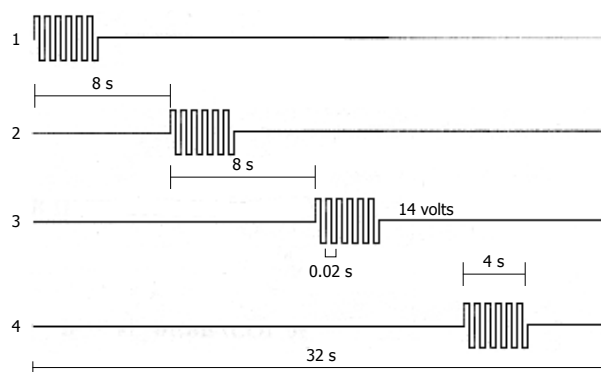


Figure 5 Characteristics of one sequential gastric pacing stimulation protocol in dogs are shown from the proximal (1) to the distal (4) electrodes, that were positioned along the gastric corpus and antrum at 4 cm interval. Four second duration pulse trains with an amplitude of 14 V and a frequency of 50 Hz were delivered in synchronized fashion with a 4 s lag between adjacent stimulus sites^[89].

a circular chain of electrodes, and to have a propagated contraction it is necessary to employ a series of these electrodes encircling both the corpus and the antrum, activating them sequentially ("neural sequential GES")^[88]. The spontaneous slow wave is overwhelmed by these electronically co-ordinated contractions. This system has the advantage of working both when spontaneous waves show a regular rhythm, but are unable to induce efficient pressure waves, and when slow waves are arrhythmic, uncoupled, completely disorganised and not responding to low-frequency pacing.

Mintchev *et al*^[89,91] with the aid of a series of 4-6 ring electrodes placed in the corpus and antrum of dogs, sequentially activated by a microprocessor (Figure 5), was able to induce strong propagated contractions, that increased GE of both liquids and solids. The effectiveness of this type of GES was also demonstrated in a gastroparetic patient at the time of laparotomy^[89]. Acute and chronic canine studies confirmed the feasibility of this microprocessor-controlled stimulation method with an implantable multichannel stimulator^[92], which may be externally controlled with radiofrequency^[93,94].

However, before initiating studies in patients with gastroparesis, chronic experiments in animal models are necessary with an implantable device to evaluate not only the long-term efficiency of this method and the possible incidence of surgical complications, but also to assess the pathophysiologic influence of electrical current pulses on neuromuscular structures of the gastric wall and the effects of the strong antral contractions in the management of gastric content. In fact, one must bear in mind the motor function of the pylorus and duodenum when antral contractions occur. If the pylorus remains open during stimulation, the strong artificial contractions may cause a rapid GE of food particles with consequent risk of maldigestion and dumping syndrome, as suggested by Hasler^[95], because the "intestinal brake" is lacking. In addition, if there is a non-propulsive motor disorder of the small intestine, an accumulation of material in the intestinal lumen may take place, which may give rise to a func-

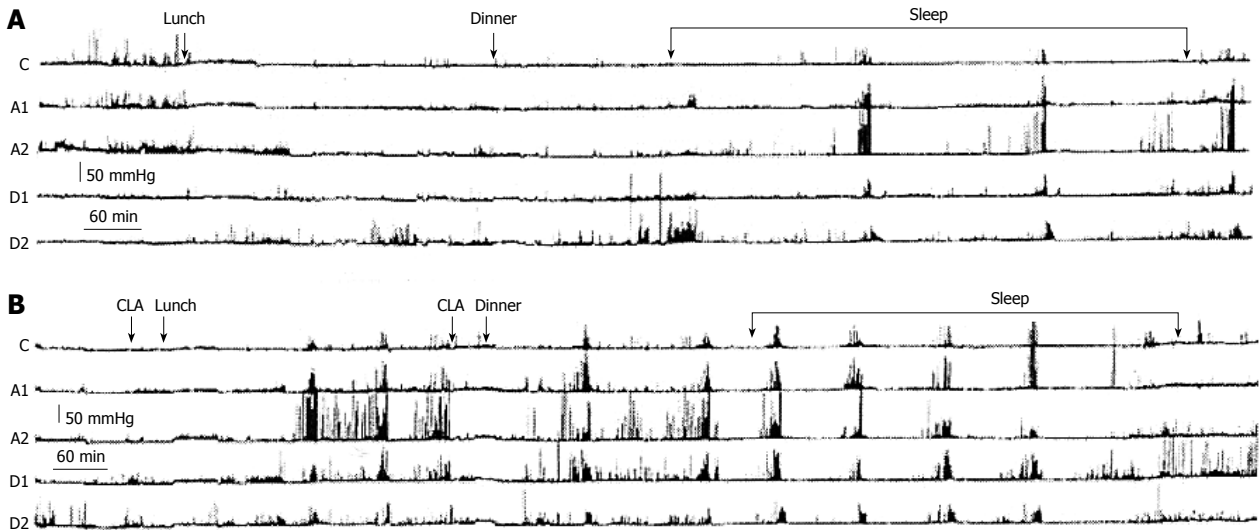


Figure 6 Two ambulatory 24-h gastroduodenal recordings (A and B) carried out on two separate days in a patient with apparently refractory gastroparesis by means of a probe with 5 miniaturized electronic pressure transducers, 5 cm apart: one in the corpus (C), two in the antrum (A1 and A2) and two in the duodenum (D1 and D2). On the first day (A) the recording was carried out without drug administration and on the second day (B) with clarithromycin (CLA) administration. A: On the first day, the postprandial gastric motor activity was very low and only three activity fronts of the Migrating Motor Complex were observed, two during the night and one early in the morning; B: On the second day, the oral administration of clarithromycin 30 min before lunch was followed about 3 h later by a burst of powerful peristaltic contractions starting in the stomach and progressing in the duodenum, followed by two others bursts at about 80 min intervals. The oral administration of clarithromycin 30 min before dinner induced after about 2.4 h a series of six bursts of powerful peristaltic waves in the stomach and duodenum at 80-100 min intervals^[103].

tional obstruction. If the pylorus does not open, as may happen in patients with diabetic gastroparesis^[96], strong artificial contractions could accumulate the gastric content against the closed pylorus with consequent abnormal antral distension and possible occurrence of pain.

Finally, we are not sure that the normalization of GE will be accompanied by the disappearance of dyspeptic symptoms, if there is visceral hypersensitivity, as happens in patients with dyspepsia despite normal GE^[97]. However, we believe that the problem of gastric stasis is crucial in gastroparesis and should be corrected in any case. In fact, besides the severe consequences on symptoms and nutrition and the negative effect on the glycaemic control of diabetes, it may cause “*per se*” gastric damage, such as gastritis (which may be erosive in diabetics), phytobezoars^[98] with possible ulceration, obstruction and gastric perforation and pharmacobezoars comprised of medications^[99].

From these considerations we believe that an in-depth experimentation of neural sequential GES in various pathophysiological conditions associated with gastroparesis is mandatory.

COMMENT AND CONCLUSION

Many investigators have been engaged in resolving the problem of gastric electrical stimulation in the last half century with different approaches and various outcomes.

Low-frequency/high-energy GES, known as gastric electrical pacing with long pulse stimulation, is able to induce a regular rhythm, restore efficient contractions, and improve GE and symptoms in some cases of gastroparesis, but requires a high quantity of electrical current, which can only be provided by a device too large and heavy to be implanted and for this reason is not suitable for clinical

studies. However, with the progress in electronic miniaturization and in the potency of batteries this method may be considered again in the future.

High-frequency/low-energy GES with short pulse stimulation, such as the Enterra system, although it does not significantly modify slow wave and motor activity and does not consistently resolve the problem of delayed GE^[34,46,60], shows, however, a good effect on nausea and vomiting with slight but significant improvement in nutritional depletion and health-related quality of life^[34,48,57,100]. However, the possibility of spontaneous improvement or a placebo effect cannot be ruled out for sure and there are many considerations regarding the clinical use of this type of GES.

First, one must keep in mind that up to 20% of patients develop more or less severe complications, sometimes lethal, related to implantation of the device^[46].

Second, 13% of patients are non-responders^[60], especially those with idiopathic gastroparesis, and the improvement in nausea and vomiting may be temporary in 50% of patients^[58] and does not include other dyspeptic symptoms, such as epigastric pain, which is an important disabling symptom compelling the patient to continue analgesic therapy^[101].

Third, the benefit in nutritional parameters is low, as the average body weight increases were from 0.9 kg^[46] to 8.4%^[34] a year.

Fourth, when an improvement is obtained, this may not be any better than that obtained by a pyloric injection of botulinum toxin, which avoids the need for surgery in up to 2/3 of patients referred to the surgeon for GES^[102]. Moreover, an aggressive drug treatment in suitable doses, taking care to recognize and avoid pseudo-refractoriness to these drugs, may spare implantation of GES. In fact,

in most of the studies examined, including those claiming the superiority of GES over medical therapy^[55,56], there was a generic statement of refractoriness to prokinetic and antiemetic drugs without specifying the kind of drugs and dosages. None of the studies considered pseudo-refractoriness due to faulty bioavailability of the drug, which may take place when it is orally administered at the usual time interval of 30 min before meals. We performed a gastroduodenal 24 h-manometric examination in a gastroparetic patient with apparent refractoriness to prokinetics and demonstrated that the drug administered 30 min before a meal took about 3 h to stimulate gastric motility (Figure 6)^[103]. Gastric contractions were almost absent during this time, while the meal stagnated in the stomach causing dyspeptic symptoms. One should remember that in these patients about 80% of the gastric content is still in the stomach 2 h after ingestion^[60], and that the prokinetic pill also takes this time to reach the intestine to be absorbed and stimulate gastric motility. Therefore, we decided to administer the prokinetic pill more than 2 h before meals to this patient and obtained a marked improvement in dyspeptic symptoms associated with an improvement in GE and a progressive gain in body weight^[103].

Fifth, among the considerations that dissuade exposing a patient with intractable gastroparesis to the Enterra system outside of a rigorous placebo-controlled study, there is also the high cost of the procedure which exceeds USD 20000 and the existence of some limitations, such as the necessity to avoid certain metal detecting security devices and magnetic resonance imaging.

With these considerations in mind, it is advisable to discontinue the use of this type of gastric stimulator for gastroparesis outside properly designed double-blind controlled studies, because it is a costly and risky procedure that does not resolve the principal problem of gastroparesis, that is GE delay, and only improves vomiting without significantly influencing other dyspeptic symptoms, such as epigastric fullness, satiety, anorexia and epigastric pain.

Sequential neural GES, which is able to induce propagated gastric contractions with consequent acceleration of GE, is the most promising method, as it affects the core of the problem of gastroparesis which is gastric stasis, rather than just mitigate the symptoms. However, there is still much research to be carried out, since to date, this method has been used only on animals and one patient with gastroparesis^[89,91], therefore it is necessary to use this type of GES in different pathophysiologic conditions of gastroparesis. The hope is that electronic technology could make possible an easily implantable device for humans, able to modulate contractile activity following physiologic necessities. Under these circumstances, GES will become able to treat gastroparesis, whereas to date none of the current technologies has been demonstrated unequivocally to consistently accelerate GE and improve all symptoms in patients with gastroparesis.

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Sang Hoon Ahn, MD, PhD, Professor, Series Editor

Quantification of HBsAg: Basic virology for clinical practice

Jung Min Lee, Sang Hoon Ahn

Jung Min Lee, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, South Korea
Sang Hoon Ahn, Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul 120-752, South Korea

Sang Hoon Ahn, Liver Cirrhosis Clinical Research Center, Seoul 120-752, South Korea

Author contributions: Ahn SH designed this article and revised it critically; Lee JM acquired information and wrote this article.

Supported by The Grant of the Bilateral International Collaborative R&D Program from the Ministry of Knowledge Economy and the Good Health R&D Project from the Ministry for Health, Welfare and Family Affairs, South Korea (A050021)

Correspondence to: Sang Hoon Ahn, MD, PhD, Professor, Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, 250 Sungsan-ro, Seodaemun-gu, Seoul 120-752, South Korea. ahnsh@yuhs.ac

Telephone: +82-2-22281930 Fax: +82-2-3936884

Received: February 22, 2010 Revised: March 23, 2010

Accepted: March 30, 2010

Published online: January 21, 2011

of HBsAg titers with respect to their biologic nature, method principles, and clinically relevant topics.

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Key words: Hepatitis B virus; Hepatitis B surface antigen; Quantitative assay; Virology

Peer reviewers: Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; Yogesh K Chawla, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Lee JM, Ahn SH. Quantification of HBsAg: Basic virology for clinical practice. *World J Gastroenterol* 2011; 17(3): 283-289
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/283.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.283>

Abstract

Hepatitis B surface antigen (HBsAg) is produced and secreted through a complex mechanism that is still not fully understood. In clinical fields, HBsAg has long served as a qualitative diagnostic marker for hepatitis B virus infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring, and there is an opportunity to make maximal use of quantitative HBsAg to elucidate its role in clinical fields. Yet, it needs to be underscored that a further understanding of HBsAg, not only from clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. It is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. In this article, we review current concepts and issues on the quantification

INTRODUCTION

Hepatitis B virus (HBV) causes a wide range of clinical consequences, from acute and chronic infection to cirrhosis and hepatocellular carcinoma, and represents a global public health problem^[1,2]. Historically, HBV dates to 1967 when an unknown antigen in Australia was recognized to be associated with hepatitis type B, which was later referred to as the hepatitis B surface antigen (HBsAg)^[3]. Since then, HBsAg has served as a qualitative diagnostic marker for HBV infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring. A number of clinical studies have evaluated the clinical utility of HBsAg and suggested its potential roles. Yet, it needs to be underscored that a further understanding of HBsAg, not only from a clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. Therefore, in this article, we review current concepts and issues on the quantification of HBsAg titers

(qHBsAg) with respect to their biologic nature, method principles, and clinically relevant topics.

STRUCTURE AND MOLECULAR VIROLOGY OF HBsAg

Components of the viral structure

HBV belongs to *Hepadnaviridae* and is composed of the envelope, core, DNA genome, and viral polymerase. It has a circular form of partially double-stranded DNA and is approximately 3200 nucleotides in length^[4,5]. A 42-45 nm long HBV spherical form (Dane particle), which is the full virion with infectivity, can be visualized (Figure 1) under electron microscopy. It has two-layered shells. The outer shell is the envelope protein referred to as hepatitis B surface (HBs) protein, which is further divided into small, middle, and large HBs proteins (SHBs, MHBs and LHBs proteins, respectively), and the inner shell is a core protein referred to as the hepatitis B core protein in which viral polymerase and the HBV genome is enclosed. In addition to the abovementioned full virion, smaller non-infectious subviral particles are present in the serum; 17-25 nm spherical particles, mainly composed of SHBs protein, constitute the most abundant form, which is as much as 10000-fold in excess of the full infectious virion^[4,6]. Filamentous (or tubular) particles are another form, with a 20 nm diameter and variable length, and are composed of SHBs, MHBs, and the LHBs protein. The form of the HBV particles appears to be determined by the proportion of LHBs protein^[7]. All three forms can be detected in serum with commercial assays and are collectively referred to as HBsAg.

Synthesis and secretion

HBV has four distinct open reading frames (ORFs) that encode the envelope, core, polymerase, and X proteins. ORF S has three internal AUG codons encoding the SHBs, MHBs, and LHBs proteins, which correspond to the S, preS2 + S, and preS1 + preS2 + S domains, respectively (Figure 2). These proteins have a common carboxyl end but different amino ends^[8].

Like all other proteins, mRNA transcription is the first event to occur. Two 2.1 kb mRNAs for the M/SHBs proteins and a 2.4 kb mRNA for the LHBs protein are formed, and take a separate pathway from viral replication. Diverse transcription factors are involved and act on promoters, enhancers, and other regulatory elements, such as the glucocorticoid responsive element^[9,10]. LHBs and M/SHBs expression are thought to be independently regulated with different promoters; a typical TATA box is present in the LHBs promotor (S promotor I, SPI), whereas the TATA-less promotor, which usually has multiple initiation sites, is associated with the M/SHBs promotor, thus accounting for synthesis of distinct proteins from one mRNA. In patients with active viral replication, the protein expression pattern shows a predominance of the M/SHBs protein in contrast to a predominance of the LHBs protein in inactive carriers^[11]. After transcription, protein synthesis and glycosylation follows at the endoplasmic reticulum (ER) membrane resulting in a 226

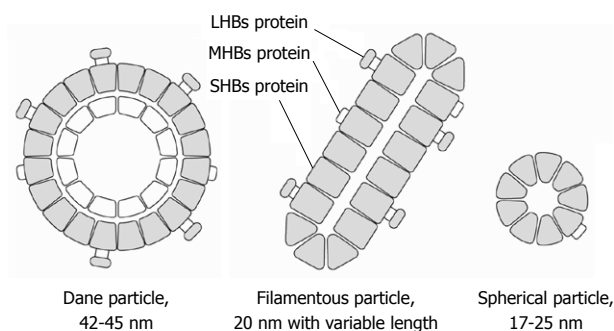


Figure 1 Schematic model of hepatitis B surface antigen structure. Three forms of hepatitis B surface (HBs) antigen (Dane particle, filamentous particle, and spherical particle) are visualized in serum by electron microscopy. These are composed of small, middle, and large hepatitis B surface proteins. LHBs: Large HBs proteins; MHBs: Middle HBs proteins; SHBs: Small HBs proteins.

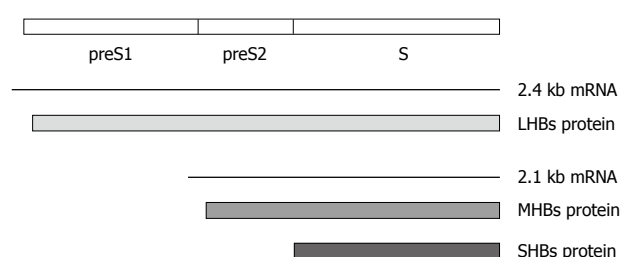


Figure 2 Schematic presentation of the S/preS1/preS2 gene, RNA transcripts, and translational products. Opening reading frame S has three internal AUG codons. Transcription to produce the 2.1 kb and 2.4 kb mRNAs first occurs after translation into small hepatitis B surface proteins (SHBs), middle hepatitis B surface proteins (MHBs), and large hepatitis B surface proteins (LHBs) ensues with different promoters.

amino acid SHBs protein, the MHBs protein with an additional 55 amino acids, and the LHBs protein with an additional 108-119 amino acids. Although the LHBs mRNA includes the M/SHBs sequence, it does not translate into the M/SHBs protein, and the ratio between the MHBs and SHBs protein is controlled by a complex mechanism, which is not fully understood^[12]. To form a full virion, a mixture of HBs proteins in a well-balanced ratio is utilized to envelop core particles in which SHBs and LHBs protein are indispensable^[13]. The virion is transported to the cell membrane through vesicles, and several conditions must be satisfied for successful secretion, because excess SHBs protein is required, whereas excess LHBs protein prevents secretion and causes dilatation of the ER with a ground-glass appearance^[14-16].

Function

The primary function of the HBs protein as a virologic structure is to enclose the viral components. It also plays a major role in cell membrane attachment to initiate the infection process. Several studies have confirmed the idea that the peptide in the preS1 domain is essential in this process, showing that it specifically binds to the human liver plasma membrane and can be inhibited by a monoclonal antibody^[17,18]. However, participation of the SHBs protein in attachment has also been suggested following identification of hepatocyte-bound endonexin II, which

specifically binds the SHBs protein^[19]. Additionally, from the host perspective, the HBs protein has the major antigenic components, including the *a* determinant, which is important for host-activated immunity. However, from a virologic perspective, it is postulated that excess HBs protein may divert such neutralizing antibody immune function away from the infectious virion^[20].

QUANTITATIVE HBsAg ASSAYS

Methods to detect HBsAg were first described in the 1970s using radioimmunoassays and enzyme immunoassays^[21,22]. Since then, various diagnostic techniques have been developed, which are mostly confined to qualitatively diagnose HBV in clinical practice. Recently, quantitative assay of HBsAg has been developed, and two commercially available assays will be briefly introduced here.

The Architect HBsAg QT (Abbott Diagnostic, Wiesbaden, Germany) is a chemiluminescent microparticle immunoassay, which is currently the method most widely used in clinical studies^[23]. The Architect HBsAg QT assay is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex, for quantitatively determining human serum and plasma HBsAg concentrations. In the first step, the sample and hepatitis B surface antigen antibody (anti-HBs) coated with paramagnetic microparticles are combined. HBsAg present in the sample binds to the anti-HBs coated microparticles. After washing, acridinium-labeled anti-HBs conjugate is added. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of HBsAg in the sample and the RLUs detected by the Architect Immunoassay System optics. The Architect HBsAg is a fully automated system and can detect as low as 0.2 ng/mL of HBsAg with a dynamic range of 0.05-250.0 IU/mL^[24].

Elecsys HBsAg II (Roche Diagnostics, Indianapolis, IN, USA) is another method for quantitatively determining HBsAg^[25]. In the first incubation step, the antigen in the sample reacts with two biotinylated monoclonal HBsAg-specific antibodies and a monoclonal/polyclonal (sheep) HBsAg-specific antibody, labeled with a ruthenium complex, to form a sandwich complex. In the second step, streptavidin-coated microparticles are added, and the complex binds to the solid phase *via* interaction with biotin and streptavidin. The results are reported as a cutoff index (signal sample/cutoff), and the sample is considered reactive if the index is greater than 1.0.

CLINICAL APPLICATION OF QUANTITATIVE HBsAg

Correlation with serum HBV DNA

Although measuring serum HBV DNA is the gold standard for monitoring viral load, it is relatively expensive and not yet readily available in some areas. By contrast, the technique for detecting qHBsAg is fairly easy and inexpensive, and the primary aim of initial clinical studies

was to determine the relationship between qHBsAg and serum HBV DNA (Table 1). In 2004, Deguchi *et al.*^[23] first reported the clinical significance of a high qHBsAg in patients who were hepatitis B e antigen (HBeAg) positive as opposed to those with an antibody positive to the hepatitis B e antigen (anti-HBe), and that qHBsAg correlated well with the serum HBV DNA level ($r = 0.862$). Although there are some contradicting results on whether qHBsAg is correlated with serum HBV DNA^[26,27], it seems that they are correlated based on a number of studies^[28-33]. Further studies are required to investigate the possibility of using qHBsAg as an aid, if not an alternative, for HBV DNA.

Correlation with covalently closed circular DNA

An important qHBsAg issue is its association with covalently closed circular DNA (cccDNA). cccDNA is a minichromosome and acts as a viral template and replenishing pool for maintaining a chronic HBV infection^[34]. Therefore, it is essential to understand the biology of cccDNA when considering HBV therapy. However, to examine cccDNA, an invasive procedure is required, and qHBsAg has been suggested as a surrogate marker for cccDNA. Werle-Lapostolle *et al.*^[29] reported a significant decrease in cccDNA, qHBsAg, and serum HBV DNA with adefovir (ADV) therapy, and that there was a strong correlation between cccDNA and other variables. This observation was supported by subsequent studies; Wurstthorn *et al.*^[35] and Chan *et al.*^[36] also showed that cccDNA was significantly correlated with qHBsAg, suggesting that serial monitoring of qHBsAg might act as an additional marker to evaluate treatment response during antiviral therapy.

Prediction of response to antiviral therapy

After the accumulation of data confirming that qHBsAg can be utilized as a viral monitor, qHBsAg has been evaluated as a predictor of virologic response. In a study by Chan *et al.*^[36] the sensitivity, specificity, and positive and negative predictive values for sustained virologic response (SVR) in patients treated with pegylated interferon (Peg-IFN) + lamivudine (LAM) were 86%, 56%, 43%, and 92%, respectively, with baseline qHBsAg concentrations less than 10000 IU/mL. According to the data of Manesis *et al.*^[31] achieving the complete elimination of HBsAg would probably require 10.6 years of effective LAM therapy or 5.4 years of a sustained response to interferon. Recently, the clinical usefulness of on-treatment qHBsAg in patients treated with Peg-IFN ± LAM has been suggested in both HBeAg positive and negative patients; a decline in qHBsAg of > 1 log IU/mL or specifically 0.5 and 1.0 log IU/mL at weeks 12 and 24, respectively, had high predictive value for SVR, and on-treatment HBsAg levels could be used as an early predictor of durable off-treatment response to Peg-IFN-based therapy^[32,33,37]. Of note is a long-term study by Marcellin *et al.*^[38] in which 35% of patients who had qHBsAg < 1500 IU/mL at week 12 eventually cleared the HBsAg by 4 years post-treatment, which supports the clinical utility of qHBsAg. Furthermore, qHBsAg was superior to cccDNA and serum HBV DNA for predicting SVR in patients undergoing Peg-IFN-based therapy with receiver

Table 1 Recent clinical studies with quantification of hepatitis B surface antigen titers in hepatitis B virus infection

Author	Antiviral therapy	Correlation	Prediction	Clinical results
Deguchi <i>et al</i> ^[23]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+)
Chen <i>et al</i> ^[28]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+) and high HBV DNA levels, whereas qHBsAg is low in low HBV DNA level CHB
Werle-Lapostolle <i>et al</i> ^[29]	ADV	cccDNA, HBV DNA	-	HBsAg and cccDNA decrease with ADV
Kohmoto <i>et al</i> ^[30]	LAM	HBV DNA	-	qHBsAg is helpful for early detection of drug resistant strains
Wursthorn <i>et al</i> ^[35]	Peg-IFN + ADV	cccDNA	-	Peg-IFN + ADV decreases cccDNA and HBsAg, which are well correlated
Chan <i>et al</i> ^[36]	Peg-IFN + LAM	cccDNA	Low baseline qHBsAg can predict SVR	Peg-IFN + LAM decreases cccDNA and HBsAg, which are well correlated
Manesis <i>et al</i> ^[31]	IFN vs LAM	HBV DNA	Low baseline qHBsAg can predict SVR	IFN induces sharper decrease in qHBsAg than LAM
Wiegand <i>et al</i> ^[27]	FAM ± LAM	HBV DNA (not correlated)	Decline of qHBsAg can predict HBsAg loss	2 log drop to below 100 IU/mL is associated with HBsAg clearance
Moucari <i>et al</i> ^[32]	Peg-IFN	HBV DNA	Early qHBsAg drop can predict SVR	qHBsAg may be useful to optimize Peg-IFN therapy
Brunetto <i>et al</i> ^[33]	Peg-IFN ± LAM vs LAM	HBV DNA	On-treatment qHBsAg decline can predict sustained HBsAg loss	qHBsAg < 10 IU/mL at week 48 and 1 log decline predict sustained HBsAg clearance to optimize treatment strategy
Lau <i>et al</i> ^[37]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	In HBeAg(+) patients, qHBsAg reduction through weeks 12, 24 and 48 were higher in patients with HBeAg seroconversion
Marcellin <i>et al</i> ^[38]	Peg-IFN ± LAM	-	qHBsAg at week 12 can predict long-term HBsAg clearance	35% of patients who had qHBsAg < 1500 IU/mL at week 12 cleared up HBsAg by 4 yr post-treatment
Lu <i>et al</i> ^[39]	Peg-IFN ± LAM	cccDNA	qHBsAg was superior to cccDNA and serum HBV DNA in predicting SVR	Area under ROC curve with qHBsAg, cccDNA and HBV DNA was 0.769, 0.734, and 0.714, respectively, for predicting SVR
Brunetto <i>et al</i> ^[65]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	On-treatment decline in HBsAg appears to be genotype dependent. Genotype B patients showed the most rapid and pronounced decline
Hou <i>et al</i> ^[66]	Peg-IFN vs LAM	-	-	Peg-IFN was superior to ADV in HBeAg seroconversion and qHBsAg decline in LAM-resistant patients

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantification of HBsAg titers; HBeAg: Hepatitis B e antigen; CHB: chronic hepatitis B; ADV: Adefovir; LAM: Lamivudine; Peg-IFN: Pegylated interferon; cccDNA: Covalently closed circular DNA; FAM: Famciclovir; SVR: Sustained virologic response; ROC: Receiver operating characteristic.

operating characteristic (ROC) curves of 0.769, 0.734, and 0.714, respectively^[39].

MOLECULAR HBsAg VARIANTS

Much of our understanding of the biologic nature of the HBs protein has been gathered from various mutation and truncated protein experimental models^[40,41], and it is worthwhile to address the relevance and consequences of HBsAg variants from a clinical point of view. Besides the lack of HBV proof-reading capacity^[42], the development of an HBsAg mutation can be attributed to immune pressure from extensive vaccination programs, injections of hepatitis B immunoglobulin (HBIG) following liver transplantation, and the overlap with a mutation in the corresponding *P* gene.

Immune escape mutants

Since the introduction of an extensive vaccination program, concerns about HBsAg variants have increased after an HBV infection occurred in infants who had received an HBV vaccination and who had mounted an adequate anti-HBs response. This was presumed to be caused by immune selection pressure, because the HBsAg *a* determinant is the major epitope for HBV vaccination^[43,44]. Changes in the amino acids within the *a* determinant, particularly between 137-147, disable surface antigen domain

recognition by neutralizing antibodies. Of importance is the G145R mutant, because it is the most common and is replication competent with stability^[45]. In a Taiwanese epidemiological study, it was reported that the prevalence of the *a* determinant mutation had increased from 7.8% to 28.1%, after 15 years of a universal vaccination program^[46]. Fortunately, in the following years, neither the percentage increase nor any significantly adverse events with an outbreak of HBV infection actually occurred; thus, a mass vaccination program is continuing with adequate justification^[47].

In addition to the extensive vaccination program, the wide use of HBIG following liver transplantation adds selection pressure to HBV. Ten of 20 patients who developed recurrent HBV infection despite hepatitis B immunoglobulin prophylaxis had amino acid substitutions involving the *a* determinant, which were mostly absent in pretransplantation clones^[48].

Overlap and mutation in the *P* gene

A mutation in the *P* gene from prolonged oral nucleos(t)ide therapy can cause an altered sequence in the corresponding *S* gene due to overlap of the two genes^[49], which is summarized in Table 2^[50]. The nucleotide at rt204 in the *P* gene is associated with resistance to LAM, telbivudine (LdT), and entecavir (ETV), and the rtM204V/I mutation typically results in a sI195M, sW196S, sW196L or a termi-

Table 2 Mutations in viral polymerase gene induced by oral antiviral agents and corresponding changes in hepatitis B surface antigen

Polymerase domain	Mutation in polymerase	Oral antiviral agents	Corresponding change in HBsAg
B	rtI169T	ETV	sF161H/L
	rtL180M	LAM, LdT	No change
	rtA181T	ADV, TFV, LAM, LdT	sW172 ¹
	rtA181T	ADV, TFV, LAM, LdT	sW172L
	rtA181V	ADV, TFV, LdT	sL173F
	rtT184A	ETV	No change
	rtT184C	ETV	sL175F + sL176V
	rtT184I	ETV	No change
	rtT184G	ETV	sL176V
	rtT184S	ETV	sL175F
	rtT184M	ETV	sL176 ¹
	rtT184L	ETV	sL175F
C	rtS202C	ETV	No change/sS193F
	rtS202I	ETV	sV194F/S
	rtS202G	ETV	No change/sS193L
	rtM204V	LAM	sI195M
	rtM204I	LAM, LdT	sW196 ¹ /S/L
D	rtN236T	ADV, TFV	After end of HBsAg
E	rtM250I	ETV	After end of HBsAg
	rtM250V	ETV	After end of HBsAg

¹Stop codon. Modified from reference^[50]. HBsAg: Hepatitis B surface antigen; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; ADV: Adefovir; TFV: Tenofovir.

nal codon in the overlapping *S* gene^[50]. In previous studies, LAM selected HBsAg mutants with reduced anti-HBs binding capacity, and secretion of HBsAg was prevented with a mutant strain due to the stop codon^[51,52]. rtI181 is another important site that confers resistance to ADV and/or LAM/LdT. Recently, Warner and Locarnini demonstrated that rtA181T caused a secretory defect and had a negative effect on secretion of the wild-type HBV virion because of a concomitant change in the envelope protein at sW172^[53]. Similarly, ETV-associated rtI169T/sF161L leads to a decrease in HBsAg immunoreactivity^[54].

The clinical significance of overlap and a common mutational substitution in the *S* and *P* gene was further extended by Kamili *et al.*^[55] who demonstrated a successful experimental infection with the rtV173L, rtL180M, and rtM204V HBV mutants that resulted in sE164D and sI195M despite high anti-HBs levels in chimpanzees^[55]. Furthermore, the possibility of a *vice versa* phenomenon with respect to an extensive vaccination program might be postulated in that HBsAg mutants from selection pressure might harbor the corresponding *P* gene mutation, resulting in primary resistance to antiviral agents and therapy failure with these agents.

Detection and variants of HBsAg

As described above, an HBsAg mutation leads to diverse effects, such as decreased secretion and reduced binding capacity to anti-HBs. Of note is that not only a mutation in the *a* determinant but also in the *S* promoter or a deletion in the preS region can cause such effects^[56,57]. These effects may hamper the diagnostic performance of commercial assays, and several reports have pointed to the

problem of not being able to detect HBV with an *a* determinant mutation^[58,59].

An occult HBV infection is defined as the persistence of the HBV genome in HBsAg negative individuals, and one of the explanations for occult HBV infection is a mutation in HBsAg and undetectability by available assays^[60]. Both the Architect HBsAg QT and Elecsys HBsAg II seem to reliably detect HBsAg mutants with high sensitivity and specificity^[24,25,61]. However, further studies are needed to validate such detection ability, because new or complex combinations of mutations can arise in this era of antiviral agents and extensive vaccination.

FUTURE PERSPECTIVES

Despite progress on qHBsAg, a number of unanswered questions still remain. Precise control mechanisms for HBsAg production in HBV are poorly understood. A discrepancy between qHBsAg and serum HBV DNA exists, although a correlation has been documented. Further research on the virologic nature of HBV could answer these two questions. Meanwhile, the role of qHBsAg in the clinical field is being actively investigated, especially as a predictor to virologic response. Of particular interest is the potential role of qHBsAg for defining the end point of oral antiviral therapy. Current American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines with respect to an end point for therapy are unsatisfactory, because reversion to HBeAg positivity does occur after terminating therapy, and the loss of HBsAg is infrequently encountered^[62,63]. In this regard, qHBsAg might be particularly helpful in patients with undetectable HBV DNA, even with a highly sensitive polymerase chain reaction assay^[64]. In contrast to undetectable HBV DNA, which provides no further information for the virologic responders, HBsAg is continuously shed and detected and, based on the observations of previous studies, qHBsAg with serial monitoring in patients with undetectable HBV DNA may be utilized to determine the end point of therapy and validate the durability of antiviral agents.

CONCLUSION

HBsAg is produced and secreted through a complex mechanism that is still not fully understood. Nevertheless, quantification of serum HBsAg is currently available and there is an opportunity to make maximal use of qHBsAg to elucidate its role in clinical fields. However, a deep understanding of the virology is necessary, and it is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. Unanswered questions need to be resolved through further qHBsAg research.

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S- Editor Wang JL L- Editor O'Neill M E- Editor Lin YP

Genomic and genetic alterations influence the progression of gastric cancer

Stefania Nobili, Lorenzo Bruno, Ida Landini, Cristina Napoli, Paolo Bechi, Francesco Tonelli, Carlos A Rubio, Enrico Mini, Gabriella Nesi

Stefania Nobili, Ida Landini, Cristina Napoli, Enrico Mini, Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, 50139, Italy

Lorenzo Bruno, Paolo Bechi, Department of Critical Care Medicine and Surgery, University of Florence, Florence, 50134, Italy
 Francesco Tonelli, Department of Clinical Physiopathology, University of Florence, Florence, 50134, Italy

Carlos A Rubio, Department of Pathology, Karolinska Institute and University Hospital, Stockholm, 17176, Sweden

Gabriella Nesi, Division of Pathological Anatomy, Department of Critical Care Medicine and Surgery, University of Florence, Florence, 50134, Italy

Author contributions: Nobili S, Bechi P, Tonelli F, Rubio CA, Mini E and Nesi G conceived and designed the study; Bruno L, Landini I and Napoli C acquired and analyzed the data; Nobili S and Nesi G wrote the paper; Bechi P, Tonelli F, Rubio CA and Mini E revised the paper critically for important intellectual content.

Supported by Ente Cassa di Risparmio di Firenze

Correspondence to: Gabriella Nesi, MD, PhD, Division of Pathological Anatomy, Department of Critical Care Medicine and Surgery, University of Florence, Viale G.B. Morgagni 85, Florence, 50134, Italy. gabriella.nesi@unifi.it

Telephone: +39-55-4478114 Fax: +39-55-4379868

Received: April 27, 2010 Revised: August 9, 2010

Accepted: August 16, 2010

Published online: January 21, 2011

In this review, we summarize the sometimes contradictory findings regarding those markers which influence the progression of gastric adenocarcinoma.

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Key words: Gastric cancer; Gene alterations; Prognosis; Molecular pathology

Peer reviewers: Dr. Thomas Wex, PhD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, Magdeburg, 39120, Germany; Hai-Yong Han, PhD, MS, BS, Division of Clinical Translational Research, The Translational Genomics Research Institute (TGen), 445 N. Fifth Street, Phoenix, AZ 85004, United States

Nobili S, Bruno L, Landini I, Napoli C, Bechi P, Tonelli F, Rubio CA, Mini E, Nesi G. Genomic and genetic alterations influence the progression of gastric cancer. *World J Gastroenterol* 2011; 17(3): 290-299 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/290.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.290>

Abstract

Gastric cancer is one of the leading causes of cancer-related deaths worldwide, although the incidence has gradually decreased in many Western countries. Two main gastric cancer histotypes, intestinal and diffuse, are recognised. Although most of the described genetic alterations have been observed in both types, different genetic pathways have been hypothesized. Genetic and epigenetic events, including 1q loss of heterozygosity (LOH), microsatellite instability and hypermethylation, have mostly been reported in intestinal-type gastric carcinoma and its precursor lesions, whereas 17p LOH, mutation or loss of E-cadherin are more often implicated in the development of diffuse-type gastric cancer.

INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related deaths worldwide, although the incidence has gradually decreased in many Western countries^[1]. Several attempts to classify gastric cancer have been made over the past decades. Most successful, and widely used, is the classification by Lauren, which, by microscopic morphology alone, distinguishes two main cancer pathogeneses, diffuse and intestinal subtypes, which clearly appear as dissimilar clinical and epidemiological entities. Although most of the genetic alterations that have been reported are observed in both intestinal and diffuse gastric cancers, it has become apparent that these two tumor types result from different genetic pathways^[2] (Table 1).

Microsatellite instability and *p53* mutation, reduced

p27 expression, cyclin E overexpression and 6.0-kb transcripts of the *c-met* gene are involved in malignant transformation from precancerous lesions to intestinal-type gastric cancer. In addition, *DCC* loss, *APC* mutations, 1q loss of heterozygosity (LOH), *p27* loss, reduced expression of tumor growth factor (TGF)- β type I receptor and *HER2* gene amplification are frequently associated with an advanced stage of intestinal-type gastric carcinoma. In contrast, LOH at chromosome 17p (*p53*) and mutation or loss of E-cadherin are more often implicated in the development of diffuse-type gastric cancer, while loss of *p27* and gene amplification of *K-sam* and *c-met* lead to disease progression and metastatic spread.

The two types of gastric carcinoma organize different patterns of interplay between neoplastic and stromal cells through the growth factor/cytokine receptor system, which has a critical role in cell growth, apoptosis, morphogenesis, angiogenesis and metastasis. Other genetic factors, such as DNA polymorphism and genetic instability, may also be implicated in the two distinct major genetic pathways of gastric carcinogenesis.

GENOMIC INSTABILITY

Two phenotypes of genomic instability are generally recognized in gastric cancer: the phenotype associated with microsatellite instability (MSI) and that which is associated with chromosomal instability (CIN). These phenotypes are not necessarily independent and may even overlap in some cases^[3].

MSI

MSI is a common feature of gastric cancer due to a deficit in the DNA mismatch repair system and derives from the presence of spontaneous DNA replication errors in simple repetitive sequences^[4]. A standard panel of microsatellite markers, including mononucleotide (*BAT26* and *BAT25*) and dinucleotide (*D2S123*, *D5S346* and *D17S250*) repeats, has been recommended and guidelines for MSI testing (Bethesda Guidelines) have been drawn up^[5]. Using the reference panel, three levels of MSI can be identified: high-level MSI (MSI-H), low-level MSI (MSI-L) and microsatellite stable (MSS). Recently, it has been established that mononucleotide repeats are instrumental in detecting MSI-H tumors because of their high sensitivity and specificity, and MSI-L has been defined as instability limited to dinucleotide loci^[6]. After the adoption of the Bethesda panel, MSI-H phenotype was reported in a range of 5%-50% of all gastric carcinomas with significant differences in various ethnic groups. MSI-H appears to be a phenotypical marker of an underlying cellular defect involving DNA mismatch repair (MMR). Functional inactivation by mutations or epigenetic mechanisms of MMR genes, including *hMLH1* and *hMSH2*, is responsible for the MSI-H phenotype in gastric cancer. Abnormal loss of protein expression of either *hMLH1* or *hMSH2* has been observed in MSI-H gastric carcinomas^[7]. In particular, altered expression of *hMLH1* has been associated with gene inactivation by promoter hypermethylation.

MSI-H gastric carcinomas follow a molecular pathway of tumor progression, characterized by the presence of multiple frameshift mutations affecting mononucleotide tracts within genes involved in cancer-related molecular networks which control cellular homeostasis at different levels. MSI-related mutations occur in many genes at variable frequencies^[4]. Genes regulating cell-cycle and apoptotic signaling are frequently targeted in MSI-H gastric carcinomas and include *TGF β R2*, *IGF1R*, *TCF4*, *RIZ*, *BAX*, *CASPASE5*, *FAS*, *BCL10* and *APAF1*^[8]. Moreover, genes involved in genomic integrity maintenance, i.e. *hMSH6*, *hMSH3*, *MED1*, *RAD50*, *BLM*, *ATR* and *MRE11*, are also frequently altered in MSI-H tumors^[9]. Several studies indicate that, in most MSI-H gastric cancers, multiple target genes are simultaneously mutated and multiple hits impact on different genes in the same pathway^[10]. In contrast, gastric carcinomas with MSS and MSI-L exhibit predominant *p53* mutations^[7].

As compared with MSS or MSI-L, gastric carcinomas with MSI-H show a significantly higher frequency of antral location, intestinal subtype, a lower incidence of lymph node metastasis and improved survival^[8,11-15].

CIN

CIN is a feature of various tumors, including gastric cancer, commonly associated with chromosomal aberrations responsible for major modifications of DNA content, i.e. changes in chromosome copy number, and also high-level LOH, gene deletions and/or amplifications^[16,17]. All these alterations may lead to oncogene activation and/or tumor suppressor gene inactivation. As with other tumors, aneuploidy is generally considered an unfavorable prognostic factor^[18-21], though contrasting results have been reported^[22-25].

High CIN levels have also been associated with a shorter survival in gastric cancer patients^[26] and high LOH frequencies have been identified at several chromosome arms, including 1p, 3p, 4p, 5q, 7p, 8p, 8q, 9p, 12p, 13q, 17p, 18q, 20q and 22q^[27-29].

The allelotype of gastric carcinoma is similar to that of colorectal and esophageal cancers, suggesting the presence of a common genetic pathway for tumor development. Some of these chromosomal segments include genes which are strongly implicated in carcinogenesis, such as the *p53* gene on chromosome 17, *DCC*, *DPC4* and *SMAD2* genes on chromosome 18, and *APC* and *MCC* genes on chromosome 5. Several studies have found that tumors with LOH at chromosome 5q, 18q or 17p had a poorer prognosis than tumors that did not show LOH at these sites^[30,31].

EPIGENETIC INSTABILITY

Epigenetic changes, such as aberrant methylation of CpG islands in promoter regions, are commonly detected in human cancers and can permanently inactivate tumor-suppressor genes and affect important pathways of cell cycle regulation and proliferation. The methylation of CpG islands may be considered a third molecular phenotype of

Table 1 Molecular genetic changes in gastric cancer

	Abnormalities	Intestinal phenotype (%)	Diffuse phenotype (%)	Local progression	Distant metastasis	Prognosis	Prolonged survival	Ref.
Microsatellite instability	Mutation, hypermethylation, reduced expression	20-30	0-10	No	NA	Good	Yes	[8,11]
Tyrosine kinases								
HER2/neu	Amplification/overexpression	10-15	0	Yes	Yes	Poor	No	[57-59]
RUNX3	Hemizygous deletion/hypermethylation/loss of expression	15-45	40-80	Yes	Yes	Poor	No	[62-64]
FHIT	Loss of protein expression (LOH, MSI)	35-65	20-80	Yes	Yes	Poor	No	[65,66]
NM23	Downregulation	3-25	30-70	Yes	Yes	Poor	Discordant results	[73,75]
VEGF	Overexpression	65	35-45	Yes	Yes	Poor	No	[77-79]
HIF-1 α	Overexpression	25-60	45-60	Discordant results	NA	NA	Discordant results	[80-82]
COX2	Overexpression	60-70	30-70	Yes	Yes	Discordant results	No	[77,83,84]
SPARC	Overexpression	70-80	25-55	Discordant results	Yes	Poor	No	[85-87]
p53	LOH/mutation/hypermethylation/overexpression	20-40	20-40	Yes	Yes	No correlation	No	[88-91]
p21	Loss	60		Yes	Yes	Poor	No	[92-94]
p27	Reduced expression	50		Yes	Yes	Poor	No	[95-97]
bcl2	LOH/overexpression	40	0	No	No	Good	Yes	[98]
BAX	Reduced expression	10	5	NA	Yes	Poor	No	[99]
pRb	Reduced expression	60	50	NA	NA	Poor	No	[1,92]
c-myc	Overexpression	45	10	Possible	Possible	Poor	No	[101-104]
	Amplification	15	5	Possible	Possible	Poor	No	
Cyclin E	Amplification/overexpression	15-20		Yes	Yes	Poor	No	[92]
E-cadherin	LOH/mutation/hypermethylation/reduced expression	0	50	Yes	Yes	Poor	No	[107,108]
MUC1	Overexpression	30-60	15-65	Yes	No	Poor	No	[65,106,110]
PRL-3	Overexpression	30-40	25-60	Yes	Discordant results	Poor	No	[112-114]
Tumor-associated proteases								[115-117]
PAI-1	Overexpression	45-75	35-50	Yes	Yes	Poor	No	
uPAR	Overexpression	40-75	30-50	Yes	NA	Poor	No	
uPA	Overexpression	65	30	Yes	NA	Poor	No	

FHIT: Fragile histidine triad; LOH: Loss of heterozygosity; MSI: Microsatellite instability; VEGF: Vascular endothelial growth factor; HIF-1 α : Hypoxia inducible factor-1 α ; COX2: Cyclooxygenase-2; SPARC: Secreted protein acidic and rich in cysteine; PRL-3: Phosphatase regenerating liver 3; PAI-1: Plasminogen activator inhibitor type 1; uPA: Urokinase-type plasminogen activator; u-PAR: u-PA receptor; NA: Not available.

gastric cancer and the tumor-related genes more commonly methylated are *APC*, *CDH1*, *MHL1*, *CDKN2A*, *CDKN2B* and *RUNX3*. It has also been widely reported that *CDKN2A*, *CDH1* and *MLH1* are more frequently inactivated by promoter methylation rather than by mutations^[32].

A series of individual methylated genes has been related to prognosis in gastric cancer. Methylation of tumor-suppressor genes, such as *CDH1*^[33], *DKK3*^[34], *PTEN*^[35] and *MGMT*^[36], of putative tumor-suppressor genes, such as *TFPI2*^[37] and *CACNA2D3*^[38], and of other tumor-related genes, such as *PCDH10*^[39] and *SOX2*^[40], has been associated with shorter disease-free and/or overall survival.

The combined use of *APC* and *CDH1* methylation markers has identified a subgroup of patients with worse prognosis^[41]. Conversely, methylation of single genes has been associated with a better prognosis in some cases. Patients showing methylation of *APC*^[42], the M1 region of *MAL* promoter^[43] and cyclooxygenase-2 (*COX2*)^[44] showed prolonged survival, compared to patients without methylation of these genes.

As with colorectal cancer, the CpG island methylator phenotype (CIMP), characterized by concurrent promoter hypermethylation of multiple genes, has also been described in gastric cancer^[45,46] and it has been shown to correlate with hypermethylation of other known cancer-related genes, such as *p16*, *bMLH1* and *THBS-1*^[45,47]. Furthermore, the CIMP status is associated with clinically useful information and patients with negative CIMP methylation have significantly shorter survival than those with high CIMP methylation^[46,48].

ALTERATIONS OF GENES INVOLVED IN MOLECULAR PATHWAYS

Genetic and genomic variations occurring in genes and molecules that participate in proliferation, invasion and metastasis (e.g. growth factors and their receptors, signal transducers, cell-cycle and apoptosis regulators, cell adhesion molecules, DNA repair genes and matrix metallo-

proteinases) may influence the prognosis of patients with gastric cancer.

Tyrosine kinases

Amplification of some tyrosine kinases (c-met, K-sam and HER2/neu) is associated with human gastric cancer progression. Alternatively, spliced transcripts and enhanced protein expression levels for some of these tyrosine kinases are correlated with the clinical outcome of gastric cancer patients^[49].

The oncogene *c-met*, encoding for the hepatocyte growth factor receptor, is preferentially amplified in diffuse-type tumors and has been described to be well correlated with stage and prognosis^[50,51]. Overexpression of c-met has also been shown to be associated with lower survival probability^[52,53].

K-sam oncogene, a member of the fibroblast growth factor receptor family, is more frequently activated in diffuse-type tumors^[2]. Overexpression of K-sam occurs in approximately 32% of diffuse-type gastric cancers, and the prognosis of K-sam-positive patients is poorer than that of K-sam-negative patients^[54].

The HER2 protein (HER2/neu or ErbB-2) is a glycoprotein with tyrosine kinase activity, homologous to the epidermal growth factor receptor. HER2 is codified by a gene located on chromosome 17q21 and does not bind to any known ligand. Some studies demonstrated that overexpression of c-erbB2 is selectively found in intestinal tumors and may serve as a prognostic marker for tumor invasion and lymph node metastasis. Overexpression of HER2 protein in gastric cancer has been reported to range from 7.4% to 38%^[55-57]. The prognostic value of HER2 expression and/or amplification has been widely investigated with controversial findings. Although most available studies indicate that the overexpression of HER2 is an independent prognostic factor associated with a shorter disease-free^[58] and overall survival^[57-59], some studies failed to confirm its prognostic role on multivariate analysis^[51] or to find a correlation between HER2 overexpression and survival parameters^[56,60]. Also associated with poor survival is the presence of HER2 amplification^[61].

RUNX3

RUNX3, a gene that codifies for a member of the runt domain-containing family of transcription factors, frequently shows loss of expression due to hemizygous deletion and hypermethylation in gastric cancer. This gene, generally expressed in only 45%-50% of gastric cancer patients^[62,63], positively regulates the expression of BIM and p21, and negatively regulates vascular endothelial growth factor (VEGF), thus affecting apoptosis, cell growth arrest and angiogenesis. The loss or substantial decrease of *RUNX3* protein expression in gastric cancer has been significantly associated with shorter survival^[62,64].

FHIT

The fragile histidine triad gene (*FHIT*) encodes a diadenosine 5',5'''-P1,P3-triphosphate hydrolase and is generally inactivated by deletion or methylation in several

tumors, including gastric cancer. The absence of *FHIT* protein has been shown to correlate with higher tumor stage and histological grade^[64], as well as with poor overall survival^[65,66].

NM23

The *NM23* gene maps to chromosome 17q21 and encodes the nucleoside diphosphate kinase A, a member of the NDP kinase family. *NM23* expression is reduced in metastatic melanoma and breast cancer cell lines^[67]. Transfection into cell lines affects invasion, motility, colonization, differentiation and liver metastasis^[68]. Decreased expression of *NM23-H1*, the human homologue, is found in advanced stages of human cancer^[69,70].

The expression of the putative metastasis-suppressor gene *NM23* in gastric carcinoma is controversial. In several studies, expression of *NM23* has been shown to be inversely correlated with the metastatic potential of gastric cancer^[71,72] and with prolonged overall survival^[73]. The results of other studies, however, suggest that *NM23* is not a metastasis suppressor gene and does not show correlation with metastasis^[74,75].

VEGF

VEGF is a pro-angiogenic factor, frequently overexpressed in tumors. Mutations of *p53*, which under physiological conditions downregulates VEGF, may be responsible for its overexpression^[76].

A correlation of the expression of VEGF with lymph node and liver metastasis has been described^[77] and patients with VEGF-positive tumors have a rather worse prognosis than those with VEGF-negative tumors^[78,79].

HIF-1 α

The hypoxia inducible factor, *HIF-1 α* , is a transcription factor that plays an essential role in cellular and systemic homeostatic responses to hypoxia. The prognostic role of *HIF-1 α* expression in gastric cancer patients is controversial: high levels have been associated with a shorter overall survival^[80], but also with no difference in survival parameters^[81]. However, its upregulation (high *HIF-1 α* mRNA or protein levels) has been found to be positively correlated with VEGF^[82] or *p53*^[80] protein expression in gastric cancer patients, and overall survival of patients with high mRNA levels of *HIF-1 α* and VEGF, as well as of *HIF-1 α* and *p53*, was shorter compared to patients with different features.

COX2

COX2 is one of the key isoenzymes in the production of prostaglandins, and is thought to be involved in carcinogenesis. Some studies indicate that *COX2* may play a role in the development of gastric cancer, and its overexpression is associated with nodal metastasis, tumor invasion and differentiation, implicating a poor prognosis^[77,83,84].

SPARC

The secreted protein acidic and rich in cysteine (SPARC or osteonectin) is a member of a family of matricellular

proteins that modulates cell-matrix interactions and cell function without participating in the structural scaffolding of the extracellular matrix. Since SPARC alters membrane permeability, cell shape, proliferation, migration and attachment, it may play a role in angiogenesis. It has been reported that its overexpression correlates with distant metastasis and poor prognosis^[85-87]. It is not clear whether SPARC overexpression is a useful marker in the prediction of lymph node metastasis development^[85].

p53

The p53 protein plays a fundamental role in cell growth and division. The function of the *p53* gene is more frequently altered due to LOH and mutation than to DNA methylation. Mutations of *p53* are present in about 40% of early and advanced, well-differentiated gastric cancers^[88]. A lower incidence of *p53* mutations has been shown in young patients compared to older patients^[89].

p53 can be investigated by immunohistochemical techniques, bearing in mind that the half-life of the p53 mutant protein is prolonged. Cells carrying the p53 mutant protein can be stained with antibodies against p53, whereas cells carrying normal p53 are negative. Sequencing of the gene after screening can also be performed in order to determine the mutation location within the gene^[90].

Overexpression of p53 often occurs in the early stages of intestinal-type tumors, and there is no significant difference between early and advanced cancers. In contrast, p53 abnormalities are not often seen in the early stages of diffuse-type tumors, but tend to occur as the disease progresses^[91].

p21

p53 cell cycle regulatory function is mediated by different effectors. One of these is a cyclin-dependant kinase inhibitor (CDK I), the p21 protein. The cell cycle check points are controlled by a cascade of phosphorylation. Protein kinases such as cyclin-dependent kinases are activated by cyclins and inhibited by CDK I, although p21 is up-regulated not only through a p53 pathway, but also through a TGF β RII pathway.

Levels of p21 expression could indicate the absence of a functional p53 protein in neoplastic cells. It has been reported that the survival of gastric cancer patients with p21-positive tumors is significantly longer than that of patients with p21-negative tumors^[92]. The expression of p21 is usually assessed in combination with p53 status and contributes to predicting the clinical outcome of gastric cancer patients^[93,94].

p27

It has been suggested that the cyclin-dependent inhibitor p27, which controls the transition from G1 to S in the cell cycle, has prognostic relevance in gastric cancer. Reduced p27 expression is detected in approximately 40%-50% of gastric cancers^[28]. Some studies have shown that tumors with a low expression of p27 protein are poorly differentiated and at an advanced stage^[95,96]. However, some authors have found no difference in overall survival of gastric can-

cer patients whether with high or low p27 expression^[97]. p53, p21 and p27 have also been analyzed in combination, confirming their role as prognostic markers^[91].

BCL2

BCL2 and *p53* are closely linked in the regulation of apoptosis. LOH at the *BCL2* locus is frequently observed in gastric cancer. The overexpression of *BCL2* may have a role in the development of gastric cancers. It has been shown that *BCL2* overexpression reduces cellular proliferative activity and correlates with a less aggressive biological behavior of the tumor. The prognostic role of *BCL2* on its own or in association with p53 has not yet been elucidated^[98].

BAX

BAX gene encodes a protein belonging to the BCL family members. Negative *BAX* protein expression has been associated with de-differentiation, lymph node metastasis and shorter survival, suggesting that *BAX* status may play a role in the development and differentiation of gastric cancer and tumor progression^[99].

pRb

pRb encodes a protein that is a negative regulator of the cell cycle. Poor prognosis of gastric cancer patients with low levels of pRb expression has been reported^[92,100].

c-myc

c-myc gene encodes a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. The c-myc protein has been shown to be significantly enhanced in well-differentiated gastric cancer^[101] and associated with a poor prognosis^[102]. Although c-myc is a short-lived protein in normal cells, its stability is increased in transformed cells through several mechanisms. One of these has recently been identified in the overexpression of a human oncoprotein, the cancerous inhibitor of protein phosphate 2A (CIP2A) that stabilizes c-myc^[103]. Interestingly, the expression of CIP2A has been associated with reduced overall survival in gastric cancer patients^[104].

Cyclin E

Cyclin E overexpression correlates with invasiveness and proliferation and may be a marker of tumor aggressiveness. Although somatic mutations of the cell cycle inhibitor *p16^{MTS}* are rare, its reduced expression is associated with depth of invasion and metastatic potential in both diffuse- and intestinal-type gastric carcinomas. However, recent data show that the survival of gastric cancer patients with cyclin E-positive tumors is not significantly shorter than that of negative patients^[92].

E-cadherin

Cell adhesion molecules are implicated in human carcinogenesis. Cadherin is a superfamily of calcium-mediated membrane glycoproteins, forming one of the four classes of adhesion molecules. E-cadherin, one of the members

of the transmembrane glycoprotein family expressed by epithelial tissues, not only acts as a cell adhesion molecule, but also plays an important role in growth development and carcinogenesis. The intact function of E-cadherin is crucial for the establishment and maintenance of epithelial tissue polarity and structural integrity. Around 25%-40% of hereditary diffuse gastric cancers are caused by heterozygous E-cadherin. The inactivation of the second allele occurs by mutation and methylation events, and this results in the complete inactivation of the protein^[105]. Reduced expression of E-cadherin correlates with infiltrative and metastatic ability in gastric cancer^[33] and the gene encoding E-cadherin, *CDH1*, was among the first to be considered as an invasion suppressor gene. Patients with E-cadherin-positive gastric cancers showed statistically significant prolonged 3- and 5-year survival rates, compared to patients with E-cadherin-negative tumors^[33,106].

It has been shown that serum soluble E-cadherin is increased in several non-neoplastic diseases and also in various cancers, including gastric tumors. E-cadherin may be a potentially useful prognostic marker and high levels of soluble E-cadherin correlate with the depth of tumor invasion, as well as inoperability^[107]. In addition, levels higher than 10000 ng/mL predict a survival of less than 3 years in more than 90% of patients^[108].

The Wnt-frizzled- β -catenin signaling pathway is frequently activated in gastric carcinoma (e.g. upregulation of *Wnt* gene expression or of genes for Wnt ligand receptors, upregulation of *RAC1* and inactivation of *APC*), leading to poor differentiation and increased tumor invasiveness^[109].

MUC1

Mucins are high-molecular weight glycoproteins containing oligosaccharides. These glycoproteins constitute the major components of the mucus that protects the gastric epithelium. Overexpression of mucin 1 (MUC1) has been linked to poor prognosis in gastric cancer patients^[65,110].

It has been reported that MUC1 may accelerate tumor invasion by the impairment of E-cadherin^[111]. The combined expression of MUC1 and E-cadherin shows that survival for gastric cancer patients with abnormal E-cadherin/MUC-positive expression was shorter than for patients with other expression patterns^[106].

PRL-3

The phosphatase regenerating liver 3 (*PRL-3*) gene encodes a protein belonging to a class of prenylated protein tyrosine phosphatases. These proteins are cell signaling molecules with a regulatory role in several cellular processes. The prognostic role of PRL-3 in solid tumors, including gastric cancer, has been recently reviewed by Bessette *et al.*^[112]. High expression of PRL-3 has been associated with several unfavorable clinical parameters, such as tumor size, depth of invasion, lymphatic invasion, advanced stage and shorter overall survival. Successive studies have confirmed these findings^[113,114].

Tumor-associated proteases

Tumor-associated proteases and their inhibitors play a

central role in tumor invasion and metastasis. The positive correlation of histological data with the urokinase-type plasminogen activator (uPA) and the plasminogen activator inhibitor type I (PAI-1) has been reported. Moreover, the independent prognostic impact of both uPA and PAI-1 on the survival of gastric cancer patients has been demonstrated. Elevated uPA and PAI-1 levels have been shown to be associated with shorter survival^[115,116]. A trend towards poor prognosis has also been observed in patients with high expression of the u-PA receptor (u-PA-R)^[113] and the uPA system may therefore be a target for novel therapeutic agents.

The prognostic role of some uPA genotypes has recently been investigated and an association was demonstrated between the exon 6 C/T polymorphism with invasive phenotype, but not with susceptibility or survival^[117].

CONCLUSION

Gastric carcinomas are histologically and genetically heterogeneous and are influenced by gene-environment interactions resulting in the activation of multiple molecular pathways. The molecular subtypes of gastric cancer include three main groups of tumors characterized by either the CIN, the MSI or the CIMP pathways. Currently, it is not clear whether or in what way knowledge of these subtypes of gastric carcinomas is of use in clinical practice, with regard to predicting specific pathways with mutational and regulatory alterations that may interfere with targeted therapies.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Lin YP

Hepatocellular carcinoma xenograft supports HCV replication: A mouse model for evaluating antivirals

Sidhartha Hazari, Henry J Hefler, Partha K Chandra, Bret Poat, Feyza Gunduz, Tara Ooms, Tong Wu, Luis A Balart, Srikanta Dash

Sidhartha Hazari, Partha K Chandra, Bret Poat, Feyza Gunduz, Tong Wu, Srikanta Dash, Department of Pathology and Laboratory Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, United States

Henry J Hefler, Feyza Gunduz, Luis A Balart, Srikanta Dash, Department of Gastroenterology and Hepatology, Tulane University Health Sciences Center, New Orleans, LA 70112, United States

Tara Ooms, Department of Comparative Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, United States

Author contributions: Hazari S performed the majority of the experiments, acquisition and analysis of data, and participated in design of the study; Hefler HJ performed some animal experiments and helped in the antiviral studies; Chandra PK performed the real time RT-PCR and RPA; Poat B and Gunduz F performed biochemical assays and tumor cell inoculation of SCID mice; Ooms T performed the intrasplenic injection of S3-GFP cells into SCID-NOD mice and gave valuable suggestions on animal handling and care during the whole study; Wu T performed the histological evaluation of subcutaneous and liver tumors, and took all the histological images; Balart LA provided valuable suggestions and edited the manuscript; Dash S conceptualized and designed the study, evaluated the overall results, and wrote the manuscript.

Supported by Funds received from the National Cancer Institute (CA127481, CA129776), Geyer Foundation, New York, Louisiana Cancer Research Consortium and Tulane Cancer Center

Correspondence to: Srikanta Dash, PhD, Professor, Department of Pathology and Laboratory Medicine and Gastroenterology and Hepatology, Tulane University Health Sciences Center, 1430 Tulane Ave., SL-79, New Orleans, LA 70112, United States. sdash@tulane.edu

Telephone: +1-504-9882519 Fax: +1-504-9887389

Received: June 9, 2010 Revised: August 3, 2010

Accepted: August 10, 2010

Published online: January 21, 2011

METHODS: We developed a stable S3-green fluorescence protein (GFP) cell line that replicated the GFP-tagged HCV sub-genomic RNA derived from a highly efficient JFH1 virus. S3-GFP replicon cell line was injected subcutaneously into γ -irradiated SCID mice. We showed that the S3-GFP replicon cell line formed human HCC xenografts in SCID mice. Cells were isolated from subcutaneous tumors and then serially passaged multiple times in SCID mice by culturing in growth medium supplemented with G-418. The mouse-adapted S3-GFP replicon cells were implanted subcutaneously and also into the liver of SCID mice *via* intrasplenic infusion to study the replication of HCV in the HCC xenografts. The tumor model was validated for antiviral testing after intraperitoneal injection of interferon- α (IFN- α).

RESULTS: A highly tumorigenic S3-GFP replicon cell line was developed that formed subcutaneous tumors within 2 wk and diffuse liver metastasis within 4 wk in SCID mice. Replication of HCV in the subcutaneous and liver tumors was confirmed by cell colony assay, detection of the viral RNA by ribonuclease protection assay and real-time quantitative reverse transcription polymerase chain reaction. High-level replication of HCV sub-genomic RNA in the tumor could be visualized by GFP expression using fluorescence microscopy. IFN- α cleared HCV RNA replication in the subcutaneous tumors within 2 wk and 4 wk in the liver tumor model.

CONCLUSION: A non-infectious mouse model allows us to study replication of HCV in subcutaneous and metastatic liver tumors. Clearance of HCV by IFN- α supports use of this model to test other anti-HCV drugs.

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Abstract

AIM: To develop a hepatocellular carcinoma (HCC) xenograft model for studying hepatitis C virus (HCV) replication in a mice, and antiviral treatment.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Tumor xenograft; SCID mouse; Interferon- α ; Antiviral agent; Virus replication

Peer reviewers: Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryō-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan; Dr. Sara Lindén, PhD, Professor, Mucosal Immunobiology and Vaccine Center, Gothenburg University, Box 435, Göteborg, 405 30, Sweden

Hazari S, Heffler HJ, Chandra PK, Poat B, Gunduz F, Ooms T, Wu T, Balart LA, Dash S. Hepatocellular carcinoma xenograft supports HCV replication: A mouse model for evaluating antivirals. *World J Gastroenterol* 2011; 17(3): 300-312 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/300.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.300>

INTRODUCTION

Hepatitis C virus (HCV) is the most common blood-borne infection that affects the liver. The majority of individuals infected with HCV end up with a chronic disease in which the virus replicates in the liver for a long period of time. There are approximately 170 million people currently infected with HCV worldwide^[1,2]. The incidence of new HCV infections each year is increasing in developing nations due to blood transfusion from unscreened donors, which makes HCV a significant worldwide public health problem. The standard treatment option for chronic HCV infection is combination of pegylated interferon- α (IFN- α) with ribavirin^[3]. However, the majority of chronic HCV patients do not clear the virus infection with this treatment, and these individuals remain at high risk for developing cirrhosis and liver cancer. There is no effective treatment available for liver cirrhosis and cancer, so the development of alternative therapeutic strategies using a small animal model system to cure chronic HCV infection is crucial.

Development of novel antiviral therapies that target the multiple steps in the HCV life cycle, including viral genome replication, assembly, and infection is now possible due to the availability of the infectious, full-length HCV cell culture systems. A number of antiviral strategies have been developed during the past 2 years, including small molecules, anti-sense oligonucleotides, siRNA, ribozymes, and recombinant antibodies that have been designed to inhibit HCV replication in cell culture models^[4,5]. Progress in the targeted delivery of these antiviral molecules to inhibit viral replication in the liver is hampered due to the lack of a small animal model for HCV infection. The development of an animal model for HCV infection has been difficult due to the fact that the virus has limited host tropism and can infect only humans and chimpanzees^[6,7]. The chimpanzee is the only natural animal model for studying HCV infection^[8]. The chimpanzee model has been used in the past to study many aspects of HCV pathobiology and infectivity using cloned viral genomes, and it remains an important animal model. However, it is a difficult and expensive animal model that cannot be used to optimize experiments that deal with the liver-targeted delivery of intracellular treatment approaches such

as siRNA or antibodies. Therefore, a small animal model for HCV infection is required to test different experimental therapies developed using HCV cell culture systems. Rodents are the preferred animal models for testing gene delivery experiments because of their size, low cost and short gestation period. Previously, researchers have developed mouse models for HCV infection using transgenic technology or direct transfection of HCV RNA into the liver^[9]. Transgenic mice are not models for HCV infection or antiviral testing because of stable integration of HCV cDNA into the mouse chromosome. It will be difficult to differentiate the viral RNA replication from the HCV RNA produced due to cellular transcription in the transgenic animal models. There has been limited success using this transgenic approach because mouse hepatocytes do not support HCV RNA replication or infection^[9,10].

To overcome this limitation, four different mouse models have been developed to study experimental HCV infection *in vivo*: the immunotolerized rat model^[11], the Trimer mouse model^[12], the uPA/SCID mouse model^[13-15], and the Fah^{-/-} Rag^{-/-} IL-2^{-/-} mouse model^[16]. The rat model for HCV infection has been developed using immunotolerized rat embryos that can allow transplantation of human hepatoma cell lines that can be infected with HCV. These models have been used to study the complete life cycle of HCV infection, and antiviral testing. In the latter three models, researchers have used highly specialized mouse strains that support transplantation of human hepatocytes into the mouse liver. These models appear to be highly relevant animal models for HCV infection. A number of investigators have been using these models to infect chimeric mice with HCV or hepatitis B virus^[13-16]. However, these models are relatively complicated to use because they require specialized surgical skills, special strains of mice, use of human hepatocytes, as well as HCV-infected serum samples. Recently, a xenograft mouse tumor model for HCV infection has been described that utilizes the mouse-adapted replicon cell line that contains the luciferase reporter^[17,18]. The replication of HCV RNA has been measured in the mouse liver and subcutaneous tumor xenograft using a whole-body real-time imaging system. This model has been used to evaluate the antiviral properties of IFN- α and protease inhibitors. There is a lot of interest in this mouse model because it is less expensive, non-infectious, and can be easily adapted to any research laboratory.

We describe here a mouse model for studying the replication of the JFH1 sub-genomic clone of HCV in subcutaneous and liver tumors using a green fluorescence protein (GFP)-labeled sub-genomic replicon in the Huh-7 cell line. The development of a mouse-adapted GFP-labeled replicon cell line, combined with the highly efficient JFH1 virus facilitates high-level replication of the HCV in subcutaneous and liver tumors of laboratory mice with a partially suppressed immune system. We have developed a mouse-adapted HCC cell clone that replicates sub-genomic HCV RNA and forms HCC xenografts in SCID mice. We show that replication of HCV in the subcutaneous and liver tumors can be assayed using several biochemical

methods by looking at GFP expression and evaluating the antiviral effects on HCV by cell colony assay, ribonuclease protection assay, and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). We showed that IFN- α successfully inhibited replication of HCV RNA in the HCC xenograft, which indicates that the model can be used to test the efficacy of different antiviral strategies that target the HCV replication cycle.

MATERIALS AND METHODS

Animals

Female SCID/bg and NOD/SCID γ mice 6-8 wk old were obtained from Charles River Laboratories (Wilmington, MA, USA) and Jackson laboratory (Bar Harbor, ME, USA), respectively. The mice were maintained in sterile conditions in a pathogen-free environment at the Department of Comparative Medicine, Tulane University Health Sciences Center. All animal experiments were carried out after receiving approval from the Institutional Animal Care and Use Committee (IACUC), Tulane University Health Sciences Center. All the SCID mice were γ -irradiated at 3 Gy (approximately 3.2 min) 1 d prior to cell transplantation. SCID/bg mice were used for subcutaneous tumor xenografts and NOD/SCID mice were used for liver tumor development. Throughout every experiment, we carefully checked the mice for their well-being, body condition and movement. All of the mice were weighed every alternate day to check for weight loss. A drop in their body weight was considered as an indication of sickness. After surgery, the mice were kept in separate sterile cages and observed carefully until they fully recovered.

Mouse-adapted replicon cell lines

We developed a stable Huh-7 cell line (S3-GFP) that replicated the HCV-GFP sub-genomic RNA of the JFH1 clone. The replicon cell line expressed a high level of GFP that could be visualized directly under a fluorescence microscope. We previously have shown that IFN- α inhibits replication of HCV in the S3-GFP cell line in a dose-dependent manner^[19]. One million GFP-replicon cells were implanted subcutaneously into the right and left flank of SCID/bg mice. Mice were followed for the development of tumors. When the tumors reached 10 mm in size, they were harvested in a sterile Petri dish. Cells were dissociated by collagen digestion and separated by low-speed (500 rev/min) centrifugation. The cell pellet was resuspended in RBC lysis buffer (eBioscience, San Diego, CA, USA) for 15 min; cells were centrifuged and the cell pellet was resuspended in DMEM with 10% FBS supplemented with G-418 (1 mg/mL). The cells were cultured and expression of GFP in the Huh-7 cells was examined over time under a fluorescence microscope. When most of the Huh-7 cells in the culture showed GFP expression, they were harvested and injected into SCID mice for tumor development. The *in vivo* passaging experiments were repeated several times until > 50% of the cells in the subcutaneous tumor were GFP-positive.

Intrasplenic infusion of mouse-adapted replicon cells

Intrasplenic infusion of replicon cells was performed using a previously described procedure^[20]. NOD/SCID γ mice were anesthetized with isoflurane under a laminar flow cabin. The surgical area was shaved and swabbed with betadine scrub. A small incision was made in the left flank in order to expose the spleen and carry out the cell injection. The spleen was accessed with a small forceps, and 10^6 replicon cells were injected into the inferior splenic pole; a monofilament suture was placed across the spleen at the site of injection to reduce spillage of cells into the abdominal cavity. The peritoneal wall and skin were separately closed using a monofilament suture and staples, respectively. After 3, 4, 5 and 6 wk, the animals were euthanized by CO₂ inhalation and their livers were removed. Part of the liver was fixed in 10% buffered saline for 72 h, processed, and embedded in paraffin. Tissue blocks were made for histological analysis after hematoxylin and eosin staining. The remaining part of the liver tissue was frozen in OCT compound for GFP expression analysis.

IFN treatment

IFN- α 2b (Intron A; Schering-Plough, NJ, USA) was diluted in PBS at a concentration of 150 IU/ μ L and stored at -70°C. Both the subcutaneous and liver tumor models were validated by intraperitoneal injection of 100 μ L IFN- α solution (total 15000 IU/mouse) three times weekly. A group of five mice was used to test the IFN antiviral effect in the subcutaneous and liver tumor models.

Histology and immunocytochemistry

The growth of HCC xenografts in the SCID mice was examined by hematoxylin and eosin staining of fixed and paraffin-embedded mouse tumor and liver specimens. Five-micrometer sections were cut from each tissue block, mounted on a glass slide, and dried over night at room temperature. All of the sections were deparaffinized in xylene, rehydrated by dipping in a graded alcohol series, and washed in PBS. To demonstrate the implantation of replicon cells in the liver, the tissue sections were stained with an antibody against human serum albumin (Dako, Carpinteria, CA, USA). The immunoreactivity of the albumin antibody was detected using the ABC detection kit using a standard laboratory protocol. To demonstrate expression of GFP in the subcutaneous and liver tumors, frozen sections were prepared. The sections were washed in PBS and stained with Hoechst dye (H33342, Calbiochem, Germany). Expression of GFP in the HCC xenograft was observed using a fluorescence microscope (Olympus) using a standard procedure^[19].

Cell colony assay

To study the replication of HCV sub-genomic RNA in the HCC xenografts, the liver was digested with collagenase and viable S3-GFP replicon cells were obtained by low-speed centrifugation. The cell pellet was suspended in 5 mL RBC lysis buffer (eBiosciences) for 15 min. The

viable tumor cells (S3-GFP) were cultured in DMEM supplemented with G-418 (1 mg/mL). Huh-7 cells that supported HCV RNA replication after IFN treatment were selected. The number of cell colonies formed in each cell culture dish was counted after Giemsa staining (Sigma, St. Louis, MO, USA).

RPA

Total RNA was isolated from the subcutaneous tumor by the GITC method and subjected to RPA for the detection of genomic positive-strand HCV RNA, using an antisense RNA probe that targeted the 5' untranslated region (UTR). For the RPA, 25 µg total RNA was mixed with a negative-strand RNA probe that targeted the 5'-UTR of HCV (10⁶ cpm) in a 10-µL hybridization solution, denatured for 3 min at 95°C, and hybridized overnight at 50°C. RNase digestion was performed in 200 µL RNase digestion buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L EDTA and 0.3 mol/L NaCl) that contained RNaseA/T1 cocktail at 1:100 dilutions (Ambion, Austin, TX, USA) for about 1 h at 37°C. It was then treated with 2.5 µL 25% SDS and 10 µL proteinase K (20 mg/mL) for 15 min. Samples were extracted with phenol:chloroform and precipitated with absolute ethanol. The pellet was suspended in 16 µL gel loading buffer, heat denatured, and separated on a 6% TBE-urea gel (Invitrogen, Carlsbad, CA, USA). The gel was dried and exposed to X-ray film (Kodak Bio-max-XAR, Rochester, NY, USA). To detect JFH1-HCV mRNA in the transfected cells, we prepared a plasmid construct called pCR-II-2a (Invitrogen), which contained the sequence of 79-297 nt of the 5'-UTR sequence of the JFH1 clone (pCR-II NT-218). This plasmid was linearized with the *Xba*I restriction enzyme. T7 RNA polymerase was used to prepare a negative-strand RNA probe for detection of positive-strand HCV RNA. The same amounts of the RNA extracts were subjected to RPA for GAPDH mRNA. We used a linearized pTRI-GAPDH-human antisense control template to prepare a probe to detect GAPDH mRNA using Sp6 RNA polymerase (Ambion). The appearance of a 218-nt fragment in the RPA indicated the presence of HCV positive-strand RNA.

RT-nested PCR and Southern blot analysis

To detect HCV RNA in the liver tumors, total RNA was isolated from the liver tissues by the GITC method^[21]. cDNA synthesis for HCV positive-strand RNA was carried out using 1 µg total cellular RNA, an outer antisense primer and avian myoblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). Amplification of the cDNA was performed in 50 µL reaction mixture that contained 250 ng outer sense primer and *Taq* DNA polymerase. The first PCR product was amplified by another round of PCR using outer and inner sets of primers, as described previously^[21]. The HCV PCR products were further subjected to Southern blot analysis by using a positive-sense HCV-specific ³²P-labeled oligoprobe. One microgram of total cellular RNA was used to amplify albumin mRNA levels by RT-PCR. The sequence of primer

and probe used in these experiments has been described previously^[21].

Real-time RT-qPCR

Real time RT-qPCR was performed to quantify HCV RNA levels in the infected cell culture using a published protocol^[22]. The 243-bp HCV DNA was amplified from the RNA extract by RT-PCR using the outer sense primer 5'-GCAGAAAGCGCCTAGCCATGGCGT-3' (67-90) and outer antisense primer 5'-CTCGCAAGCGCCCTATCAGGCAGT-3' (287-310). cDNA synthesis was performed from positive-strand HCV RNA using an outer antisense primer that targeted the highly conserved 5'-UTR region of HCV in a 20-µL volume. Two micrograms of total cellular RNA were mixed with 1 µL outer antisense primer (200 ng/µL), denatured at 65°C for 10 min, and annealed at room temperature. AMV reverse transcriptase (10 U) (Promega) was added and incubated at 42°C for 60 min in the presence of 50 mmol/L Tris, pH 8.3, 50 mmol/L EDTA, 500 nmol/L dNTP, 250 nmol/L spermidine, and 40 U RNasin (Promega). cDNA was stored at -20°C until use. SYBR Green real-time PCR amplification was performed in a 20-µL volume that contained 10 µL SYBR Green ER qPCR SuperMix, 1 µL (250 ng/µL) of sense and antisense primer with 4 µL cDNA and 4 µL distilled water. All samples were run in triplicate. The amplification was carried out using the standard program recommended by Bio-Rad Laboratories that included: a first cycle at 50°C for 2 min, 95°C for 8 min, and an additional 50 cycles in which each cycle consisted of a denaturation step at 95°C for 10 s, and annealing and extension steps at 60°C for 30 s. At the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (0.1°C/s) up to 95°C. Amplification, data acquisition, and analysis were performed on CFX96 Real Time instrument (Bio-Rad Laboratories) using CFX manager software (Bio-Rad Laboratories).

RESULTS

HCC xenografts formed subcutaneously support HCV replication

We prepared a chimeric clone of the sub-genomic HCV of JFH1 2a virus by fusing it with GFP in a frame with one of the non-structural proteins of HCV (NS5A). Huh-7 replicon cells that replicated the HCV-GFP sub-genomic RNA emitted a green fluorescent signal when exposed to a specific wavelength of light. This allowed direct visualization of HCV RNA replication. These replicon cells were cultured for > 1 year and were found to have stably maintained GFP expression with > 60%-80% of cells positive by flow analysis. We have shown that IFN-α inhibits replication of HCV RNA and inhibits GFP expression in a dose-dependent manner in the replicon cells^[19]. With this GFP-based replicon cell line, we sought to establish an HCC xenograft in SCID mice using the mouse-adapted replicon cells as described previously^[18]. The experimental steps involved in generating the *in vivo*

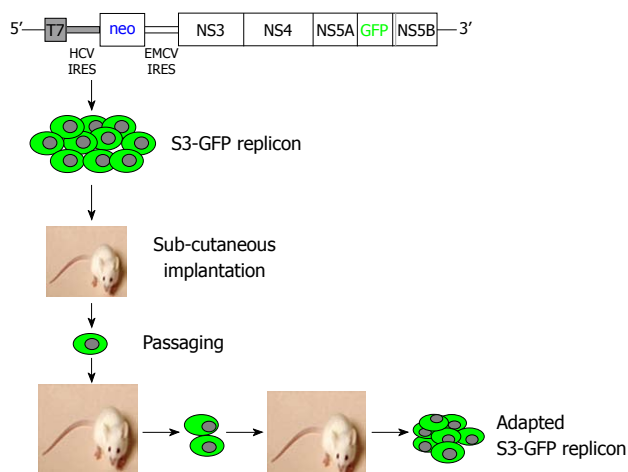


Figure 1 Summary of overall experimental plan to generate a mouse-adapted hepatitis C virus-green fluorescence protein replicon cell line. A chimeric replicon clone was prepared by inserting the green fluorescence protein (GFP) coding sequences with the NS5A sequences of the hepatitis C virus (HCV) sub-genomic clone. Huh-7 cells were transfected with a transcribed sub-genomic HCV-RNA replicon. A stable Huh-7 cell line with replicating HCV GFP chimera RNA (S3-GFP replicon) was developed. The replicon cell line was implanted in SCID mice for tumor development. Subcutaneous tumor that developed in SCID mice was collected and cells with replicating HCV-GFP were selected by culture in growth medium that contained G-418 (1 mg/mL). The *in vivo* adaptation process was repeated to generate a mouse-adapted S3-GFP replicon cell line that demonstrated 50% GFP expression in subcutaneous tumors.

adapted GFP replicon cells are summarized in Figure 1. S3-GFP replicon cells were injected subcutaneously into the right and left flank of SCID mice after γ irradiation. Tumors were harvested when they were 10 mm in size and cells were isolated and cultured in selection medium. After the first passage, we found that expression of GFP in the replicon cells was very faint and only a few cells (< 10%) showed bright GFP positivity. The sub-genomic HCV RNA has a neomycin gene selection marker that allows the selection of tumor cells that support HCV replication. Therefore, replicon cells were cultured with medium that contained G-418, to select for increased intracellular GFP expression. After 1 wk, a high level of GFP expression was seen in most of the cells in culture. This experiment suggested that *in vivo* tolerance occurred at the cellular level as well as at the level of intracellular virus replication in the replicon cells. We were able to select a homogeneous population of Huh-7 cells with stable GFP expression after the first passage in SCID mice. To obtain a GFP replicon cell line with increased growth and replication capacity, *in vivo* passaging experiments were repeated three additional times. Figure 2 suggests that *in vivo* passaging of replicon cells allowed for the selection of replicon cells with increased replicative ability. The expression of GFP in the frozen tumor sections was examined under a fluorescence microscope after nuclear staining. The levels of viral RNA replication in the subcutaneously formed HCC xenografts were very high and GFP expression was observed in most of the replicon cells (Figure 2). We generated a highly adapted GFP replicon cell line (S-3GFP/*in vivo*) that formed a HCC xenograft in SCID/bg mice within 2 wk.

HCC xenografts in the liver support HCV replication

It is important to develop an HCC xenograft in the liver because HCV replicates in the liver of the human host. Furthermore, a liver tumor model is needed to assess and optimize the targeted delivery of antiviral therapy. We wanted to determine whether the mouse-adapted replicon cells that formed a subcutaneous tumor could also form HCC in NOD/SCID mouse liver. The Huh-7 cells were infused into the liver by intrasplenic injection, and examined for liver tumor development at weekly intervals. The growth of mouse-adapted Huh-7 cells in the liver was slow compared to the subcutaneous model, and took almost 4 wk to reveal diffuse HCC nodules throughout the liver (Figure 3C and D). Normal mouse liver and histology are shown in Figure 3A and B. To examine whether HCV replication in liver tumors occurred at a similar level as that seen in the subcutaneous model, frozen sections of the liver tumors were examined for GFP expression. To our surprise, the level of HCV RNA replication was low in the liver tumor, and we could not see a very high level of GFP expression in the HCC xenografts formed in the SCID mouse liver. The loss of GFP expression was not due to an alteration in the open reading frame of GFP in the sub-genomic HCV RNA, because we could recover HCV GFP RNA levels when cells were isolated from the xenografts and cultured in medium supplemented with G-418. The negative GFP expression was due to the low level of HCV replication in the liver tumors. These results suggested that the liver microenvironment significantly suppressed HCV RNA replication compared to that in the subcutaneously formed tumors. We then examined whether these HCV GFP replicon cells needed to adapt to the liver microenvironment to support high-level replication of HCV. The mouse-adapted GFP replicon cells were therefore adapted to the liver microenvironment by another three passages. At each passage, we could recover HCV GFP expression in culture, which suggested that HCV RNA replication in the liver tumors was not completely lost. We then examined whether GFP expression could be seen in the HCC xenografts formed in the mouse liver, using the cells that had undergone three passages. We were able to see GFP expression in tumor nodules in the liver after three passages (Figure 3E and F). The replication of HCV RNA in the liver tumors was also confirmed by cell colony assay after culturing tumor cells in medium that contained G-418 (1 mg/mL). The replicon cells derived from the liver tumors formed G-418-resistant cell clones, which suggested that HCV replication occurred within the HCC xenografts in SCID mice. Liver tumor supporting HCV RNA replication was confirmed by RT-nested PCR followed by Southern blot analysis. These results suggest that we have developed a mouse-adapted S3-GFP replicon cell line that forms liver tumor after splenic injection. The level of HCV RNA in the liver tumor was determined by real time RT-qPCR that was in the range of 10^4 copies/ μ g of total cellular RNA. The subcutaneous tumor tissue showed a mean titer of 10^5 copies/ μ g of total cellular RNA. There is one log reduction in the HCV RNA titer in

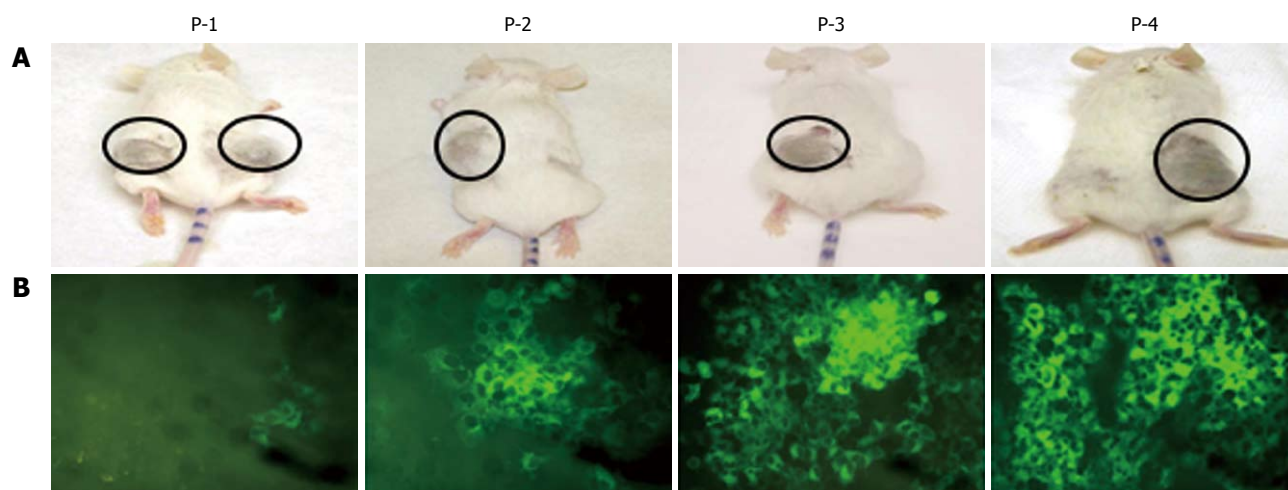


Figure 2 Intracellular expression of hepatitis C virus-green fluorescence protein in the subcutaneous tumor of SCID mice. Mice were injected subcutaneously with 10^6 hepatitis C virus-green fluorescence protein (GFP) replicon cells. Tumor growth was monitored on a weekly basis. A: Tumorigenicity of Huh-7 replicon cells in γ -irradiated SCID mice; B: Expression of GFP in tumor cells during the *in vivo* passage. The number of GFP positive cells was low ($< 10\%$) after the first passage. There was a gradual increase in GFP expression in the subcutaneous tumors after each passage. After four passages, the number of GFP-positive cells increased significantly ($> 50\%$).

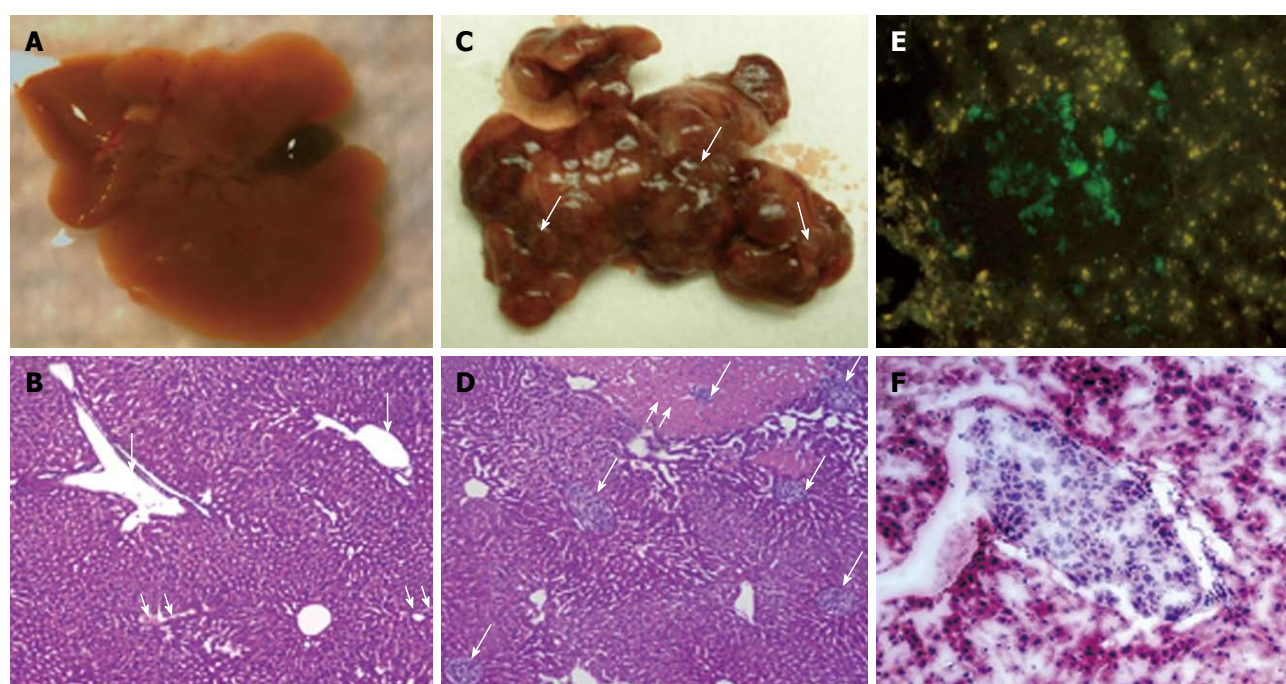


Figure 3 Human hepatocellular carcinoma xenograft in SCID/NOD mouse liver. A: Gross appearance of normal mouse liver with distended gall bladder; B: Light microscopic appearance of normal liver stained with hematoxylin and eosin ($4 \times$ magnification). The portal tracts are shown by single arrows and the central veins are marked with double arrows; C: Gross appearance of mouse liver with metastatic nodules of hepatocellular carcinoma (HCC). Note the distended white-tan areas of metastasis and infarction in the mouse liver (single arrows); D: Microscopic metastasis of HCC diffusely infiltrating the liver through the portal venous system (single arrows). Note the areas of infarction secondary to the tumor emboli in the portal vein (double arrow). Human hepatocytes can be easily discriminated from the mouse hepatocytes by their size and pale color; E: Expression of hepatitis C virus-green fluorescence protein (GFP) fusion protein in the S3-GFP liver tumors in the mouse; F: Hematoxylin and eosin staining (frozen section) of HCC tumor in the mouse liver at 4 wk after intrasplenic infusion of S3-GFP replicon cells.

the HCC tumor formed subcutaneously *vs* those formed in the liver (Figure 4). The HCV RNA titer in the liver tumor model is comparable to that found in chronically infected human liver tissue^[23]. We hypothesize that this might be due to the fact that there are differences in the innate immune pressure on HCV RNA replication in the peripheral compared with internal organs such as the liver.

There is evidence to support our observations that the liver has its own innate immune system that might suppress replication of HCV^[24-26].

IFN- α inhibits HCV replication in the HCC xenograft formed subcutaneously

To investigate the ability of IFN- α to inhibit HCV RNA

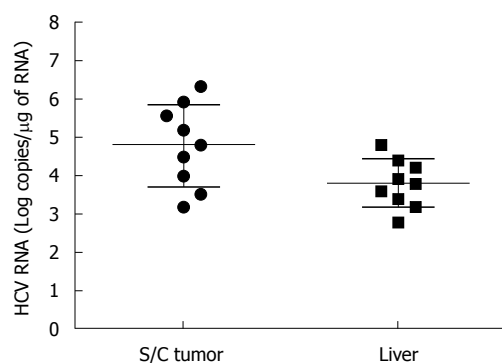


Figure 4 Comparison of hepatitis C virus RNA level between hepatocellular carcinoma xenografts formed subcutaneously and in the liver. RNA extract was prepared using the GITC method. The hepatitis C virus (HCV) RNA level was measured by real-time polymerase chain reaction using 1 μ g total RNA isolated from the tumor samples, and mentioned as log copies/ μ g RNA. HCV RNA levels were 10-fold higher in the tumors formed subcutaneously compared to those that were formed in the liver. The titer of HCV in the liver tumor model was comparable to that in the infected human liver.

replication in the subcutaneous HCC xenografts, the mice at 2 wk post-implantation were treated with IFN- α at 15000 IU/mice three times weekly. The mice were sacrificed at different time points after initiation of IFN- α treatment and the tissues were processed for HCV expression. IFN treatment decreased GFP expression in a time-dependent manner, with > 50% decrease in GFP expression after 1 wk (Figure 5B and E), with complete inhibition after 2 wk (Figure 5C and F). The untreated sections showed expression of GFP (Figure 5A and D). To show that the hepatocytes that support HCV replication are human hepatoma cells, immunostaining of HCC cells was performed using an antibody to human serum albumin. The results presented in Figure 5G-I clearly showed that the HCC xenografts were positive for human albumin. The level of albumin expression did not change due to IFN- α treatment. To ensure that IFN- α treatment inhibited viral RNA replication and reduced the level of HCV RNA in the HCC xenografts, total RNA was extracted from the tumor samples and processed for RPA. The results of this analysis are shown in Figure 6, which indicated that HCC xenografts in SCID mice supported a high level of HCV RNA replication, and the levels of HCV RNA in the tumors were completely inhibited after 2 wk of IFN- α treatment. The results shown in Figure 6A and B suggested that HCV RNA level in the subcutaneous HCC tumors increased from 18 to 28 d after tumor development. IFN treatment successfully inhibited virus replication within the tumors and the HCV-RNA titer decreased significantly after 2 wk of treatment, and remained below the limits of detection at 4 wk. We examined the possibility that IFN- α treatment could have an effect on tumor growth, which could have affected the measured HCV RNA levels in the tumors. The tumor size and histology of IFN- α -treated and untreated animals were compared (Figure 7). We did not notice any difference in the tumor growth or histology between the treated and untreated

animals. The dose of IFN- α treatment used in the *in vivo* experiments only inhibited HCV RNA replication, but did not inhibit the tumor growth. Taken together, these results indicated that HCC xenografts that formed subcutaneously in the SCID mice supported a high level of HCV RNA replication, and that IFN- α directly inhibited HCV replication in the tumors. All mice were doing well throughout the IFN- α treatment experiment and their body weight did not change significantly. Mice tolerated the IFN- α treatment well.

IFN- α completely inhibits replication of HCV 2a sub-genomic RNA in the HCC xenograft in mouse liver

We demonstrated the usefulness of this model by evaluating whether HCV replication in the liver tumor model could be inhibited by IFN- α . Mice were treated with intraperitoneal injection of IFN- α three times weekly at 15000 IU/mouse, starting at 4 wk after S3-GFP transplantation. Control animals received saline injections at similar time intervals. At different treatment intervals, mice were sacrificed and liver tissue was collected. The ability of IFN- α to inhibit HCV replication was confirmed by four independent assays. First, hepatocytes were isolated from the liver and cultured in the presence of growth medium that contained G-418 (1 mg/mL). The number of S3-GFP replicon cell colonies in the plate was compared to assess the antiviral effects of IFN- α against HCV replication in the xenograft tumors. Figure 8A shows that 4 wk IFN- α treatment successfully inhibited replication of HCV in the liver tumors. Second, the intracellular HCV RNA in the tumors tissue was examined by using RT-nested PCR and Southern blot analysis that targeted the highly conserved 5'-UTR region of the HCV genome. We showed that HCV RNA was detectable in the liver tumors of untreated mice over 5 wk, by RT-nested PCR and Southern blot analysis. IFN- α treatment completely inhibited HCV RNA replication in the liver tumors and remained undetectable at 4 and 5 wk, using the highly sensitive RT-nested PCR and Southern blot analysis (Figure 8B). IFN- α treatment did not alter the cellular albumin RNA levels. Third, expression of GFP in the liver tumors after IFN- α treatment was negative after 4 wk (Figure 8C). Fourth, the level of HCV RNA in the treated and untreated tumor samples was assessed by real-time PCR. The results of this analysis suggested that HCV levels decreased significantly in the tumors and remained below the detection limit after 4 wk of IFN- α treatment (Figure 8D). These data clearly showed that IFN- α inhibited HCV RNA replication in the liver tumor model and there was a significant difference in the level of HCV replication in the liver tumors between the untreated and IFN- α -treated mice. To demonstrate that the decrease in the HCV RNA replication after IFN- α treatment was not due to the direct effect of IFN on tumor growth in these HCC xenografts, histological examination of liver tumor in IFN- α -treated and untreated animals was performed. The histological pictures shown in Figure 9 indicate that there was no evidence of cellular necrosis in the tissue

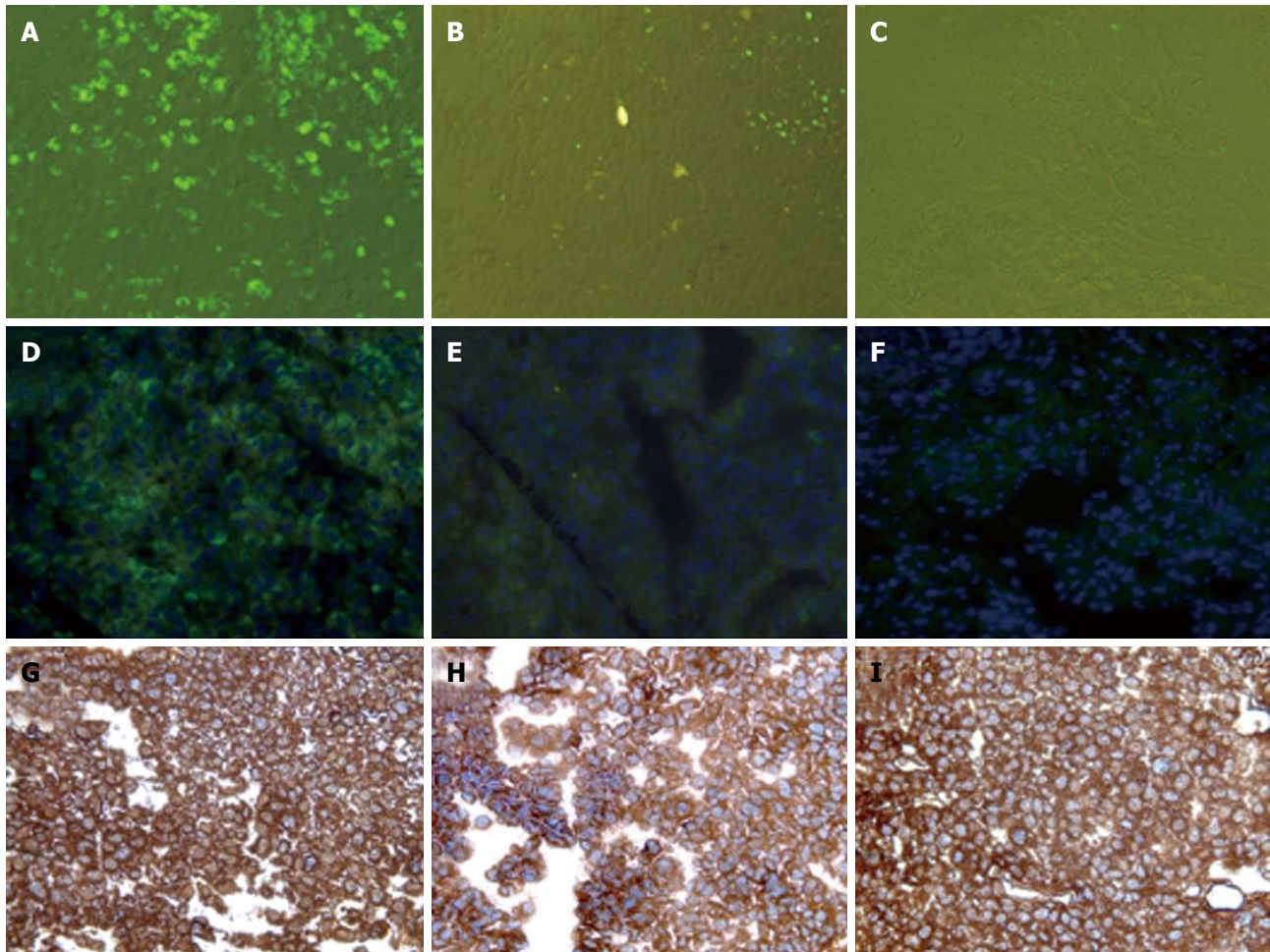


Figure 5 Interferon- α inhibits hepatitis C virus-green fluorescence protein expression in human hepatocellular xenografts formed subcutaneously in SCID mice. Mouse-adapted replicon cells were injected subcutaneously into SCID mice, which then developed visible tumors after 2 wk. The mice were injected intraperitoneally with a total dose of 15 000 IU interferon- α (IFN- α) in 100- μ L volumes, three times weekly. Tumors were harvested after 1 and 2 wk of IFN treatment and examined for green fluorescence protein (GFP) expression and viral RNA by RPA and real-time polymerase chain reaction. A, D: Expression of GFP in the frozen sections of hepatocellular carcinoma (HCC) xenografts before IFN- α treatment; B, E: Expression of GFP 1 wk after IFN-treatment; C, F: Expression of GFP after 2 wk IFN-treatment. The middle panel shows DAPI staining of the nucleus. IFN inhibited hepatitis C virus RNA replication and GFP expression in the liver tumors at 7 and 14 d; G-I: Intracytoplasmic expression of human albumin in the HCC xenografts formed by subcutaneous injection of *in vivo* adapted Huh-7 replicon cells.

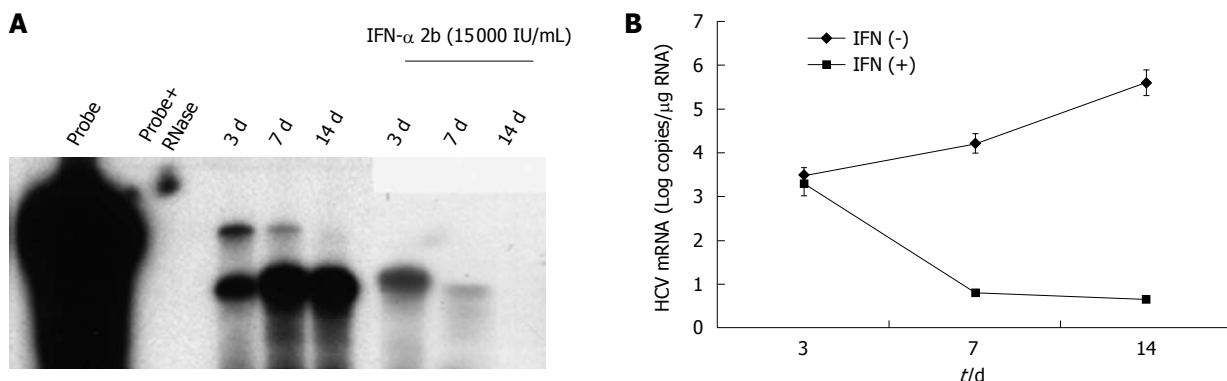


Figure 6 Intracellular hepatitis C virus RNA levels in subcutaneous tumors. A: RPA measuring intracellular hepatitis C virus (HCV) RNA levels in hepatocellular xenografts formed subcutaneously. There was a gradual increase in HCV RNA levels in the subcutaneous tumors at 3, 7 and 14 d post-tumor development. Two weeks of interferon (IFN) treatment completely inhibited HCV RNA replication in the subcutaneous tumors, as demonstrated by a negative RPA signal at 14 d of IFN- α treatment; B: Real-time reverse transcription polymerase chain reaction showing HCV RNA levels in the subcutaneous tumors before and after IFN- α treatment. IFN treatment decreased HCV RNA levels in the tumors, which remained below the detection limits after 2 wk.

sections of mice that received IFN- α treatment. These results indicated that the reduction of HCV RNA levels

in the tumor was due to a specific antiviral mechanism of IFN- α .

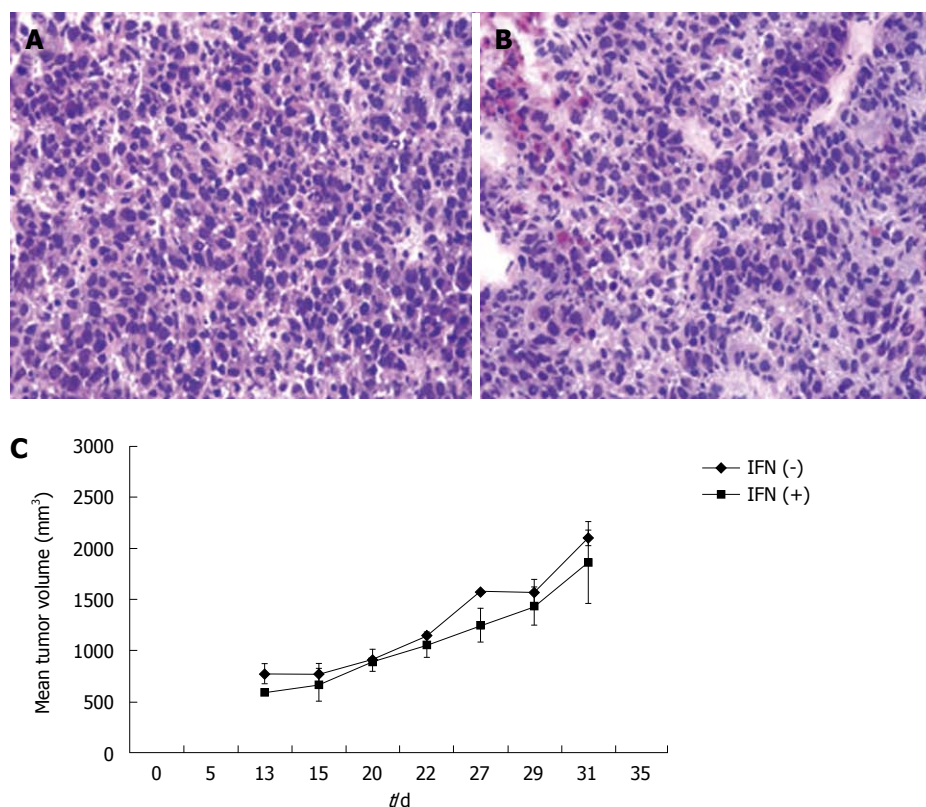


Figure 7 Interferon treatment has no effect of tumor growth in SCID/bg mice. A, B: Histology of tumor sections of experimental mice before (A) and after (B) 2 wk of interferon- α (IFN- α) treatment showed no evidence of tumor cell necrosis or apoptosis (10 \times magnification); C: Kinetics of tumor growth between two groups of mice during IFN- α treatment. The mean tumor volume remained the same between these two groups of mice.

DISCUSSION

Cell culture and animal models are required for the development of novel experimental therapies for chronic HCV infection. A number of antiviral strategies have been developed that inhibit HCV replication in cell culture systems^[4,5]. The successful use of different therapeutic agents to inhibit chronic HCV infection requires further validation using an easily accessible small animal model. Our laboratory has developed multiple siRNA targets in the 5'-UTR region of the HCV genome, and has shown that intracellular delivery of these siRNAs can completely degrade HCV RNA in cell culture^[27]. We have also developed recombinant antibodies targeted to the viral NS3 helicase, and have shown that intracellular expression of these antibodies inhibits viral helicase activity and virus replication in a cell culture model^[28]. The next step of this research is to test the effectiveness of these different antiviral strategies that target inhibition of viral replication, using an animal model system. Therefore, development of a small animal model for HCV infection was essential to optimize targeted, hepatic delivery of siRNA and recombinant antibodies. Mice and other rodents are not susceptible to natural HCV infection, thus, there are several alternative approaches that have been employed during the past few years to replicate HCV infection using mouse models. The advantages and disadvantages of these models to study HCV infection in mouse models are discussed.

The most promising small animal model for studying HCV infection is now the human liver chimeric mouse. The principle of developing this mouse model is based on the availability of specific mouse strains in which human hepatocytes can be efficiently transplanted into the mouse liver. Two specific mouse strains are currently being used for development of the humanized mouse liver. One is the SCID-Alb/uPA mouse and the other is the Fah^{-/-} Rag^{-/-} IL-2^{-/-} mouse. Both mouse strains have high liver regeneration capacity due to their liver injury and support a high degree of human hepatocyte transplantation. The humanized mouse liver has been shown to have normal hepatic architecture and support HCV replication after natural infection. The studies conducted by Mercer *et al.*^[13] and Tateno *et al.*^[29] have used a mouse strain called SCID-Alb/uPA. Using this mouse strain, Mercer *et al.*^[13] have shown that the humanized mouse liver contains > 50% human hepatocytes, and Tateno *et al.*^[29] have shown that humanized mouse liver contains > 92% human hepatocytes. The Alb/uPA transgenic mouse strain shows severe hepatotoxicity due to overexpression of the urokinase plasminogen (*uPA*) gene in hepatocytes, which leads to continuous liver regeneration. Human hepatocytes that lack this transgene preferentially survive after transplantation and are stably maintained in this mouse liver. This mouse strain has been crossed with an immunodeficient mouse (SCID or Rag2) to generate the SCID-Alb/uPA mouse strain that tolerates transplantation of human hepa-

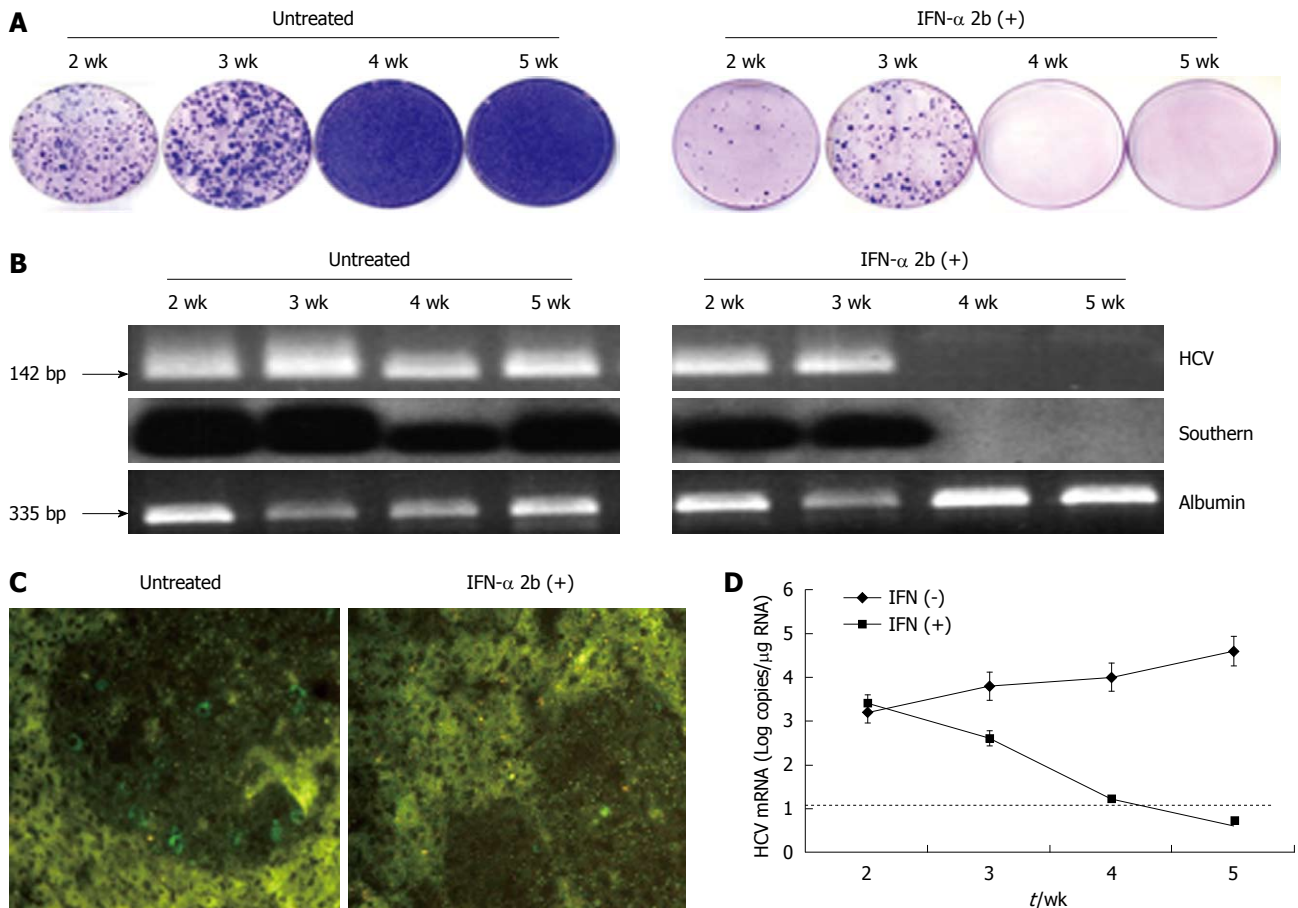


Figure 8 Antiviral effect of interferon- α in the liver tumor model. S3-green fluorescence protein (GFP) cells were implanted in the liver of NOD/SCID mice by intrasplenic injection. After 3 wk, interferon- α (IFN- α) treatment was started. The time shown represents the weeks after IFN treatment. A: Colony assay of viable tumor cells isolated from the liver tumor model at different time points with or without IFN- α treatment. Tumor cells isolated from the entire mouse liver were cultured in the medium that contained 1 mg/mL G-418. The replicon cells with hepatitis C virus (HCV) survived the treatment and formed cell colonies. Left panel: there was an increase in the number of cell colonies between 4 and 5 wk. Right panel: IFN- α treatment completely inhibited HCV replication and cell colony formation at 4 wk; B: RT-nested polymerase chain reaction (PCR) and Southern blot analysis for HCV and albumin in the RNA extracts of the liver tumor. Left panel shows the results without treatment. Right panel shows IFN- α treatment; C: Expression of HCV-GFP in the liver tumors before and after IFN treatment. IFN treatment after 4 wk completely inhibited HCV-GFP expression in the S3-GFP tumors in the SCID mouse liver; D: Real-time reverse transcription PCR (RT-PCR) showed that the levels of HCV in the liver tumor remained undetected after 4 wk of IFN- α treatment. The dotted line indicates the limit of detection for real time RT-PCR assay.

tocytes well. Human hepatocytes can be successfully transplanted into the SCID-Alb/uPA mice within 1 or 2 wk after birth. Several studies have now successfully used this model to replicate HCV infection and have tested several antiviral molecules, including IFN- α and protease inhibitors. The study conducted by Bissig *et al*^[16] has used Fah^{-/-} Rag2^{-/-} IL-2^{-/-} mice and has shown that the humanized mouse liver contains > 80% human hepatocytes. This mouse strain lacks fumaryl acetoacetate, which is an enzyme that is required for tyrosine amino acid metabolism. Mice that lack this enzyme show severe hepatotoxicity due to accumulation of toxic metabolites. This mouse strain has been bred with Rag2^{-/-} IL-2^{-/-} mice to generate an immunodeficient mouse strain that supports transplantation of adult human hepatocytes. Recently, Bissig *et al*^[16] have used this mouse model to replicate HCV infection from cell culture and from the serum of chronically infected patients. They also have shown that pegylated IFN- α and Debio 025 (cyclophilin inhibitor) inhibit HCV replication in this chimeric mouse model. The investigators have

shown that these chimeric mice sustain viral replication for > 6 mo, without any substantial side effects. There is no doubt that these chimeric mouse models are extremely challenging technically, complicated to use, and expensive. Despite the technical challenges, these models hold promise for studying the long-term effect of HCV infection on the development of liver fibrosis and cancer.

We described here an HCC xenograft mouse model in which human tumor cells were implanted in the liver of an immunodeficient mouse. This model is relatively simple compared to the human liver chimeric mouse model. This model can be used for experimental verification of the efficacy of targeted antiviral therapies against HCV. The model utilizes SCID/bg and NOD/SCID mice with a partially suppressed innate immune system (natural killer cell response) and adaptive immune system (both B- and T-cell mediated response)^[30]. In an earlier study, Zhu *et al*^[18] have performed similar experiments to generate a mouse-adapted HCV Con-1 replicon cell line using the SCID/bg mice. The *in vivo* adaptation of HCV is not possible using

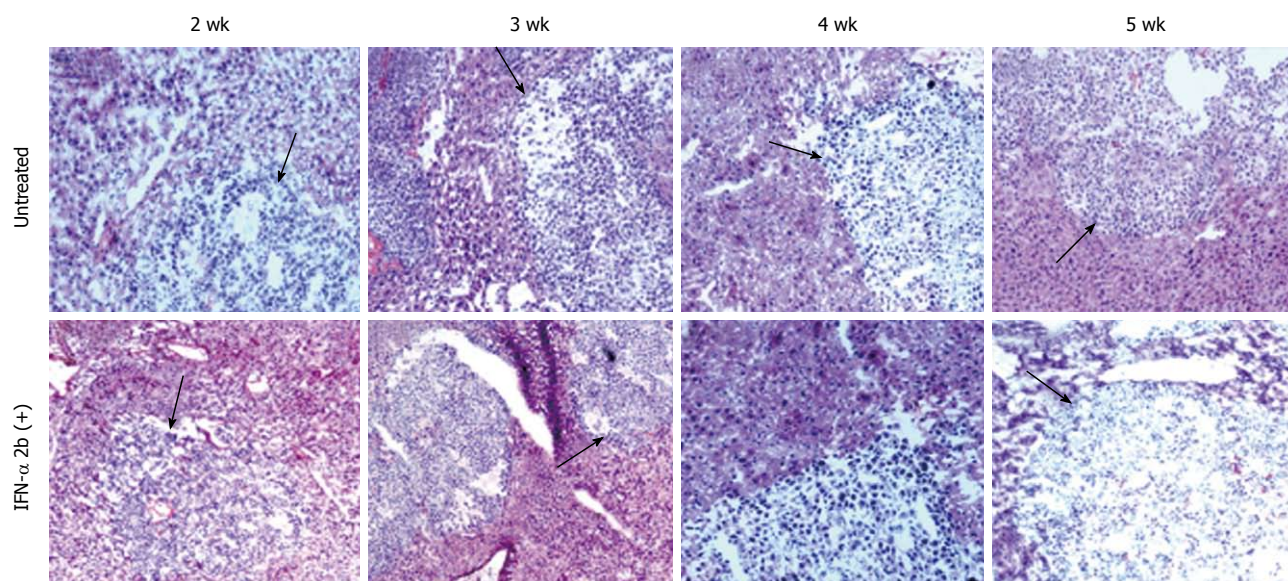


Figure 9 Histological evaluation of liver sections after hematoxylin and eosin staining that indicates that interferon- α treatment did not cause any tumor necrosis or reduce the size of tumor nodules in the SCID mice liver. The upper panel shows the untreated mouse liver sections after 2-5 wk of tumor development. The black arrows indicate the hepatocellular carcinoma (HCC) tumor in the mouse liver. Similarly, the lower panel shows the interferon- α -treated mouse liver sections with HCC tumor at different time points (10 \times magnification).

this approach due to the low-level replication capacity of the HCV Con-1 replicon clone. The authors had to re-engineer the sub-genomic clone with reinsertion of HCV sub-genomic RNA with a highly efficient internal ribosome entry site, and a highly adapted mutation in the non-structural gene, to sustain high-level replication of HCV RNA within the tumor. They have successfully measured HCV replication in this mouse model by measuring luciferase activity using non-invasive whole-body imaging. They have shown that IFN- α and protease inhibitor BILN-2061 reduce HCV RNA replication in the subcutaneous and liver tumor models. The authors have not shown whether these treatments lead to complete clearance of HCV in these tumors models. Another study by Guévin *et al*^[31] also has demonstrated that full-length HCV replication can be achieved in HCC tumor xenografts formed subcutaneously using immunodeficient mouse strains. The authors have made mouse-adapted Huh-7 cells by multiple passages in SCID mice. The authors have shown that the xenograft tumors in the SCID mouse produce infectious virus particles. The infectious virus produced in the xenograft tumors can be serially passaged in SCID mice. The authors have shown that IFN- α and protease inhibitor BILN-2061 inhibit replication of the cell culture HCV cc strain JFH1 in the mouse model.

The mouse model described here differs in some aspects compared to the two previously described tumor xenograft models. We realized that high-level replication of HCV in the SCID mouse liver might be possible by using the highly efficient JFH1 clone. We used the JFH1 virus clone that replicated at a much higher level in the HCC xenografts formed in the SCID mice. The GFP-labeled replicon cell line showed that replication of HCV occurred in the HCC tumors, and could be determined by

direct examination under fluorescence microscopy. The replicon cell line is similar to chronically infected human hepatocytes, except that these cells do not produce the infectious virus, which makes this animal model safer to use. We have used a number of biochemical methods (colony assay, RPA, real-time RT-PCR and RT-nested PCR and Southern blot analysis) that quantitatively evaluate HCV replication in the subcutaneous tumors in an accurate manner. If necessary, this model can also be adapted using a luciferase-based, mouse-adapted HCV replicon cell line so that the whole-body imaging technique can be used to monitor HCV replication in the mouse model. The diffuse metastasis of HCC throughout the liver lobes after intrasplenic infusion of replicon cells mimics *in vivo* distribution of chronically infected hepatocytes in human liver. We demonstrated replication of HCV RNA in multiple HCC nodules in the portal tracts of the SCID mice livers. This liver tumor model developed by intrasplenic injection of replicon cells provides a better assessment of the biodistribution and pharmacokinetics of IFN- α when compared to direct injection of replicon cells to the liver, where HCC tumors are formed only in selected areas of the organ. We noticed that the level of HCV replication in the liver tumor model was 10-fold lower when compared to that in the subcutaneous model. This could be due to the innate antiviral pressure against HCV between the peripheral organ and liver microenvironment. The dynamics of viral RNA replication in the liver tumor model are comparable to those of chronically infected human liver. As a practical validation of this mouse model, we showed that IFN- α treatment inhibited HCV RNA replication and abolished GFP expression in the subcutaneous HCC xenografts within 2 wk, which suggests that the mouse model can be used to test the success of other antiviral

strategies against HCV. We showed that IFN- α completely inhibited viral replication in the liver tumor model, and HCV RNA in liver tumors was undetectable by using a highly sensitive RT-nested PCR. The sensitivity of this RT-nested PCR assay was found to be in the range of 1-10 copies of HCV RNA^[32]. Using this mouse model, we showed that IFN- α administration completely eliminated HCV sub-genomic RNA replication, which supports the practical use of this animal model to test other antiviral strategies against the background of a growing tumor. This mouse model, therefore, can be used practically to optimize methods for targeted delivery of siRNA and recombinant antibodies that are designed to inhibit HCV replication in tumor xenografts in the mouse liver.

The SCID mouse model described here can be adapted to any research laboratory because it is relatively simple, safe and less expensive, thus allowing it to be used in large numbers for testing. This mouse model can be utilized to establish the success of targeted liver delivery methods of a variety of antiviral strategies within a short period of time. *In vivo* experiments that address the toxicity of different antiviral approaches with this animal model can be tested using a large number of mice to achieve statistical power of significance. This model deals with non-infectious HCV and does not depend on the use of donated or harvested human hepatocytes or infected serum samples. We have also developed *in vivo* adapted Huh-7 cells by elimination of HCV replicon replication. Tumor xenografts that use this Huh-7 cell line in SCID mice can be used to study the infectivity of JFH1 virus and other relevant clinical HCV strains. This will allow us to test the efficacy of neutralizing monoclonal antibodies directed against the HCV envelope protein. There are also some limitations in the tumor xenograft mouse models for HCV that are described here. For example, this model cannot be used for investigation of immunopathogenesis of chronic HCV infection or vaccine development because the SCID mice have defective B- and T-cell responses. Nevertheless, this animal model will be useful for antiviral evaluation and testing the potential of intracellular delivery of siRNA and recombinant antibodies that are directed at inhibition of HCV replication in the liver.

ACKNOWLEDGMENTS

We thank Jeanne Frois and Mallory Schexnayder for critically reading the manuscript. The authors thank Takaji Wakita for providing the JFH-1 and pSGR clone. The authors thank Mathew Burrow for assisting us during the experiments with tumor implantation.

COMMENTS

Background

Hepatitis C virus (HCV) is the most common blood-borne infection that affects the liver. Several antiviral strategies have been developed during the past 2 years, including small molecules, antisense oligonucleotides, siRNA, ribozymes, and recombinant antibodies that are designed to inhibit HCV replication in cell culture models. A small animal model for HCV is required to test different experimental therapies developed using HCV cell culture systems.

Research frontiers

Development of a small animal model for studying HCV infection has been difficult because the virus only infects humans and chimpanzees. The only available animal model is a chimeric humanized mouse model. This model is very costly, technically very challenging, and very expensive. The authors developed an hepatocellular carcinoma (HCC) xenograft tumor model in SCID mice using a replicon cell line that replicates highly efficient JFH1 virus. The replicon cells were transfected with a green fluorescent protein (GFP)-tagged HCV sub-genomic clone so that the antiviral effect could be observed by GFP expression. The replication of HCV could be inhibited by interferon- α (IFN- α), which indicates that the model can be used for antiviral screening. The HCC xenograft mouse model is less expensive, non-infectious, and can be easily adapted to any research laboratory.

Innovations and breakthroughs

The authors developed an S3-GFP replicon Huh-7 cell line that was highly adapted to mice and formed human HCC xenografts in SCID mice. We showed that HCV replicated in the HCC xenografts formed subcutaneously and in the liver. The antiviral effect of IFN- α showed clearance of HCV in this mouse model.

Applications

This model can be used to optimize methods for liver-targeted delivery of several antiviral strategies against HCV replication.

Peer review

The paper is well written and the scientific content is worth publishing.

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S- Editor Cheng JX L- Editor Kerr C E- Editor Zheng XM

Octreotide induces caspase activation and apoptosis in human hepatoma HepG2 cells

Nikos J Tsagarakis, Ioannis Drygiannakis, Antonis G Batistakis, George Kolios, Elias A Kouroumalis

Nikos J Tsagarakis, Ioannis Drygiannakis, Antonis G Batistakis, Elias A Kouroumalis, Liver Research Laboratory, School of Medicine, University of Crete, Heraklion, 71003, Greece
George Kolios, Laboratory of Pharmacology, School of Medicine, Democritus University of Thrace, Alexandroupolis, 68100, Greece
Elias A Kouroumalis, Department of Gastroenterology and Hepatology, University Hospital of Heraklion, Heraklion, 71003, Greece

Author contributions: Tsagarakis NJ, Kouroumalis EA and Kolios G designed the research; Tsagarakis NJ, Drygiannakis I and Batistakis AG performed the research; Tsagarakis NJ, Drygiannakis I and Kouroumalis EA analyzed the data; Tsagarakis NJ, Kouroumalis EA and Kolios G wrote the paper.

Supported by Research funds of the Liver Research Laboratory, School of Medicine, University of Crete, Greece

Correspondence to: George Kolios, MD, PhD, Laboratory of Pharmacology, School of Medicine, Democritus University of Thrace, Dragana, Alexandroupolis, 68100, Greece. gkolios@med.duth.gr

Telephone: +30-25510-30368 Fax: +30-25510-30368

Received: July 4, 2010 Revised: September 25, 2010

Accepted: October 2, 2010

Published online: January 21, 2011

Abstract

AIM: To investigate the role of octreotide on cellular proliferation and apoptosis of human hepatoma (HepG2) cells.

METHODS: We studied cellular proliferation, apoptosis and the possible internal caspase-mediated apoptosis pathway involved, after treatment of HepG2 carcinoma cells with octreotide in comparison with the apoptosis caused by tumor necrosis factor- α (TNF- α). Activities of caspase-3, caspase-9, caspase-8 and caspase-2 were studied, while apoptosis was investigated through detection of DNA fragmentation and through identification of apoptotic cells with the annexin-V/propidium iodide flow cytometric method.

RESULTS: After an initial increase in HepG2 cellular proliferation, a significant inhibition was observed with

10^{-8} mol/L octreotide, while TNF- α dose-dependently decreased proliferation. Early and late apoptosis was significantly increased with both substances. Octreotide significantly increased caspase-3, caspase-8 and caspase-2 activity. TNF- α significantly increased only caspase-2. Cellular proliferation was decreased after treatment with octreotide or TNF- α alone but, in contrast to TNF- α , octreotide decreased proliferation only at concentrations of 10^{-8} mol/L, while lower concentrations increased proliferation.

CONCLUSION: Our findings are suggestive of caspase-mediated signaling pathways of octreotide antitumor activity in HepG2 cells, and indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

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Key words: Octreotide; Hepatocellular carcinoma; Apoptosis; Caspases; Somatostatin

Peer reviewer: Dr. Chao-Hung Hung, MD, Associate Professor, Division of Hepatogastroenterology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital, 123 Ta Pei Road, Niao Sung, Kaohsiung 833, Taiwan, China

Tsagarakis NJ, Drygiannakis I, Batistakis AG, Kolios G, Kouroumalis EA. Octreotide induces caspase activation and apoptosis in human hepatoma HepG2 cells. *World J Gastroenterol* 2011; 17(3): 313-321 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/313.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.313>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause approximately half a million deaths annually^[1]. There are striking differences in the incidence of HCC related to

age, gender, race, and geographic region, with hepatitis C virus (HCV) infection acquired 2-4 decades previously explaining at least half of the observed increase in HCC^[2]. The survival rates remain generally dismal (median 8 mo)^[2]. Undoubtedly, the best available treatment for all liver tumors is complete surgical resection. However, the synthetic somatostatin analogue octreotide has been found effective in inhibiting tumor growth in a variety of experimental models^[3,4]. Octreotide did not influence hepatic or portal blood flow, although significantly increased reticuloendothelial system activity^[5,7]. Apart from stimulation of the reticuloendothelial system, octreotide may have other mechanisms of action, to inhibit the growth of hepatic tumors. One of the mechanisms suggested may be a direct antiproliferative effect, through receptor-mediated growth inhibition^[8]. *In vitro* studies have shown that octreotide demonstrates high affinity binding towards somatostatin receptors sstr2, sstr3 and sstr5, while no binding affinity is found towards receptors sstr1 and sstr4^[9-11]. It has been reported that octreotide inhibits the proliferation and induces apoptosis of different HCC cell lines *in vitro*^[12-18]. The mechanisms of apoptosis induction however are not well understood.

Tumor necrosis factor- α (TNF- α) is a well established as a means of apoptosis induction in a variety of cell types through specific responsive receptors, but other cells require transcriptional arrest^[19]. Hepatoma cells treated with TNF- α and cycloheximide (CHX) undergo apoptosis, which is preceded by a strong activation of c-jun N-terminal kinase^[20]. The human HCC cell line, SMMC-7721, was insensitive to TNF- α cytotoxicity, but quickly underwent apoptosis in the presence of TNF- α and CHX^[21].

Apoptosis is a complex process characterized by caspase activation, cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation^[22-25]. Caspases are cysteine-containing aspartic acid-specific proteases and all have similar site-specific proteolytic activity. Caspases are divided into 3 distinct groups based on their substrate specificities. Group I (YVADase) includes caspase-1, -4 and -5 which are involved in cytokine production. Group II (DEVDase) caspases, e.g. caspase-3 and -7 are the main effector caspases during apoptosis. These are cleaved by group III (IETDase) caspases (e.g. caspase-6, -8, -9 or -10) early in the onset of apoptosis^[26]. Upon activation, group II caspases act on various cell proteins^[26]. Most, but not all, events in apoptosis appear to require a caspase-mediated proteolytic step^[25].

Octreotide has been clinically used for treatment of HCC, with conflicting results. Both increased survival^[27,28] and no effect^[29,30] have been reported. Although negative studies have been criticized^[31], the mechanisms by which octreotide may act have not been adequately clarified. Apoptosis may be a fundamental mechanism. In this study, we examined the effect of octreotide on cellular proliferation, apoptosis and caspase activation in HepG2 HCC cells. The model of TNF- α -induced apoptosis was chosen for comparison with octreotide in a study of the biological behavior of caspases, after treatment of HepG2 cells with octreotide.

MATERIALS AND METHODS

Octreotide was from Novartis (Basel, Switzerland) and was used at concentrations of 10^{-10} mol/L to 10^{-7} mol/L, to identify the optimal concentration for inhibition of cell proliferation. Incubations with TNF- α (R&D Systems, Minneapolis, USA) were made at concentrations from 0.1 to 100 ng/mL (0.1, 1, 10, 20, 100 ng/mL). According to the proliferation curve, the suitable concentrations for further experiments were 10^{-8} mol/L for octreotide and 20 ng/mL for TNF- α . These concentrations were used for all further combinations and measurements of apoptotic features.

Cell culture and incubation conditions

The HepG2 cell line is a human hepatocyte carcinoma cell line derived from a well-differentiated human hepatoblastoma and was purchased from the European Collection of Cell Cultures (ECACC, Porton Down, UK). HepG2 cells are maintained in continuous culture in our laboratory in RPMI supplemented with 10% fetal bovine serum (FBS, Gibco, Paisley, UK), at 37°C and in an atmosphere of 5% CO₂. For experiments, HepG2 cells were seeded in 24-well plates at a density of 2×10^4 /cm². Twenty four hours before the experiment, they were cultured in fresh medium without FBS, and then treated with different concentrations and combinations of all substances. Incubations were made at 37°C in 5% CO₂. Supernatants were collected and stored in -80°C, while cell extracts were used for measurements of caspase activity. Control medium was complete media with 10% FBS. HepG2 cells cultured in control medium are referred to as control cells.

Proliferation assays

For measurement of growth inhibition, the sulforhodamine B colorimetric assay (SRB Assay; Biotium Inc., Hayward, CA, USA) was used, as previously described^[32,33]. HepG2 cells were plated in 96-well plates, at an initial density of 5×10^3 cells, with 200 μ L medium per well. All substances were added to cultures 1 d after seeding, in order to obtain best attachment of the cells at the beginning of the experiments. Cells were grown for a total of 6 d, with a change of medium and substances on the third day after treatment. Measurements were made as described in the original protocol. Briefly, 50 μ L of 50% trichloroacetic acid were placed into the 200 μ L medium and plates were stored at 4°C for 30 min. After washing 5 times with deionized water, plates were left to dry for 24 h at room temperature. Then, 70 μ L of 0.4% sulforhodamine B in 1% acetic acid were placed in every well and left at room temperature for 20 min. Before air drying for a second time, plates were washed 5 times with 1% acetic acid. At the end of the procedure, 200 μ L of unbuffered Tris-base solution (pH 10.5) were added to each well and measurements were made at 490 nm, subtracting the background at 620 nm. The mean of the optical densities of 8 different controls was considered to be 100% and all other values were expressed as a percentage of the controls.

Detection of apoptosis

DNA fragmentation: For detection of apoptosis, a sandwich, one step, colorimetric enzyme-linked immunosorbent assay, the Cell Death Detection ELISA Plus kit (Roche Diagnostics, Mannheim, Germany) was used. The assay allows for the specific determination of histone-complexed DNA fragments (mono and oligonucleosomes) from the cytoplasm of cells, after the induction of apoptosis. Briefly, after induction of apoptosis and 24-h incubation, the cells were pelleted by centrifugation (200 *g*, 10 min) and the supernatants (containing DNA from necrotic cells that leaked through the membrane during incubation) were discarded. Cells were resuspended and incubated for 30 min in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation. Aliquots of the supernatants were transferred to a streptavidin-coated well of a microtiter plate with 2 monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated), so that nucleosomes in the supernatant created antibody-nucleosome complexes, which were continuously bound to the microtiter plate by the streptavidin. All samples were then incubated with peroxidase substrate and absorbance was measured at 405 nm. The mean of the optical densities of 8 different controls was considered to be 100% and all other values were expressed as a percentage of the controls.

Annexin-V/propidium iodide staining: For better evaluation of apoptotic features, the modified Annexin-V Apoptosis Detection Kit (BioVision, Mountain View, CA, US) was used, which is based on the observation that soon after initiation of apoptosis, cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface, but they also shrink, increasing their side scatter (SS) and reducing their forward scatter (FS) characteristics. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin-V, that has a high affinity for PS. Cells that have bound Annexin-V-fluorescein isothiocyanate (FITC) (early apoptotic) show green staining in the plasma membrane, while cells that have lost membrane integrity will show red staining [propidium iodide (PI)] throughout the nucleus and a halo of green staining (FITC) on the cell surface (late apoptotic or necrotic cells).

After treatment and 24-h incubation in 24-well plates, adherent HepG2 cells were gently trypsinized and washed once with serum-containing medium. Then, 1.5×10^5 cells were collected by centrifugation (98 *g*, 5 min) and resuspended in 300 μ L of $1 \times$ Binding Buffer. After gentle pipetting to resuspend the cell pellets, 3 μ L of Annexin-V-FITC and 3 μ L of 50 μ g/mL PI, were added, followed by a 5-min incubation at room temperature in the dark. Annexin-V-FITC binding was analyzed by flow cytometry (Epics Elite) (Ex = 488 nm; Em = 530 nm) using a FITC signal detector and PI staining by the phycoerythrin emission signal detector. Debris was excluded by scatter gating (forward *vs* side). At least 10000 events were counted for each sample.

Caspase activity

The activities of caspase-3, caspase-9, caspase-8 and caspase-2 were measured. For the evaluation of caspase activity, colorimetric activity assay kits (Chemicon, Temecula, CA, USA) were used. The assays are based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA (caspase-3), LEHD-*p*NA (caspase-9), IETD-*p*NA (caspase-8) and VDVAD-*p*NA (caspase-2), respectively. Briefly, after treatment and 24-h incubation, supernatants were collected and cells were resuspended in 250 μ L of chilled lysis buffer and incubated on ice for at least 10 min. After centrifugation (5 min, 10000 *g*), supernatants (cytosolic extracts) were transferred to a fresh tube and placed on ice. The protein concentration for each sample set was assayed with BIORAD Protein assay (BIORAD, Munchen, Germany)^[34]. Samples were incubated for 2-3 h at 37°C and measured at 405 nm, as indicated. The absorbance of *p*NA from every sample was compared with the uninduced controls and values were expressed as μ mol/L of *p*NA per microgram of cytosolic protein (μ mol/L per microgram).

Statistics

Statistical analysis was performed using Microsoft Excel 2007 and Instat software (GraphPad software inc., San Diego, California, USA). Results are expressed as mean \pm standard error of the mean (SE). The Kolmogorov and Smirnov test was used to check the Gaussian distribution of data. Statistical comparisons were performed using one-way analysis of variance with Tukey's *post hoc* comparisons. The non parametric Kruskal-Wallis test was used instead if Bartlett's test indicated a significant difference between standard deviations. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of octreotide on HepG2 proliferation

Octreotide caused an initial increase in HepG2 proliferation ($165.2\% \pm 6.2\%$ and $127\% \pm 3\%$ with octreotide 10^{-10} mol/L and 10^{-9} mol/L, respectively) followed by a significant inhibition at a concentration of 10^{-8} mol/L (concentration expected in the blood of patients receiving treatment), reducing cellular proliferation to $77.5\% \pm 1.9\%$ of control (Figure 1). No difference was observed at a concentration of 10^{-7} mol/L ($108.3\% \pm 3.8\%$).

The effect of TNF- α was also examined, at concentrations from 0.1 ng/mL to 100 ng/mL. At the low concentrations of 0.1 and 1 ng/mL, TNF- α had no significant effect on the proliferation of HepG2 cells ($96.9\% \pm 5\%$ and $90.6\% \pm 4.5\%$, respectively), after 6 d of incubation. A marked inhibitory effect was detected with 10, 20 and 100 ng/mL TNF- α , which reduced cellular proliferation to $69.4\% \pm 4\%$, $69.6\% \pm 2.3\%$ and $61.4\% \pm 1.7\%$ of control, respectively (Figure 2). However, because TNF- α at a concentration of 20 ng/mL had the optimum inhibitory effect in another HCC cell line (SMMC-7721 cells)^[19], this concentration was selected for further experiments.

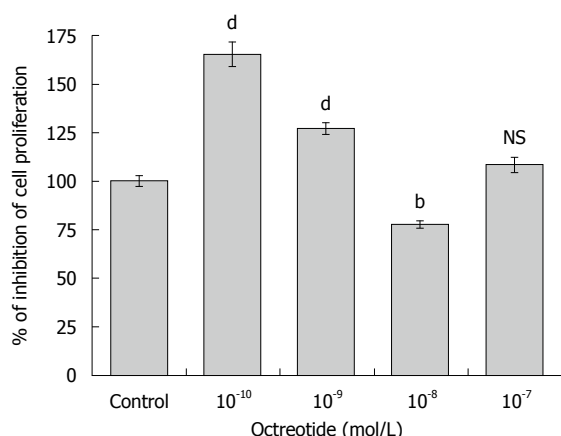


Figure 1 Octreotide at a concentration of 10^{-8} mol/L had a statistically significant inhibitory effect on cellular proliferation of HepG2 hepatocellular carcinoma cells, compared to untreated cells. Lower concentrations caused an initial increase in proliferation. The results represent the mean of 8 different experiments \pm SE ($^bP < 0.01$, $^dP < 0.001$). NS: Not significant.

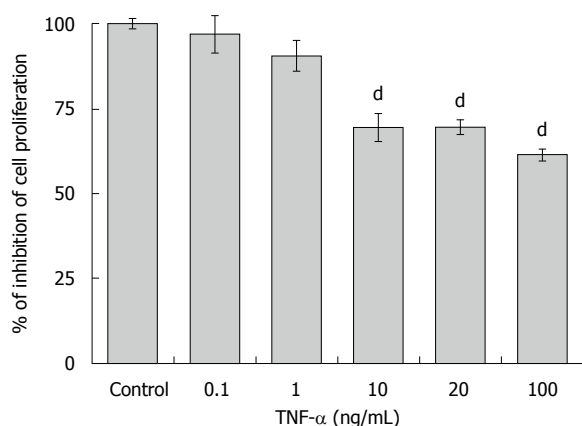


Figure 2 Tumor necrosis factor- α at concentrations of 10, 20 and 100 ng/mL had a statistically significant inhibitory effect on cellular proliferation of HepG2 cells, compared to untreated cells. The results represent the mean of 8 different experiments \pm SE ($^dP < 0.001$). TNF- α : Tumor necrosis factor- α .

Effect of octreotide on HepG2 apoptosis

Apoptosis was detected based on determination of histone-complexed DNA fragments (mono and oligonucleosomes) from the cytoplasm of apoptotic cells. Similar non significant detection of DNA fragmentation was noted after 24-h treatment of HepG2 cells with either octreotide or TNF- α ($115.2\% \pm 6.95\%$ and $115.2\% \pm 8.17\%$, respectively) (Figure 3).

Necrotic cells should be visualized as double positive cells of large dimensions, as they rapidly lose membrane integrity and swelling occurs before destruction, or as fractured membranes of low FS and SS, PI only positive or double positive, but in the place where debris is usually detected and excluded. We also observed that all double positive cells had very small dimensions (data not shown), although they were still intact. We considered double positive cells as cells that followed the apoptotic rather than the necrotic procedure, having increased their SS and decreased their FS characteristics, but still retaining relatively small dimensions. That was the reason why we followed

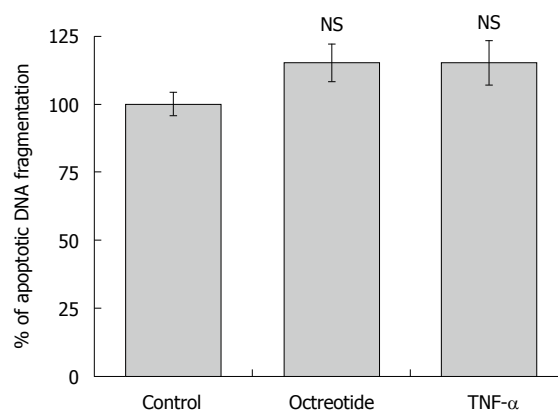


Figure 3 Detection of DNA fragmentation revealed a non significant increase in DNA fragments, after 24-h treatment with octreotide or tumor necrosis factor- α ($n = 8$). TNF- α : Tumor necrosis factor- α ; NS: Not significant.

a specific gating strategy for analysis. Octreotide caused a significant increase in early apoptosis ($7.2\% \pm 1.4\%$, $P < 0.01$, Annexin-V positive cells) and a highly significant increase in late apoptosis ($15.3\% \pm 2.7\%$, $P < 0.001$, Annexin-V/PI double positive cells). TNF- α significantly increased early ($12.5\% \pm 1.4\%$, $P < 0.001$) and more so late apoptosis ($26.4\% \pm 4\%$, $P < 0.001$, Figure 4). All comparisons were made with untreated HepG2 cells used as control cells ($0.5\% \pm 0.3\%$ and $2 \pm 0.3\%$ for early and late apoptosis, respectively) (Figure 4).

Effect of octreotide on caspase activity in HepG2 cells

Caspase-3 activity was significantly increased after treatment of HepG2 cells with octreotide (4.71 ± 0.81 $\mu\text{mol/L}$ per microgram protein, $P < 0.01$) alone, while after TNF- α only, a non significant increase was found (3.28 ± 0.55 $\mu\text{mol/L}$ per microgram protein), compared with uninduced cells (1.87 ± 0.24 $\mu\text{mol/L}$ per microgram protein) (Figure 5). A small but not significant increase in caspase-9 activity was detected after treatment of HepG2 cells with TNF- α (2.44 ± 0.33 $\mu\text{mol/L}$ per microgram protein) or octreotide alone (2.42 ± 0.77 $\mu\text{mol/L}$ per microgram protein) (Figure 5), compared to uninduced cells (1.56 ± 0.21 $\mu\text{mol/L}$ per microgram protein). TNF- α caused a non significant increase in caspase-8 activity (0.9 ± 0.18 $\mu\text{mol/L}$ per microgram protein) compared with control cells (0.51 ± 0.06 $\mu\text{mol/L}$ per microgram protein) (Figure 5). However, octreotide caused a significant increase (1.3 ± 0.1 $\mu\text{mol/L}$ per microgram protein, $P < 0.01$). TNF- α and octreotide caused a significant increase in caspase-2 activity (1.73 ± 0.17 $\mu\text{mol/L}$ per microgram protein and 1.7 ± 0.18 $\mu\text{mol/L}$ per microgram protein, respectively, $P < 0.001$), compared with control cells (0.8 ± 0.09 $\mu\text{mol/L}$ per microgram protein) (Figure 5).

DISCUSSION

Clinical studies of non-neuroendocrine tumors demonstrate that octreotide can inhibit the growth of a variety of tumors, either directly, through binding on the sstrs of tumor cells, or indirectly, through an immunomodulatory or an antiangiogenic effect^[35-37]. Several reports indicate that

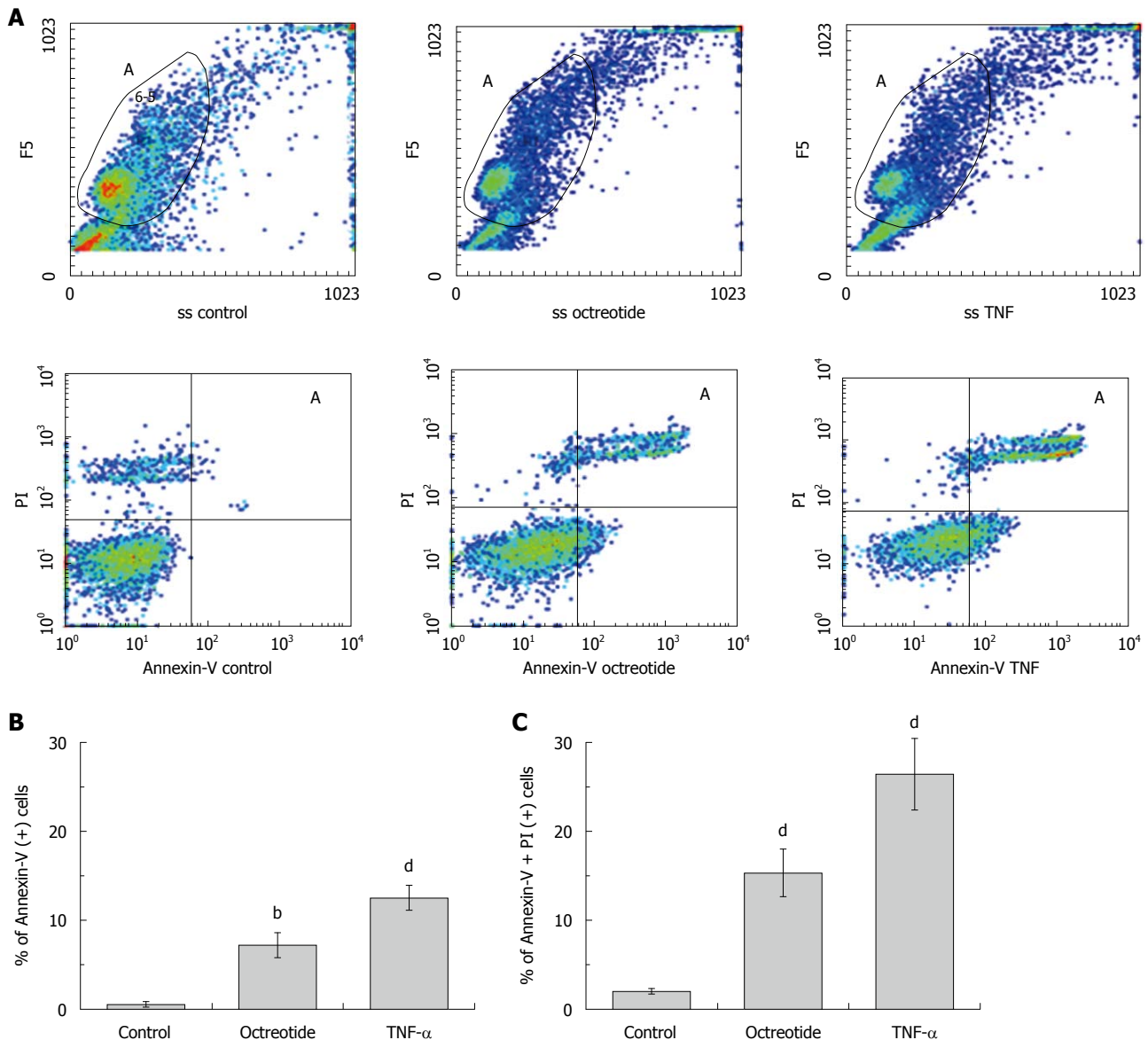


Figure 4 The apoptotic effect of octreotide and tumor necrosis factor- α alone is shown. A: Octreotide and tumor necrosis factor- α (TNF- α) significantly increased early (Annexin-V only positive, right lower quadrant) and more so late apoptotic cells [Annexin-V and propidium iodide (PI) positive, right upper quadrant]. Every sample was analyzed with the same gating strategy (Gate A) to exclude debris and non-specific binding of Annexin-V, while control refers to uninduced HepG2 cells; B: Annexin-V positive cells; C: Annexin-V/PI positive cells. B and C: Mean of 8 different experiments \pm SE ($^bP < 0.01$, $^dP < 0.001$).

octreotide inhibits the proliferation and induces apoptosis of HCC cells *in vitro*^[12-18]. In this study we confirmed that octreotide inhibits HepG2 proliferation^[12,15,18], but only at a concentration of 10^{-8} mol/L, although an initial increase at lower concentrations was observed.

In contrast to these findings, there are also reports that proliferation of HCC cells or hepatic stellate cells is not affected by octreotide^[38,39]. In the study of Reynaert *et al*^[39], shorter periods of culture compared to ours were used, while activation of sstrs was achieved with individual synthetic agonists, therefore a possible combined effect of concomitant receptor activation may have been missed. Similarly, clinical trials have demonstrated a survival benefit of patients with inoperable HCC treated with octreotide^[27,28], but also negative studies have been published^[29,30] and recently criticized^[31]. Interestingly, in our study, lower concentrations of octreotide increase proliferation and

this is possibly an additional reason for divergent results in both clinical trials and *in vitro* studies of octreotide in HCC. Our findings also indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

Octreotide binds mainly to sstr2, sstr3 and sstr5^[40], the presence of which has been recently documented in HepG2 cells^[12,41]. The antiproliferative effect of octreotide is thought to be mediated by sstr2^[42] and sstr5^[43]. Even when a significant amount of sstr2 binding in cellular membranes is not evident, it is possible that octreotide is internalized either along with sstr2 or alone as reported by Dournaud *et al*^[44] and Hornick *et al*^[45]. Recently a desensitization of sstr2 has been reported after short term incubation of an HCC cell line with octreotide, which is probably reversed after long term incubation^[46]. We have previously reported an IC₅₀ of 1.25 nmol/L for the

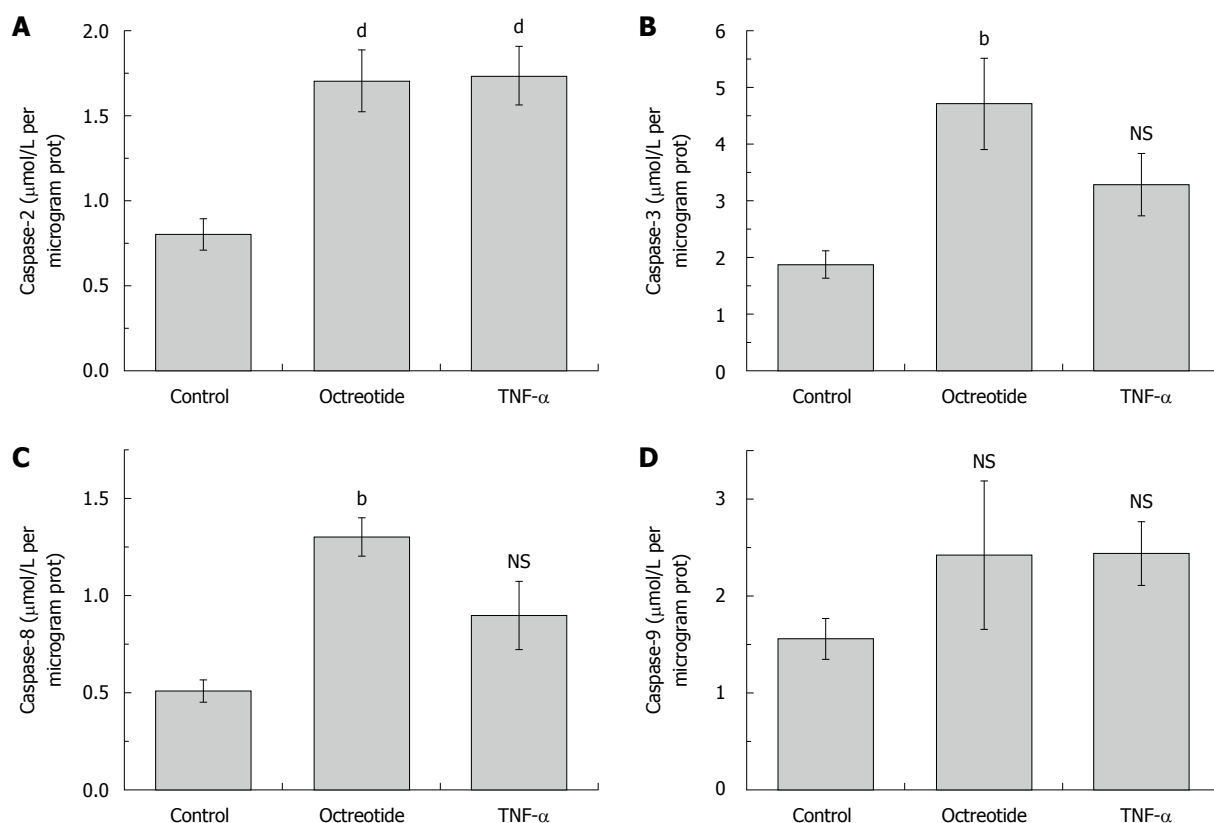


Figure 5 Caspase-2 (A), -3 (B), -8 (C) and -9 (D) activities, after treatment of HepG2 cells with 10^{-8} mol/L octreotide and 20 ng/mL tumor necrosis factor- α , compared to untreated cells. The results represent the mean of 10 different experiments \pm SE ($^bP < 0.01$, $^dP < 0.001$). TNF- α : Tumor necrosis factor- α ; NS: Not significant.

antiproliferative effect of octreotide for HepG2 cells^[41] which is within the range of IC_{50} for sstr2 but it is lower from the IC_{50} reported for sstr5. In the case of sstr5, it is possible that a biological effect can be achieved without activation of the total number of receptors.

The antiproliferative effect of octreotide may be due to either cell necrosis or cell apoptosis^[47]. Therefore, we investigated the apoptotic effect of octreotide, particularly in association with caspase activation, comparing this effect with the well-described pathways of TNF- α -mediated apoptosis. The machinery of apoptosis includes death receptors, adaptor proteins and proteolytic enzymes (caspases). Death receptors belong to the tumor necrosis factor receptor gene superfamily. Among these receptors, TNF receptor-1 (TNFR1) and Fas (CD95) are the most extensively characterized, and both are abundantly expressed in liver^[20,48]. TNF- α at 20 ng/mL induced apoptosis in human hepatoma cell line SMMC-7721 *in vitro*, which was exacerbated by the hypoxanthine-xanthine oxidase system and CHX, but alleviated by superoxide dismutase, suggesting that TNF- α -induced apoptosis may be due to oxidative stress^[49]. The SMMC-7721 cell line was insensitive to TNF- α cytotoxicity and underwent apoptosis quickly in the presence of TNF- α and CHX^[21]. In accordance with this study, the optimal concentration of TNF- α was also found to be 20 ng/mL for HepG2 cells in our study.

Our findings with flow cytometry showed that both octreotide and TNF- α induced a significant early and late apoptosis of HepG2 cells. DNA fragmentation measurements also demonstrated a non significant induction

of apoptosis. This possibly means that flow cytometry is a more sensitive method for quantification of apoptosis.

The mechanism by which octreotide induces apoptosis is not well understood. Changes in sstr expression because of downregulation or possible heterodimerization^[27,50] of a receptor, together with changes in the expression of regulatory proteins required for correct trafficking of specific sstr subtypes, could affect the direct antitumor effect of octreotide, which has been previously demonstrated in models expressing sstr2^[51,52]. Mediated by sstr2, octreotide upregulates tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), death receptor 4 (DR4) and TNFR1, and down-regulates Bcl-2, which results in apoptosis^[53]. Mediated by sstr3, octreotide upregulates p53^[47] or induces Bcl-2-associated protein Bax^[47,54] and acidic endonuclease, resulting in apoptosis. In addition to these mechanisms, in this study we demonstrated a direct effect of octreotide on caspase activation. The effect of octreotide on caspase-mediated apoptosis, is limited to caspase-3 activation, as the main effector caspase supportive of apoptosis and it was detected in primary pheochromocytoma cells^[55], in radiation-induced intestinal damage^[56] and in activated lymphocytes^[57]. Interestingly, decreased caspase-3 mRNA expression in Kupffer cells also indicates a possible additional beneficial effect of octreotide in HCC, through an antiapoptotic effect on Kupffer cells^[58].

We suggest a caspase-mediated apoptotic pathway after treatment of HCC cells with octreotide, where, unlike TNF- α induced apoptosis, 3 out of 4 caspases tested were significantly increased. The activation of all caspases

indicates a possible mitochondria-dependent apoptotic pathway. In contrast, findings from TNF- α -induced apoptosis possibly indicate a different pathway.

A TNF- α -mediated pathway is reported to activate caspase-8, which promotes cleavage of various downstream caspases, including caspases-3, -6 and -7. Caspase-8 can also cleave the Bcl-2 homologue Bid to reveal an active truncated Bid fragment inducing cell death through a mitochondrial pathway^[59-62]. However in our study, caspase-8, caspase-3 and caspase-9 were all increased by TNF- α , but this was not statistically significant. Furthermore, caspase-2 was significantly increased by TNF- α , a finding not reported before. In a previous study, we presented a TNF- α -induced increase of caspase-2, but this increase did not reach the statistical significance^[63]. In the present study, with an increased number of experiments, we found a significant increase in caspase-2 by TNF- α in HepG2 cells. This may be related to inefficient cleavage of Bid, so that all caspases can be significantly activated^[59].

Caspase-2 seems to play critical and specific roles in programmed cell death^[64]. It has been difficult to assign caspase-2 to the effector or initiator caspase groups. Cytokine-induced and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation^[65]. In our study, caspase-2 appeared to be activated independent of significant (octreotide) or non-significant (TNF- α) activation of the mitochondria-mediated pathway. This may have been the result of intracellular events (such as pH or stress) or feedback activation by effector caspases (such as caspase-3).

Thus, our findings suggest that in HepG2 cells octreotide probably causes apoptosis by a mitochondrial apoptosis pathway, sequentially implicating caspase-8, -2, -9 and -3, although further experiments are required to define the exact initiator pathway. TNF- α on the other hand seems to induce caspase-2 activation, possibly mediated through oxidative stress, as suggested before^[65]. The non-significant activation of the extrinsic pathway (caspase-8) or of the intrinsic pathway (caspase-9), perhaps due to inefficient Bid cleavage, is maybe the cause of the resistance observed in previous studies and of the eliminated TNF- α -mediated apoptotic effects observed in our study.

In summary, our results support the induction of a caspase-mediated apoptotic pathway by octreotide in HCC cells, implicating both the receptor-mediated and the mitochondrial-apoptotic pathway. The correlation of specific apoptotic, caspase-mediated pathways, with the expression of sstrs in HCC cells needs more investigation to better define and clarify the intracellular mechanisms of the antiproliferative effects of octreotide.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause approximately half a million deaths annually. Undoubtedly, the best available treatment for all liver tumors is complete surgical resection. However, the synthetic somatostatin analogue octreotide has been found effective in inhibiting tumor growth in a variety of experimental models.

Research frontiers

Apart from stimulation of reticuloendothelial system, octreotide may have other mechanisms of action, to inhibit the growth of hepatic tumors. It has been reported that octreotide inhibits the proliferation and induces apoptosis of different HCC cell lines *in vitro*. The mechanisms of apoptosis induction however are not well understood.

Innovations and breakthroughs

Several reports indicate that octreotide inhibits the proliferation and induces apoptosis of HCC cells *in vitro*. In this study, the authors confirmed that octreotide inhibited HepG2 proliferation at a concentration of 10^{-8} mol/L. Interestingly, lower concentrations of octreotide increased proliferation and this is possibly an additional reason for divergent results in both clinical trials and *in vitro* studies of octreotide in HCC. Also, their results support the induction of a caspase-mediated apoptotic pathway by octreotide in HepG2 cells, implicating both a receptor-mediated and mitochondrial-apoptotic pathway.

Applications

The findings of the present study indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations. Also, based on the recently documented presence of sstr2, sstr3 and sstr5 in HepG2 cells, the need for further correlation of specific apoptotic, caspase-mediated pathways, with the expression of somatostatin receptors in HCC cells, is highlighted, to better define and clarify the intracellular mechanisms of the antiproliferative effects of octreotide.

Peer review

The authors evaluated the role of octreotide on cellular proliferation and apoptosis of HepG2 cells. Their results support the induction of a caspase-mediated apoptotic pathway by octreotide in HepG2 cells, implicating both a receptor-mediated and a mitochondrial-apoptotic pathway. They, also, indicated that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

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S- Editor Sun H L- Editor Cant MR E- Editor Lin YP

Effect of heme oxygenase-1 on renal function in rats with liver cirrhosis

Shi-Bin Guo, Zhi-Jun Duan, Qing Li, Xiao-Yu Sun

Shi-Bin Guo, Zhi-Jun Duan, Qing Li, Xiao-Yu Sun, Department of Gastroenterology, the First Affiliated Hospital of Dalian Medical University, Dalian 116001, Liaoning Province, China
 Author contributions: Guo SB and Duan ZJ designed the experiments; Guo SB performed the experiments, analyzed the data and wrote the manuscript; Li Q and Sun XY performed the experiments and collected the data; Duan ZJ revised the manuscript.
 Supported by National Natural Science Foundation of China, No. 30970886; and Science and Technology Project of Dalian, No. 2008E13SF193

Correspondence to: Zhi-Jun Duan, Professor, Department of Gastroenterology, the First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China. cathydoctor@yahoo.com

Telephone: +86-411-83635963 Fax: +86-411-83632383

Received: August 17, 2010 Revised: September 25, 2010

Accepted: October 2, 2010

Published online: January 21, 2011

Abstract

AIM: To investigate the role of heme oxygenase-1 (HO-1) in pathogenesis of experimental hepatorenal syndrome (HRS).

METHODS: Rats were divided into liver cirrhotic group, zinc protoporphyrin IX (ZnPP) treatment group, cobalt protoporphyrin (CoPP) treatment group and sham group. Biliary cirrhosis was established by bile duct ligation in the first three groups. Rats in the ZnPP and CoPP treatment groups received intraperitoneal injection of ZnPP and CoPP, respectively, 24 h before sample collection. Expression of HO-1 mRNA in kidney was detected by reverse-transcription polymerase chain reaction, while protein expression was determined by immunohistochemical analysis. Hematoxylin and eosin staining was performed to observe liver cirrhosis and renal structure. Renal artery blood flow, mean arterial pressure and portal vein pressure, 24 h total urinary volume, serum and urine sodium concentrations, and creatinine clearance rate (Ccr) were also measured.

RESULTS: The HO-1 mRNA and protein expression levels in kidney, 24 h total urinary volume, renal artery blood flow, serum and urine sodium concentration and Ccr were lower in cirrhotic group than in sham group ($P < 0.05$). However, they were significantly lower in ZnPP treatment group than in cirrhotic group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$).

CONCLUSION: Low HO-1 expression level in kidney is an important factor for experimental HRS.

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Key words: Heme oxygenase-1; Carbon monoxide; Hepatorenal syndrome; Zinc protoporphyrin IX; Cobalt protoporphyrin; Bile duct ligation; Biliary cirrhosis

Peer reviewer: Dr. Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire, SY3 8XQ, United Kingdom

Guo SB, Duan ZJ, Li Q, Sun XY. Effect of heme oxygenase-1 on renal function in rats with liver cirrhosis. *World J Gastroenterol* 2011; 17(3): 322-328 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/322.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.322>

INTRODUCTION

Renal dysfunction is very common in patients with advanced liver disease or cirrhosis. Its severity ranges from electrolyte-balance disturbances and water retention to hepatorenal syndrome (HRS)^[1], which is a unique form of renal failure associated with liver cirrhosis or portal hypertension^[2]. Although HRS represents a functional form and sometimes a reversible form of renal failure without significant changes in renal histology during the course of decompensated cirrhosis^[3,4], it is a poor prognostic indica-

tor for patients with liver cirrhosis, who show an increased risk of morbidity and mortality^[2,3]. So far, no effective strategies are available for the treatment or prevention of HRS. Instead, patients are usually managed by maintaining their adequate hemodynamic status and intravascular volume. A better understanding of the pathophysiological mechanism underlying HRS helps to guide its treatment.

It is currently believed that marked renal vasoconstriction and predominant peripheral arterial vasodilation play a critical role in the pathogenesis of HRS^[5-7]. Previous studies have shown that nitric oxide (NO), a potent vasodilator, plays an important role in the development of hyperdynamic syndrome and peripheral vasodilation during cirrhosis^[8]. Increased NO level and synthetase activity in patients with liver cirrhosis have adverse effects on the functions of renal tubules and glomeruli^[9], and inhibition of NO synthetase prevents the development of renal failure in an animal model of HRS^[10,11]. Carbon monoxide (CO), a byproduct of heme oxygenase-1 (HO-1), shares many characteristics with NO. Endogenous CO is an activator of soluble guanylate cyclase and relaxes vascular smooth muscle in a cGMP-dependent or a cGMP-independent manner^[12,13]. Studies have shown that the HO-1/CO system plays an important role in the control of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme^[12-14]. HO-1 is also involved in the prevention of renal failure after renal ischemia^[15] or glycerol-induced acute renal injury in rats^[16].

Metalloporphyrins constitute a class of compounds in which the central iron of heme is replaced by other metals such as cobalt and zinc^[17]. These metalloporphyrins inhibit or induce HO-1. This study was to evaluate the expression of HO-1 in kidneys of rats with experimental HRS and the functional role of HO-1 in the pathogenesis of HRS by manipulating its activity *via* intraperitoneal injection of either zinc protoporphyrin IX (ZnPP), a specific HO-1 enzyme inhibitor, or cobalt protoporphyrin (CoPP), a specific HO-1 enzyme inducer.

MATERIALS AND METHODS

Animals

Healthy male Sprague Dawley rats, weighing 200-220 g, were obtained from the Laboratory Animal Center of Dalian Medical University.

Reagents

ZnPP and CoPP (Sigma, St Louis, MO, USA) were dissolved in 0.2 mol/L of NaOH, adjusted to a pH of 7.4 and diluted in 0.85% NaCl with a final concentration of 1 mg/mL as previously described^[18]. Rabbit anti-mouse HO-1 antibody (Boster Biological Technology, Wuhan, China), anti-rabbit IgG (MaxVision™2, Maixin Biotechnology, Fuzhou, China), TaKaRa RNA polymerase chain reaction (PCR) kit (AMV) Version 3.0 (TaKaRa Biotechnology, Dalian, China) were used in the study.

Animal model and grouping

The rats were randomly divided into sham group ($n = 8$),

cirrhotic group ($n = 10$), ZnPP treatment group ($n = 9$) and CoPP treatment group ($n = 8$). They were well fed and housed for 3 d before any experimental protocols. Biliary cirrhosis was induced by bile duct ligation (BDL)^[19,20] in rats of the cirrhotic group, ZnPP and CoPP treatment groups. The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with a 4-0 silk suture, and the antibiotic benzathine benzylpenicillin powder was sprinkled over the closed incision. The rats were continuously fed and housed for a further 4-wk period after surgery, and samples were collected. Rats in sham group underwent laparotomy with the bile duct isolated but not ligated. Rats in ZnPP and CoPP treatment groups received an intraperitoneal injection with ZnPP^[21] or CoPP (30 mg/kg body weight) once, 24 h before sample collection. Rats in the 4 groups were housed in metabolic cages for the last 24 h, and urine was collected to measure its volume and the sodium and creatinine (Cre) levels.

Sample collection

Four weeks after surgery, the rats were anesthetized with ether and their portal vein, right carotid artery, and renal artery were isolated. Renal artery blood flow was measured by ultrasound (LOGIQ7, GE, USA). A catheter, connected to a pressure transducer (BL-420F biological experimental system, Chengdu Technology and Market Co. Ltd., China), was placed in the carotid artery for measurement of mean arterial pressure (MAP), then 1 mL of arterial blood was collected in a heparinized syringe through an arterial catheter to measure carboxyhemoglobin (COHb) using a RapidLab 1245 blood gas analyzer (Siemens, USA), as an index for the CO level in arterial blood. The catheter was placed in the portal vein to measure portal vein pressure (PVP). Then, 4 mL of blood was collected from the rats to measure serum levels of bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Cre and sodium with a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co., Tokyo, Japan). Urine levels of Cre and sodium were also measured using this machine. The Cre clearance rate (Ccr) was calculated as urine Cre \times urine volume/serum Cre. The left kidney and one liver lobe were excised, some of their tissues were fixed in a 10% neutral formalin solution and embedded in paraffin, while the remaining tissues were preserved at -80°C for PCR.

Reverse-transcription PCR analysis

Total RNA was extracted from kidneys following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm ($A_{260 \text{ nm}} = 1$ for 40 $\mu\text{g/mL}$ RNA), and the purity of RNA was assessed by determining the ratio of the optical density obtained at 260 and 280 nm (pure RNA: $A_{260 \text{ nm}}/A_{280 \text{ nm}} = 2.0$) using a Shimadzu UV-1206 spectrophotometer (Shimadzu, Japan). The primer sequences for HO-1 are 5'-ACTTTCAGAAAGGGTCAGGTGTCC-3' (forward) and 5'-TTGAGCAGGAAGGCGGTCTTAG-3'

Table 1 Effect of bile duct ligation and cobalt protoporphyrin or zinc protoporphyrin IX treatment on serum and urine levels of creatinine and sodium, and creatinine clearance rate (mean \pm SD, $n = 8-10$ per group)

	Sham group	Cirrhosis group	CoPP group	ZnPP group
Serum Cre ($\mu\text{mol/L}$)	30.4 \pm 1.81	36.3 \pm 6.27 ^a	33.5 \pm 5.98	45.3 \pm 8.92 ^c
Urine Cre ($\mu\text{mol/L}$)	7.18 \pm 1.15	8.08 \pm 2.50	4.49 \pm 1.51	6.31 \pm 1.20
Serum sodium (mmol/L)	142.86 \pm 3.44	138.75 \pm 0.96 ^a	142.64 \pm 5.43 ^c	136.57 \pm 1.40 ^c
Urine sodium (mmol/L)	91.50 \pm 12.12	71.33 \pm 10.07 ^a	109.15 \pm 64.93	66.25 \pm 11.8
Ccr (mL/min)	0.23 \pm 0.02	0.12 \pm 0.05 ^a	0.14 \pm 0.04	0.07 \pm 0.01 ^c
AST (IU/L)	156.8 \pm 18.28	237.2 \pm 95.13 ^a	467.14 \pm 222.28 ^c	209.11 \pm 65.77

^a $P < 0.05$ vs sham group; ^c $P < 0.05$ vs cirrhotic group. CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin IX; Cre: Creatinine; Ccr: Creatinine clearance rate; AST: Aspartate aminotransferase.

(reverse) and the product size is 524 bp, while the primer sequences for β -actin are 5'-GGAGTCAACGGATT-TGGT-3' (forward), 5'-GTGATGGGATTTCCATTG-3' (reverse) and the product size is 226 bp. An aliquot of each mixture was used for reverse-transcription (RT)-PCR amplification using reagents purchased from Takara Bio Inc (Dalian, China). PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were photographed and the density of each product band was quantified. The results were expressed as the ratio of the band density for HO-1 mRNA to that of β -actin mRNA.

Immunohistochemical analysis

Kidney and liver tissues were fixed in a 10% neutral formalin solution and embedded in paraffin wax and cut into sections. Some sections were routinely stained with HE while the other sections underwent deparaffinization, rehydration and inactivation, and were incubated with rabbit-anti-mouse HO-1 monoclonal antibody (1:50) at room temperature for 60 min, and then with secondary antibody (MaxVisionTM2) at room temperature for 15 min. The sections were mounted after staining. The primary antibody was replaced by phosphate-buffered saline to serve as a negative control. Five high-power microscopic fields were randomly chosen per slide and the yellow material in cytoplasm was considered to represent a HO-1-positive cell. Cell staining was assigned to 4 scores: 4 = > 75% positive cells, 3 = 50%-75% positive cells, 2 = 25%-50% positive cells, and 1 = < 25% positive cells. Cell staining intensity was scored based on its color as follows: 0 = no staining, 1 = faint yellow, 2 = light brown, and 3 = dark brown^[22]. The final score was defined as staining intensity \times percentage of positive cells. The mean score of five fields was used to compare the four groups.

Statistical analysis

Data analysis was performed using the SPSS 10.0 software (Chicago, IL, USA). Analysis of variance or Wilcoxon statistical methods were used to determine statistical significance. All measurements in this study were expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

Biochemical examination

The serum level of AST in biliary cirrhotic group was

237.2 \pm 95.13 IU/L, which was significantly higher than that (156.8 \pm 18.28 IU/L) in sham group ($P < 0.05$). The serum level of Cre was significantly higher and the Ccr was significantly lower in biliary cirrhotic group than in sham group ($P < 0.05$). The serum Cre level was significantly higher in ZnPP treatment group ($P < 0.05$) and slightly lower in CoPP treatment group than in cirrhotic group ($P > 0.05$). The serum and urine sodium levels were significantly lower in cirrhotic group than in sham group ($P < 0.05$). The serum sodium concentration was significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$, Table 1).

Hemodynamic parameters and arterial blood gas levels

The PVP was significantly higher and the MAP was significantly lower in cirrhotic group than in sham group ($P < 0.01$). However, no significant difference was found in PVP and MAP in ZnPP and CoPP treatment groups compared with cirrhotic group. The COHb level in arterial blood was significantly higher in cirrhotic group than in sham group ($P < 0.05$) while significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$). The renal artery blood flow was significantly lower in cirrhotic group than in sham group ($P < 0.01$), while significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$). Furthermore, the 24 h urine volume was significantly smaller in cirrhotic group than in sham group ($P < 0.05$), while significantly smaller in ZnPP treatment group and significantly larger in CoPP treatment group than in cirrhotic group ($P < 0.05$, Table 2).

Histopathological analysis of liver and kidney in cirrhotic and sham rats

Liver and kidney tissue samples from cirrhotic and sham rats were stained with HE to examine the histopathological changes. The bridging necrosis of hepatic cells was observed in livers of rats 4 wk after BDL, particularly in portal areas, nodular regeneration of hepatocytes, collapse, and disorganization of the hepatic lobular structure, numerous lymphocytes infiltrating the portal area and around the central vein, and the formation of pseudolobules surrounded by fibrous septa. In contrast, except for vascular dilation and congestion of the mesenchyme, no

Table 2 Effects of bile duct ligation and cobalt protoporphyrin or zinc protoporphyrin IX treatment on hemodynamic parameters, carboxyhemoglobin and 24-h urine volume (mean \pm SD, $n = 8-10$ per group)

	Sham group	Cirrhosis group	CoPP group	ZnPP group
PVP (mmHg)	9.24 \pm 0.76	15.56 \pm 2.36 ^b	17.28 \pm 1.20	13.71 \pm 1.39
MAP (cmH ₂ O)	118.83 \pm 8.09	59.23 \pm 12.19 ^b	52.75 \pm 5.76	67.76 \pm 7.66
COHb (%)	0.23 \pm 0.05	0.50 \pm 0.20 ^a	0.83 \pm 0.39 ^c	0.23 \pm 0.06 ^c
RABF (mL/min \cdot 100 g)	3.89 \pm 0.09	3.58 \pm 0.04 ^b	3.76 \pm 0.06 ^c	3.50 \pm 0.08 ^c
Urine (mL/24 h)	15.00 \pm 2.23	10.93 \pm 1.92 ^a	13.5 \pm 1.10 ^c	8.50 \pm 1.10 ^c

^a $P < 0.05$, ^b $P < 0.01$ vs sham group; ^c $P < 0.05$ vs cirrhosis group. CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin IX; PVP: Portal vein pressure; MAP: Mean arterial pressure; COHb: Carboxyhemoglobin; RABF: Renal arterial blood flow.

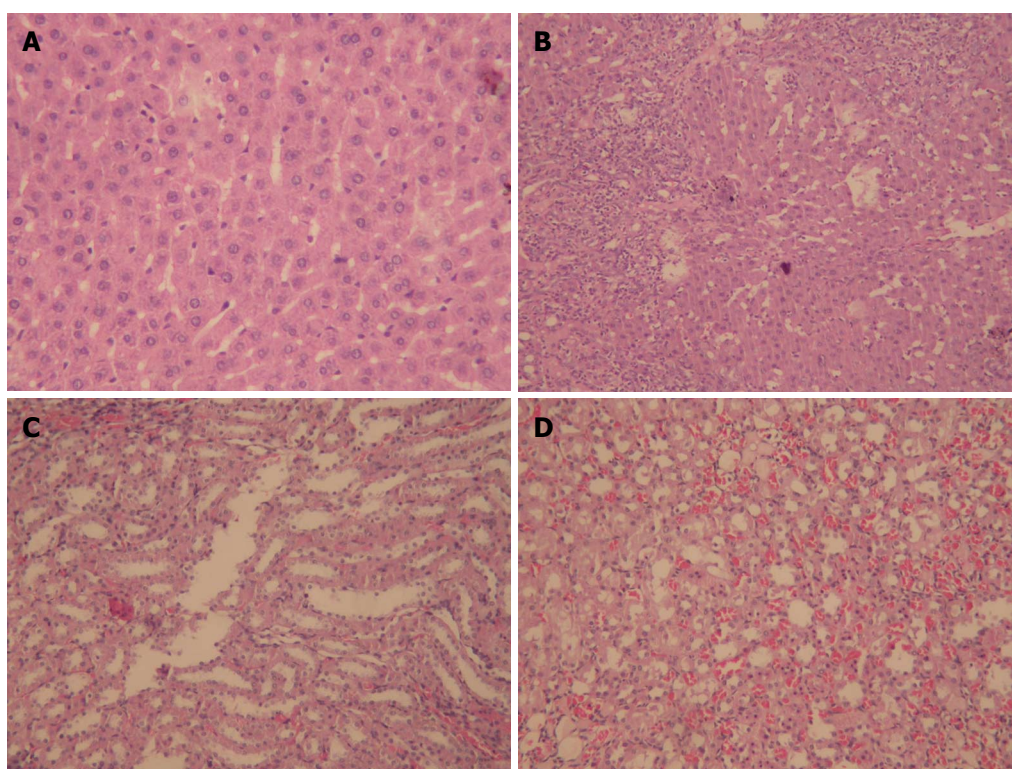


Figure 1 Representative photomicrographs of rats in cirrhotic and sham groups (magnification 200 \times , HE staining). A: Normal liver structure; B: Liver cirrhosis; C: Normal kidney structure; D: Renal structure in cirrhotic group.

obvious pathological changes were observed in kidneys of cirrhotic group compared with sham group (Figure 1).

Renal HO-1 mRNA expression level

RT-PCR showed that the expression level of HO-1 mRNA in kidney was significantly lower in cirrhotic group than in sham group ($P < 0.01$). Furthermore, renal HO-1 mRNA expression was significantly decreased in ZnPP treatment group ($P < 0.05$) and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$) (Figure 2).

Immunohistochemical detection of HO-1 protein in kidney and liver

To localize the HO-1 protein expression in kidneys, immunohistochemistry was performed using specimens from the four groups. The HO-1 protein was mainly expressed in the distal renal tubules, which is similar to reported

findings^[23] (Figure 3). The intensity and percentage of cells expressing HO-1 protein in kidney were also detected. Mild staining was observed in renal tissue samples from sham group, with a score of 1.21 ± 0.33 . The HO-1 score was 0.79 ± 0.25 in cirrhotic group, which was significantly lower than that in sham group ($P < 0.01$). The HO-1 score (0.21 ± 0.25) was lower in ZnPP treatment group and higher in CoPP treatment group (2.46 ± 0.46) than in cirrhotic group ($P < 0.05$). The staining intensity and percentage of cells expressing HO-1 protein were also evaluated in liver of cirrhotic and sham groups. Unlike the HO-1 protein expression in kidney, the mean hepatic HO-1 score was significantly higher in cirrhotic group than in sham group (4.63 ± 0.74 vs 0.63 ± 0.52 , $P < 0.01$).

DISCUSSION

Renal dysfunction is very common in patients with ad-

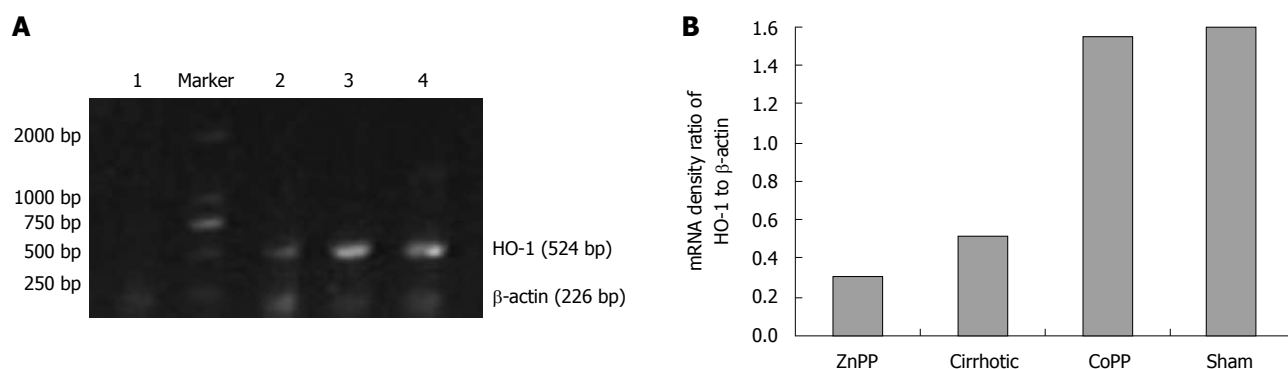


Figure 2 Expression of heme oxygenase-1 mRNA in kidney. A: Representative reverse-transcription polymerase chain reaction data showing the heme oxygenase-1 (HO-1) mRNA expression levels in kidneys from zinc protoporphyrin IX (ZnPP) treatment group (lane 1), cirrhotic group (lane 2), cobalt protoporphyrin (CoPP) treatment group (lane 3), and sham group (lane 4); B: Quantitative data showing the ratio of band density of the corresponding HO-1 mRNA to that of β-actin mRNA.

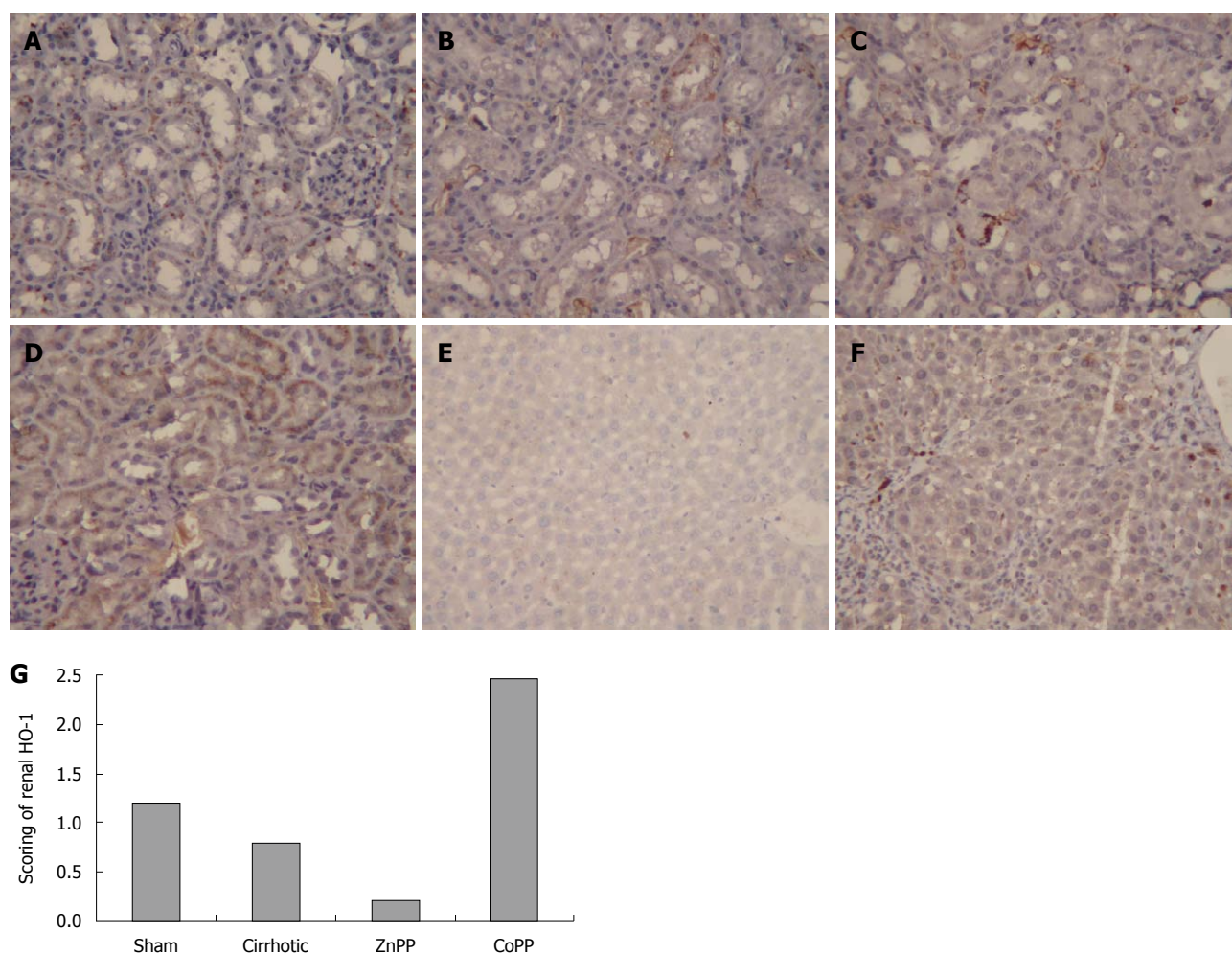


Figure 3 Expression of heme oxygenase-1 protein in kidney and liver. Immunohistochemical staining of renal heme oxygenase-1 (HO-1) protein in rats of sham group (A), cirrhotic group (B), zinc protoporphyrin IX (ZnPP) treatment group (C), cobalt protoporphyrin (CoPP) treatment group (D), and immunohistochemical staining of hepatic HO-1 protein in rats of sham group (E) and cirrhotic group (F) in the upper part (magnification 200 ×), and quantitative scoring (G) of immunohistochemical staining of renal HO-1 protein expression in each group in the lower part.

vanced cirrhosis, and its severity varies from electrolyte-balance disturbances and water retention to HRS. HRS is considered a functional renal failure because no structural damage has been observed in kidney, and can be reversed in some cases^[4]. However, it carries a worse prognosis of patients with cirrhosis and increases their risk of mortal-

ity^[2,3]. It has been reported that the annual incidence of HRS in patients with liver cirrhosis and ascites is about 8%^[24]. Typical features of HRS include oliguria, hyponatremia, azotemia, and hyponatruiria. Although the pathophysiological mechanism underlying HRS is still incompletely understood, marked renal vasoconstriction in the presence

of splanchnic and systemic vasodilation may play an important role, and may thus reduce the renal arterial blood flow and the glomerular filtration rate, resulting in oliguria and an increased serum Cre concentration^[6-8]. Studies have shown that the HO-1/CO system plays an important role in the control of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme^[12-14]. In addition, HO-1 is also involved in the prevention of renal failure after renal ischemia^[15] or glycerol-induced acute renal injury in rats^[16]. In this study, the relation between expression of HO-1 in kidney and renal arterial blood flow and renal function was investigated in cirrhotic rats.

To evaluate the hepatic, renal, and systemic changes in rats 4 wk after BDL, the MAP, PVP, COHb in arterial blood, serum levels of AST and ALT, Cre and sodium, urine sodium and Cre were measured. The changes in hepatic and renal histology were also examined, and the expression levels of HO-1 mRNA and protein in liver and kidney were evaluated. The Ccr level was also measured as an index of glomerular filtration rate and renal function. The serum level of AST was significantly higher in cirrhotic group than in sham group, indicating that BDL causes marked liver injury, with liver cirrhosis confirmed with HE staining of liver specimens. In addition, the MAP level was significantly lower in BDL rats than in time-matched sham rats, indicating that a hyperdynamic state occurs. Furthermore, the PVP was significantly higher in BDL rats than in time-matched sham rats, indicating that portal hypertension exists. Oliguria, hyponatremia, hyponatremia, increased Cre concentrations and decreased Ccr were also observed in BDL rats. All these findings show that experimental HRS was established in BDL rats. Accompanied with the decreased renal arterial blood flow and renal function, the HO-1 mRNA and protein levels in kidney of rats were significantly lower in cirrhotic group than in sham group, indicating that production of CO is decreased in kidney, because CO is mainly generated by degrading heme due to HO. HO has constitutive and inducible isoforms^[25,26]. HO-1, a 32-kDa inducible protein^[27], catalyzes the rate-limiting step in oxidative degradation of heme to biliverdin, releasing equimolar amounts of CO and iron^[26]. The HO-1/CO system plays a vital role in many activities, including anti-oxidative stress, anti-inflammation, inhibition of cellular proliferation, and regulation of cytokine expression. CO, a gaseous messenger similar to NO, can activate soluble guanylate cyclase leading to production of cGMP^[28] which mediates various physiological functions^[29] including vasodilation^[30]. CO can also relax vascular smooth muscle in a cGMP-independent manner^[12,13]. HO-1 activity is the primary source of circulating CO^[31], and HO-1 contributes to vasodilation mainly through HO-1-derived CO^[32]. Thus, the declined HO-1 expression in kidney may be responsible for a decrease in vasodilation. In addition, oxidants can cause localized renal vasoconstriction^[33]. Thus, the antioxidant action of HO-1 and its products can preserve renal arterial blood flow. Decreased HO-1 expression in kidney of BDL rats impairs their ability to buffer locally produced oxidants, thus leading to decreased renal arterial blood flow and deteriorated renal function.

Surprisingly, the COHb level was significantly higher

in cirrhotic group than in sham group, suggesting that there is more CO in circulation, since it is predominately bound to hemoglobin in the form of COHb^[34]. This large amount of CO may be produced by increased HO-1 expression in other organs, such as liver, because the HO-1 expression level in liver was higher in cirrhotic group than in sham group. Thus, we speculate that the decreased HO-1 expression in kidney and suppression of locally produced CO contribute to the decreased renal arterial blood flow and renal dysfunction in cirrhotic rats.

To evaluate the functional consequences of HO-1 changes, BDL rats were treated with either ZnPP or CoPP. The PVP and MAP were measured to evaluate systemic effects of ZnPP and CoPP treatment, renal arterial blood flow, 24 h total urinary volume, serum and urine levels of sodium and Cre were also measured to evaluate the effects of ZnPP and CoPP on HRS. The expression level of HO-1 mRNA and protein in kidney was lower in ZnPP treatment group and higher in CoPP treatment group than in cirrhotic group, without obvious changes in PVP and MAP, while the renal arterial blood flow was significantly lower and the renal function was more severely impaired in ZnPP treatment group than in cirrhotic group, as demonstrated by the decreased 24 h total urinary volume, Ccr, and serum level of sodium. In contrast, the renal arterial blood flow and 24 h total urinary volume were higher in CoPP treatment group than in cirrhotic group. However, unlike ZnPP treatment, CoPP treatment did not significantly affect serum Cre or Ccr compared with cirrhotic group. Nevertheless, increasing the treatment time or the CoPP dose may have elicited the different results in our study. Because ZnPP or CoPP treatment did not significantly affect MAP or PVP, changes in renal artery vascular tone were not considered to represent systemic vascular effects of HO-1 inhibition or HO-1 induction.

In conclusion, renal HO-1 expression, renal arterial blood flow, Ccr, 24 h total urinary volume, serum and urine sodium concentrations are lower in cirrhotic rats than in sham rats. Inhibition of renal HO-1 activity decreases renal arterial blood flow and aggravates renal dysfunction in rats with HRS. Meanwhile, activation of renal HO-1 activity had the opposite effects. Taken together, decreased HO-1 expression in kidney plays an important role in the pathogenesis of experimental HRS.

COMMENTS

Background

Renal dysfunction is very common in patients with advanced cirrhosis, and its severity ranges from electrolyte-balance disturbances and water retention to hepatorenal syndrome (HRS). HRS is a poor prognostic indicator for patients with liver cirrhosis, and can increase its morbidity and mortality in such patients. At present, the pathophysiological mechanism underlying HRS is still incompletely understood.

Research frontiers

Intense renal vasoconstriction in combination with peripheral arterial vasodilation plays an important role in occurrence of HRS. Studies have shown that the heme oxygenase-1 (HO-1)/carbon monoxide system is a crucial component in regulation of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme. HO-1 is also central to the prevention of renal failure after renal ischemia or glycerol-induced acute renal injury in rats. In this study, we in-

vestigated the expression of HO-1 in the kidney of experimental rats with HRS and evaluated the functional role of HO-1 in the pathogenesis of HRS.

Innovations and breakthroughs

The characteristics of HRS became evident in rats 4 wk after bile duct ligation (BDL), including reduced creatinine clearance rate and fluid retention, without changes in renal histology. The decreased renal arterial blood flow and renal function were accompanied with decreased renal expression of HO-1 at mRNA and protein levels. To evaluate the functional consequences of the changes in HO-1 expression, the authors treated BDL rats with either zinc protoporphyrin IX (ZnPP), a specific HO-1 inhibitor or cobalt protoporphyrin (CoPP), a HO-1 inducer. ZnPP treatment significantly reduced the renal arterial blood flow and further worsened the renal function, while CoPP treatment had the opposite effects. The relation between HO-1 and renal arterial blood flow and renal function was systematically evaluated by treating BDL rats with ZnPP and CoPP.

Applications

The mechanisms underlying renal dysfunction in patients with advanced liver disease or cirrhosis are complicated and remain incompletely understood. However, the findings in this study indicate that decreased renal HO-1 expression plays an important role in the pathogenesis of experimental HRS.

Terminology

HRS, a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in the absence of any apparent clinical cause for renal insufficiency, corresponds to a functional alteration without histological changes in renal tissue. HO-1 is heme oxygenase-1, a rate-limiting enzyme that is also known as heat shock protein 32, and can be induced by CoPP and inhibited by ZnPP *in vivo*.

Peer review

This paper, written in rather good English, is quite important and interesting, which shows that decreased HO-1 expression in the kidney plays an important role in the pathogenesis of experimental HRS, as it demonstrated that the renal HO-1 expression, renal arterial blood flow, creatinine clearance rate, 24 h total urinary volume, serum and urine sodium concentrations were lower in cirrhotic rats than in sham rats, thus inhibition of renal HO-1 activity decreases renal arterial blood flow and aggravates renal dysfunction in rats with HRS.

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Low red blood cell levels of deglycating enzymes in colorectal cancer patients

Maria Notarnicola, Maria Gabriella Caruso, Valeria Tutino, Vito Guerra, Giovanni Misciagna

Maria Notarnicola, Maria Gabriella Caruso, Valeria Tutino, Laboratory of Biochemistry, National Institute for Digestive Diseases, 70013 Castellana G, Bari, Italy

Vito Guerra, Giovanni Misciagna, Laboratory of Epidemiology and Biostatistics, National Institute for Digestive Diseases, 70013 Castellana G, Bari, Italy

Author contributions: Notarnicola M and Caruso MG contributed equally to this work; Caruso MG and Misciagna G designed the research; Notarnicola M and Tutino V performed the research; Guerra V analyzed the data; Notarnicola M and Caruso MG wrote the paper.

Correspondence to: Giovanni Misciagna, MD, PhD, Laboratory of Epidemiology and Biostatistics, National Institute for Digestive Diseases, IRCCS "S. de Bellis", Via Turi, 27, 70013 Castellana G, Bari, Italy. gmisciag@libero.it

Telephone: +39-80-4994650 Fax: +39-80-4994313

Received: June 28, 2010 Revised: July 28, 2010

Accepted: August 5, 2010

Published online: January 21, 2011

Abstract

AIM: To investigate Glyoxalase I and fructosamine-3-kinase (FN3K) activity in red blood cells from patients with colorectal adenomas and cancer.

METHODS: Thirty three consecutive subjects with one or more histologically confirmed colorectal adenomatous polyps, 16 colorectal cancer patients and a group of 11 control subjects with normal colonoscopy were included in the study. Glyoxalase I and FN3K activities were measured in red blood cells using a spectrophotometric and radiometric assay, respectively.

RESULTS: A significant reduction in both Glyoxalase I and FN3K activity was detected in patients with tumors compared to patients with adenomas and the controls. Erythrocyte Glyoxalase I activity in colorectal cancer was approximately 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter vs 0.128 ± 0.19 mmol/min per milliliter of red

blood cells, $P = 0.003$, Tukey's test). FN3K activity in red blood cells from patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter vs 38.6 ± 31.7 pmol/min per milliliter of red blood cells, $P = 0.04$, Tukey's test).

CONCLUSION: These findings suggest that deglycating enzymes may be involved in the malignant transformation of colon mucosa.

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Key words: Colorectal cancer; Enzymatic activity; Fructosamine-3-kinase; Glycation; Glyoxalase I

Peer reviewer: Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Notarnicola M, Caruso MG, Tutino V, Guerra V, Misciagna G. Low red blood cell levels of deglycating enzymes in colorectal cancer patients. *World J Gastroenterol* 2011; 17(3): 329-333 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/329.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.329>

INTRODUCTION

The enzymatic defense against glycation involves enzymatic activities, such as the glyoxalase system, amadoriase and fructosamine 3 kinase, which suppress the formation of glycation adducts and repair sites of early glycation^[1].

Glyoxalase I together with Glyoxalase II constitutes the glyoxalase system, a ubiquitous detoxification pathway which protects against cellular damage caused by potent cytotoxic metabolites, such as methylglyoxal. Methylglyoxal is a physiological substrate, derived from glycolysis, via degradation of triose phosphate intermediates, lipid peroxidation, and fragmentation of glycated proteins^[2].

As a highly reactive metabolite, methylglyoxal has a strong ability to cross-link with protein amino groups to form stable products called advanced glycation end products, and to attack guanine residues of DNA leading to DNA glycation^[3]. The cytotoxicity of methylglyoxal is due to its mutagenic and antiproliferative properties and to its ability to trigger apoptosis^[4], *via* oxidative signaling^[5]. Experimental evidence shows that the glyoxalase system is involved in the regulation of cellular growth^[6,7]. Altered expression of this system is involved in several human disorders, including cancer^[8-11]. Over-expression of Glyoxalase I is associated with clinical multidrug resistance in tumors of high incidence and mortality, such as carcinomas of the lung, breast and prostate and Glyoxalase I inhibitors provide effective therapy for these tumors^[1,12].

Fructosamine-3-kinase (FN3K) is an intracellular deglycating enzyme that phosphorylates fructosamines on the third carbon of their deoxyfructose moiety. The fructosamine 3-phosphates so formed are unstable and their spontaneous decomposition leads to the regeneration of the free amine^[13,14]. This enzyme seems to catalyze a repair mechanism offering selective cell advantage.

We previously evaluated *FN3K* gene expression in colorectal cancer patients, and showed that *FN3K* gene expression was significantly lower in colon cancer tissue than in the corresponding surrounding normal mucosa^[15]. Moreover, we found that *FN3K* activity is particularly downregulated in tumors located on the left side of the colon^[16].

The adenoma-carcinoma sequence in the colon represents one of the most characterized models of human tumor progression. The transition from normal to malignant phenotype implies the activation of pathways that underlie aberrant clone expansion^[17].

Alterations of metabolic pathways, such as changes in the balance between glycation and enzymatic anti-glycation defense, are considered to be crucial for sustaining tumor development^[18]. The risk of colorectal adenoma increases with serum levels of fructosamine^[19]. The decline in expression of deglycating enzymes may be the key to increased protein glycation in the tumor phenotype.

In this study, we evaluated the levels of Glyoxalase I and *FN3K* activity in red blood cells from patients with colorectal adenomas and cancer.

MATERIALS AND METHODS

Subjects

The study included thirty three consecutive subjects (18 males and 15 females, mean age 67.6 ± 11.7 years) with one or more histologically confirmed colorectal adenomatous polyps removed after complete endoscopy, and sixteen colorectal cancer patients (6 males and 10 females, mean age 68.1 ± 6.4 years) undergoing colon surgery. A group of eleven control subjects (6 males and 5 females, mean age 45 ± 5.8 years) with normal colonoscopy, performed in the same endoscopy unit during the same period, was also included.

Written informed consent was obtained from all the participants.

Measurement

Anthropometric measurements were obtained by the participants wearing scrub suits without shoes. Body weight was measured using a calibrated scale (Detecto; model 437). Standing height was measured with a vertical metal ruler. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2).

Ficoll-Paque separation

Participants were fasted for 12 h prior to examination. Blood samples taken from the subjects by venous puncture were collected in tubes containing EthyleneDiamineTetraacetic Acid (K-EDTA) anticoagulant or a serum separator gel. Blood serum was shipped to the central laboratory for routine analyses. For *in vitro* isolation of erythrocytes, blood samples with K-EDTA were quickly layered on the Ficoll-Paque solution and centrifuged at 400 *g* for 40 min at 20°C. The lymphocytes and plasma were then removed and the erythrocytes were recovered from the bottom layer and washed with 4-volumes of phosphate-buffered saline. Isolated red blood cells were stored at -80°C until assayed. All the analyses were performed within 6 mo.

Glyoxalase I activity assay

Glyoxalase I enzymatic activity was measured in frozen erythrocytes, according to the method described by Thornalley^[20] with minor modifications. The frozen red blood cell pellet was lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT, 1 $\mu\text{g}/\text{mL}$ leupeptin and was well mixed. The samples were centrifuged for 10 min at 2000 *g* and the supernatant was used for the enzymatic activity assay. Aliquots of 50 μL of supernatant were incubated with 100 μL of reaction mix containing 7.9 mmol/L methylglyoxal, 1 mmol/L glutathione, 14.6 mmol/L magnesium sulfate and 182 mmol/L imidazole HCl, pH = 7.0. The activity of Glyoxalase I was determined by monitoring the increase in absorbance at 240 nm due to the formation of *S*-D-lactoylglutathione for 2 min at 25°C. One unit of activity was defined as the formation of 1 mmol of *S*-D-lactoylglutathione/min per milliliter of blood red cells.

Fructosamine-3 kinase activity assay

FN3K enzymatic activity was measured in frozen erythrocytes lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT and 1 $\mu\text{g}/\text{mL}$ leupeptin. The samples were centrifuged for 10 min at 2000 *g* and 50 μL of supernatant were incubated with 100 μL of reaction mix [5 mmol/L glucose, 10 mmol/L Tris-HCl (pH 7.8), 1 mmol/L DTT, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 2 mmol/L D-[1-¹⁴C]-glucose (49.5 mCi/mmol) for 40 min at 37°C.

Subsequently, 30 μL aliquots of the samples were spotted on cation-exchange papers (P81; Whatman), which were washed three times with ice-cold 75 mmol/L H_3PO_4 and then once with alcohol and once with acetone. After drying, the papers were counted for radioactivity in the presence of a scintillant. *FN3K* activity was expressed as picomoles of incorporated D-[1-¹⁴C]-glucose/min per milliliter of red blood cells. Parallel samples were as-

sayed to evaluate total and non-specific radioactivity. The enzyme activity assay was validated using samples in the presence of the FN3K inhibitor, 1-deoxy-1-morpholino-fructose.

Statistical analysis

The mean and standard deviation were calculated for each group. Groups were compared using one-way analysis of variance and Tukey's Multiple Comparison test. Differences in the means were considered statistically significant if the *P*-value was < 0.05. Analysis of covariance was used to model, controlling for glycemia, a potential variable confounder.

RESULTS

The clinical characteristics of all subjects studied are shown in Table 1. There was a weak increase in glycemia levels from the controls to the adenoma and cancer patients. No difference in mean BMI values between the groups was observed.

Table 2 summarizes the data for Glyoxalase I and FN3K activity levels detected in red blood cells from controls, adenoma and tumor-bearing patients.

There was a significant reduction in Glyoxalase I activity in red blood cells from patients with tumor compared to controls, and a trend in decreasing activity from controls to adenoma and cancer patients. Erythrocyte Glyoxalase I activity in colorectal cancer was about 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter *vs* 0.128 ± 0.19 mmol/min per milliliter of red blood cells, *P* = 0.003, Tukey's test). A significant reduction in FN3K activity levels in erythrocytes from colon cancer patients with respect to controls and adenoma patients was also observed. FN3K activity in the red blood cells of patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter *vs* 38.6 ± 31.7 pmol/min per milliliter of red blood cells, *P* = 0.04, Tukey's test).

The differences in enzymatic activities among the control, adenoma and cancer groups were controlled for fasting glycemia using analysis of covariance. The absolute value of association did not decrease; there was only an increase in standard error of the coefficients and consequently a decrease in the *P*-value of the null hypothesis. In any case, the inverse association of both enzymes with cancer was still statistically significant (two tails, *P* < 0.05).

DISCUSSION

This study provides evidence of a role for the deglycating enzymes, Glyoxalase I and FN3K, in colorectal cancer development. The trend of decreased Glyoxalase I activity from the controls to adenoma and cancer patients strengthens the association between this deglycating enzyme and colon cancer, and suggests that its decrease in activity can support the evolution of the malignant process.

The glyoxalase system has received considerable atten-

Table 1 Clinical characteristics of subjects enrolled in the study (mean \pm SD)

	Control subjects	Adenoma patients	Cancer patients
<i>n</i>	11	33	16
Age (yr)	45 \pm 5.8	67.6 \pm 11.7	68.1 \pm 6.4
Female/male	5/6	15/18	10/6
Glycemia (mmol/L)	4.87 \pm 0.2	5.82 \pm 1.2	7.06 \pm 2.4
BMI (kg/m ²)	25.6 \pm 7.7	27.8 \pm 6.3	27.1 \pm 3.1

BMI: Body mass index.

Table 2 Red blood cell levels of Glyoxalase I and fructosamine-3-kinase activity in controls, adenoma and tumor-bearing patients (mean \pm SD)

	Glyoxalase I	FN3K
Controls (<i>n</i> = 11)	0.173 \pm 0.25	29.05 \pm 14.6
Adenomas (<i>n</i> = 33)	0.128 \pm 0.19 ^a	38.6 \pm 31.7 ^b
Tumors (<i>n</i> = 16)	0.022 \pm 0.01 ^a	19.55 \pm 6.4 ^b

^{a,b}*P* < 0.05, Tukey's test. The glyoxalase enzymatic activity is expressed as mmol/min per milliliter of red blood cells and the enzymatic activity of FN3K is expressed as pmol/min per milliliter of red blood cells. FN3K: Fructosamine-3-kinase.

tion regarding its possible relationship with cancer. In an animal model of carcinogenesis, an appreciable decrease in rat liver glyoxalase activity was found after the development of hepatoma^[21]. Some authors also showed that glyoxalase activity in the blood of tumor-bearing animals was much lower than glyoxalase activity in the blood of normal animals^[22]. The presence of Glyoxalase I gene polymorphism, which may result in a decrease in glyoxalase activity, increases breast cancer risk^[23]. This gene polymorphism seems to have a role not only in the development of breast cancer, but also in the progression of neoplasia^[23].

Recently, a significant reduction in *FN3K* gene expression was detected in colorectal cancer with respect to normal pair-matched tissue^[15,16], suggesting that decreased FN3K expression is related to the malignant phenotype.

This study also suggests that there are functional modifications of FN3K in colon cancer development, since reduced FN3K activity is detectable in the progression from adenoma to cancer.

Epidemiological studies clearly indicate that the risk of several types of cancer (including pancreas, liver, breast, colorectal, and urinary tract) is increased in diabetic patients^[24,25]. Higher levels of serum glucose were present in our patients with colon cancer compared to those with adenoma and the controls. The inverse association of both enzymes with cancer was maintained after controlling for fasting glycemia. However, we doubt the necessity to control for glycemia, because it may be part of the causal chain between the enzymes and the neoplasia, as an intermediate variable. Hyperglycemia may correlate with the development of adenoma and invasive colon cancer^[26], and non-enzymatic glycation is one of the principal

mechanisms by which hyperglycemia contributes to cellular damage^[27].

The enzymatic defense against glycation suppresses damage to biological macromolecules, if this defense in normal physiological states is at a low level, cellular injury occurs. Glycation proteins with associated functional impairment of repair enzymes has a critical role in the activation of pathways of cellular transformation^[18,28].

The findings of this study provide evidence that deglycating enzymes may be involved in increasing the risk of precancerous lesions and malignant transformation of colon mucosa.

Further studies on a large cohort of patients with colorectal cancer will be designed to translate our findings into clinical practice, allowing the development of a fast and accurate blood test to diagnose colorectal cancer at different stages.

ACKNOWLEDGMENTS

The authors thank Dr. Maria Teresa Rotelli, Department of Emergency and Transplantation of Organs, University of Bari, for her help in the enrollment of patients.

COMMENTS

Background

Protein glycation is a spontaneous reaction involving reducing sugars with the amino groups of proteins. In this reaction the ϵ -amino group of lysine condenses with the carbonyl of a sugar to form a Schiff's base, which then slowly undergoes an Amadori rearrangement to become a ketoamine or, if the reacting sugar is glucose, fructosamines. The fate of fructosamines is to continue to react slowly until they become "advanced glycation end products" which are thought to play a role in the pathophysiology of several human disorders, including cancer. Protein glycation contributes to the morbidity and mortality of diseases which have a major social impact (diabetes, heart disease and endstage renal disease) and is suspected to contribute to other diseases (Alzheimer's disease, arthritis and ageing). It is now recognized that there is an enzymatic defense against glycation - a group of enzymes which suppress the physiological levels of potent glycating agents and repair glycated proteins, such as Glyoxalase I and fructosamine-3-kinase.

Research frontiers

Alterations of metabolic pathways, such as changes in the balance between glycation and enzymatic anti-glycation defense, are considered to be crucial in the activation of pathways of cellular transformation. This study identifies molecular targets which can be used not only for colorectal cancer diagnosis, but also for its prevention and treatment.

Innovations and breakthroughs

The study of circulating levels of deglycating enzymes in red blood cells from patients with colorectal cancer is certainly an innovation that might help to diagnose colorectal cancer using a fast and partially invasive method.

Applications

Further studies on a large cohort of patients with colorectal cancer will be designed to translate our findings into clinical practice, allowing the development of a fast and accurate blood test to diagnose colorectal cancer at different stages.

Peer review

This is a nicely presented concise research study on the assessment of Glyoxalase I and fructosamine-3-kinase activity in red blood cells from colorectal cancer patients. The experimentation is well performed. A clinical relevant experimental group of cancer and healthy patients are investigated in this study. Statistical methods are careful chosen and resulted in significant clinical relevant observations. The study of deglycating enzymes in red blood cells may be translate in clinical practice by the development of a fast accurate test to diagnose colorectal cancer in different stages.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Lin YP

Bones and Crohn's: No benefit of adding sodium fluoride or ibandronate to calcium and vitamin D

Jochen Klaus, Max Reinshagen, Katharina Herdt, Christoph Schröter, Guido Adler, Georg BT von Boyen, Christian von Tirpitz

Jochen Klaus, Katharina Herdt, Christoph Schröter, Guido Adler, Georg BT von Boyen, Department of Internal Medicine I, University of Ulm, Albert Einstein Allee 23, 89081 Ulm, Germany
 Max Reinshagen, Department of Internal Medicine I, Städtisches Klinikum Braunschweig, Salzdahlumer Straße 90, 38126 Braunschweig, Germany

Christian von Tirpitz, Medizinische Klinik, Kreisklinik Biberach, Ziegelhausstraße 50, 88400 Biberach, Germany

Author contributions: Klaus J and von Tirpitz C contributed equally to this work; Klaus J, Reinshagen M and von Tirpitz C designed the research, and wrote the paper; Klaus J, Reinshagen M, Adler G, von Boyen GBT and von Tirpitz C performed the research; Klaus J, Herdt K, Schröter C and von Tirpitz C analyzed the data.

Correspondence to: Jochen Klaus, MD, Department of Internal Medicine I, University of Ulm, Albert Einstein Allee 23, 89081 Ulm, Germany. jochen.klaus@uniklinik-ulm.de

Telephone: +49-731-50044727 Fax: +49-731-50044610

Received: August 21, 2010 Revised: October 15, 2010

Accepted: October 22, 2010

Published online: January 21, 2011

Abstract

AIM: To compare the effect of calcium and cholecalciferol alone and along with additional sodium fluoride or ibandronate on bone mineral density (BMD) and fractures in patients with Crohn's disease (CD).

METHODS: Patients ($n = 148$) with reduced BMD (T-score < -1) were randomized to receive cholecalciferol (1000 IU) and calcium citrate (800 mg) daily alone (group A, $n = 32$) or along with additional sodium fluoride (25 mg *bid*) (group B, $n = 62$) or additional ibandronate (1 mg *iv*/3-monthly) (group C, $n = 54$). Dual energy X-ray absorptiometry of the lumbar spine (L1-L4) and proximal right femur and X-rays of the spine were performed at baseline and after 1.0, 2.25 and 3.5 years. Fracture-assessment included visual reading of X-rays and quantitative morphometry of vertebral bodies (T4-L4).

RESULTS: One hundred and twenty three (83.1%) patients completed the first year for intention-to-treat (ITT) analysis. Ninety two (62.2%) patients completed the second year and 71 (47.8%) the third year available for per-protocol (PP) analysis. With a significant increase in T-score of the lumbar spine by $+0.28 \pm 0.35$ [95% confidence interval (CI): 0.162-0.460, $P < 0.01$], $+0.33 \pm 0.49$ (95% CI: 0.109-0.558, $P < 0.01$), $+0.43 \pm 0.47$ (95% CI: 0.147-0.708, $P < 0.01$) in group A, $+0.22 \pm 0.33$ (95% CI: 0.125-0.321, $P < 0.01$); $+0.47 \pm 0.60$ (95% CI: 0.262-0.676, $P < 0.01$), $+0.51 \pm 0.44$ (95% CI: 0.338-0.682, $P < 0.01$) in group B and $+0.22 \pm 0.38$ (95% CI: 0.111-0.329, $P < 0.01$), $+0.36 \pm 0.53$ (95% CI: 0.147-0.578, $P < 0.01$), $+0.41 \pm 0.48$ (95% CI: 0.238-0.576, $P < 0.01$) in group C, respectively, during the 1.0, 2.25 and 3.5 year periods (PP analysis), no treatment regimen was superior in any in- or between-group analyses. In the ITT analysis, similar results in all in- and between-group analyses with a significant in-group but non-significant between-group increase in T-score of the lumbar spine by 0.38 ± 0.46 (group A, $P < 0.01$), 0.37 ± 0.50 (group B, $P < 0.01$) and 0.35 ± 0.49 (group C, $P < 0.01$) was observed. Follow-up in ITT analysis was still 2.65 years. One vertebral fracture in the sodium fluoride group was detected. Study medication was safe and well tolerated.

CONCLUSION: Additional sodium fluoride or ibandronate had no benefit over calcium and cholecalciferol alone in managing reduced BMD in CD.

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Key words: Crohn's disease; Bone mineral density; Vertebral fracture; Cholecalciferol; Calcium; Ibandronate; Sodium fluoride

Peer reviewer: Pär Erik Myrelid, MD, Department of Surgery, Unit of Colorectal Surgery, Linköping University Hospital, Linköping, 58185, Sweden

Klaus J, Reinshagen M, Herdt K, Schröter C, Adler G, von Boyen GBT, von Tirpitz C. Bones and Crohn's: No benefit of adding sodium fluoride or ibandronate to calcium and vitamin D. *World J Gastroenterol* 2011; 17(3): 334-342 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/334.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.334>

INTRODUCTION

Inflammatory bowel disease (IBD) patients are at risk of reduced bone mineral density (BMD), especially in Crohn's disease (CD)^[1-5]. Genetic, endocrine, metabolic and nutritional factors contribute to CD-associated osteoporosis, and inflammation *per se* may exert an important risk since inflammatory mediators such as the pro-inflammatory cytokines tumour necrosis factor (TNF)- α , interleukin (IL)-1 β or IL-6 and other TNF-related cytokines such as receptor activator of nuclear factor κ B (RANK) and its ligand, RANKL or osteoprotegerin, are directly involved in the disease process^[6-13].

The prevalence of a reduced BMD in IBD patients is up to 38% with some 15% suffering from osteoporosis^[1-5,7]. Thus, of approximately 300 000 patients with IBD in Germany^[14], up to 45 000 may have an increased fracture risk. The high prevalence of up to 21.7% in osteoporosis-related vertebral fractures is of clinical relevance^[1,2,15-18].

Different strategies to improve BMD and to prevent osteoporosis-related fractures have been examined. Hormone replacement therapy (HRT) and bisphosphonates are established in postmenopausal osteoporosis and bisphosphonates in steroid-induced osteoporosis^[19-21]. In particular, the efficacy of bisphosphonates has received special interest in large clinical trials^[22-24]. Bisphosphonates have reduced the fracture risk considerably in patients with postmenopausal osteoporosis^[25,26]. Sodium fluoride can also increase BMD but its efficacy in reducing fractures remains controversial^[27-29].

To this day, few studies have evaluated the management of reduced BMD in IBD patients. Calcium and vitamin D administration can inhibit the rate of bone loss^[30]. HRT is an effective treatment to prevent bone loss in postmenopausal women with CD^[31]. In a previous study, we demonstrated the efficacy of sodium fluoride in increasing BMD in CD patients^[32]. Other studies reported a significant increase in BMD with the administration of iv pamidronate (30 mg every 3 mo)^[33], alendronate (10 mg/d)^[34] or etidronate periodically (400 mg orally for 14 d)^[35]. However, the primary end-point in all studies was BMD and only small cohorts with limited follow-up were investigated; the prevalence and incidence of vertebral fractures was not evaluated.

Our aim was to assess the effectiveness of cholecalciferol and calcium alone or with additional sodium fluoride or ibandronate in a larger CD patient population and longer follow-up period. The primary endpoint was to assess the efficacy of the 3 therapeutic approaches to improve BMD (in-group change). Secondary endpoints were

to compare the 3 therapies for the best improvement in BMD (between-group change), fracture rate and safety.

MATERIALS AND METHODS

Patients

The 148 randomized outpatients had a diagnosis of CD based on histological, endoscopic, radiological or clinical criteria and a reduced BMD of the lumbar spine: T-score < -1, i.e. osteopenia according to World Health Organization (WHO) criteria as published in 1994^[36]. Disease-related data on previous and current state of health were recorded using a standardized questionnaire throughout the study including adverse effects and serious adverse effects reporting. Disease activity was estimated using the CD activity index (CDAI)^[37]. Cumulative lifetime steroid-dose was estimated and expressed in grams of prednisolone equivalent. Nutritional status was assessed by body mass index (BMI). Exclusion criteria included: age < 18 years, chronic renal insufficiency (creatinine > 1.5 mg/dL), known primary hypo- or hyperparathyroidism, untreated thyroid disease, and any known medication, e.g. previous treatment with either sodium fluoride or bisphosphonates, or a condition affecting BMD other than glucocorticoid therapy. None of the patients was pregnant and female patients planning pregnancy were excluded.

Ethics

The study was approved by the Ethics Committee of the University of Ulm/Germany, and conducted in accordance with the 1975 Helsinki Declaration, as revised in 1983. All participants gave written informed consent before inclusion.

Protocol, assignment and masking

Patients were randomized to treatment group A, B or C, taking study medication as follows: (1) 1000 IU cholecalciferol (Vigantolekten®, Merck, Darmstadt, Germany) and 800 mg calcium citrate (Calcitrat®, Merckle, Ulm, Germany) daily (group A); (2) additional 25 mg of slow-release sodium fluoride (Nafril®, Merckle, Ulm, Germany) *bid* (group B); and (3) additional ibandronate 1 mg iv 3-monthly (Bondronat®, Roche, Basle, Switzerland) (group C). A random 1:2 allocation sequence, basic cholecalciferol and calcium (A) and additional sodium fluoride or additional ibandronate (B or C), was computer-generated and the sequences were concealed until intervention was assigned. Baseline examination included dual energy X-ray absorptiometry (DXA) of the spine and femur and plain radiographic imaging of the thoracic and lumbar spine in 2 planes. Follow-up examinations were conducted at 3-mo intervals. In group B, sodium fluoride was taken daily for 12 mo, followed by a 3-mo fluoride-free period. The second and third 12-mo cycle started at month 15 and 30. Follow-up DXA and plain radiography of the spine were performed after 12, 27 and 42 mo, i.e. 1.0, 2.25 and 3.5 years. With the last patient in study in June 2005, this patient completed the 3.5-year study period in January 2009 (last patient out).

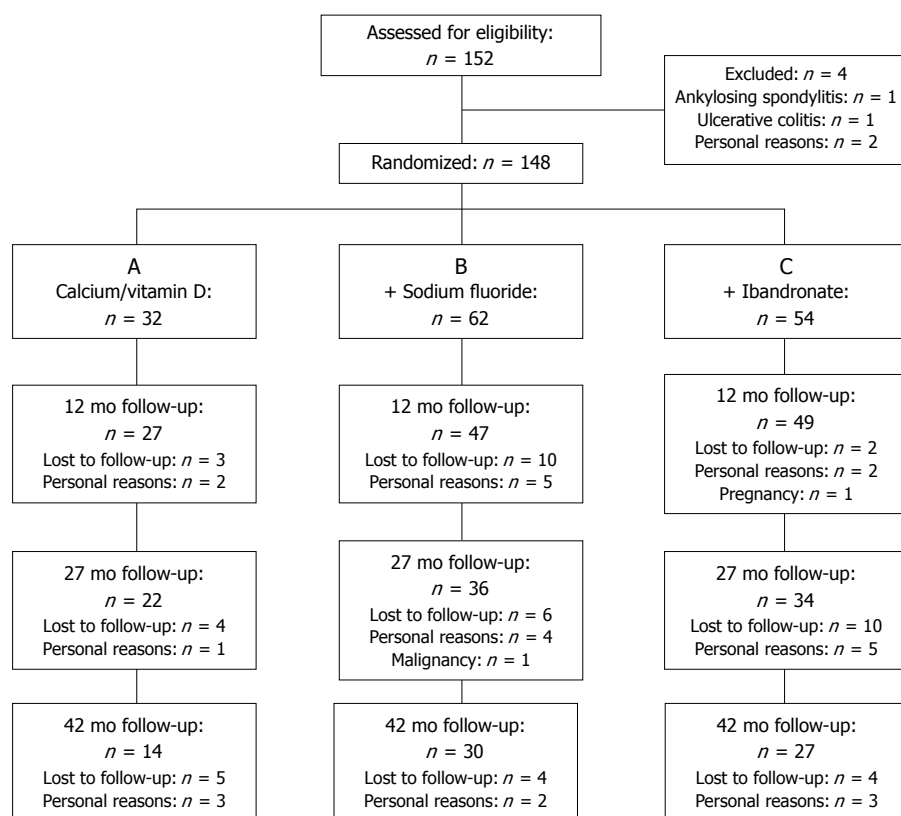


Figure 1 CONSORT diagram.

Bone densitometry

BMD of the spine (L1-L4) was assessed by DXA (Hologic QDR1000, Hologic Inc., Waltham/MA). At the proximal right femur, 4 sites (femoral neck, trochanter and intertrochanteric area, Ward's triangle) were measured; an average (total femur) was obtained from the first 3 sites. Average BMD values for L1-L4 and total femur were used for calculations. The manufacturer supplied the normal values. BMD was expressed as absolute values (g/cm^2) and as number of standard deviations from the peak bone mass of a young adult gender-matched reference population (T-score). According to the WHO recommendation for postmenopausal women as published in 1994, reduced BMD was defined as a T-score < -1.0 ^[36]. Patients with e.g. major sclerosis of the aorta, osteophytes and scoliosis on X-rays precluding accurate measurements of lumbar BMD by DXA were excluded.

Quantitative morphometry

Morphometric methods have been developed for standardized assessment of vertebral deformities in studies of spinal osteoporosis^[38]. The use of a fixed percentage reduction in vertebral height is the simplest and most practical method to study vertebral deformities^[39]. In this study, visual reading of X-rays and the quantitative morphometry (QM) of the vertebral bodies were standardized according to criteria of the European Vertebral Osteoporosis Study^[40]; only the threshold value was set from 25% to 20%. QM was performed using 6-point digitization to calculate the anterior (Ha), mid (Hm), and posterior (Hp)

height of the vertebral bodies T4-L4 (Figure 1). A vertebra was classified deformed if at least one ratio (Ha/Hp, Hm/Hp, Hp/Hp-up and Hp/Hp-low) was below the threshold value. For every vertebra considered deformed quantitatively, a radiological differential diagnosis was performed for the etiology, distinguishing osteoporotic, degenerative, traumatic and other reasons. Differential diagnosis prevents overestimation of prevalent osteoporotic fractures due to deformations of other etiology, since 45.9% and 30.9% of spinal deformities in men and women are reported to be of non-osteoporotic origin^[41].

Laboratory testing

A patient's hematocrit was determined for the calculation of the CDAI, and other inflammation-related parameters [leukocytes, platelets, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)] were obtained. Regarding bone metabolism, we focused on calcium phosphate homeostasis and investigated calcium and phosphate as well as the 25(OH)- and 1,25(OH)₂-vitamin-D₃ serum levels and parathyroid hormone. All laboratory tests were performed in the DIN EN ISO 15189:2007 accredited "Zentrale Einrichtung Klinische Chemie" of the University Hospital of Ulm, Germany. Laboratory technology and standard values can be checked at <http://www.uniklinik-ulm.de/index.php?id=1159>.

Statistical analysis

Results are presented as mean \pm SD. Qualitative variables were expressed as frequencies and percentage. The Mann-

Whitney rank sum test was used to test the effect of each therapy on BMD and biochemical markers after 12, 27 and 42 mo compared to baseline. The Student *t*-test for unpaired observations was used to compare between-group differences. Intention-to-treat (ITT) analysis was performed for all patients with at least one DXA during follow-up. Two-tailed tests for significance were used in the statistical analyses and $P \leq 0.05$ was considered significant. The Statistical Package SAS V6.11 was used for analysis.

RESULTS

Participant flow and follow-up

The CONSORT diagram shows the number of patients randomly assigned and receiving intended treatment, the patient flow through each year of the study, the number completing the study protocol, and the number analyzed for the primary outcome (Figure 1). One hundred and forty-eight patients with a T-score < -1.0 were ITT analysis, 92 (62.2%) completed the 27-mo and 71 (47.8%) the 42-mo study period and were available for per-protocol (PP) analysis. Reasons for withdrawal were failure to attend follow-up [48 patients (32.4%)] and personal reasons [27 patients (18.2%), withdrawal of written informed consent ($n = 7$), referred to primary care ($n = 10$), moving house ($n = 7$), unknown ($n = 3$)]. One patient was excluded due to a malignancy (testicular cancer), retrospectively present before randomization; he recovered completely.

Baseline characteristics

Baseline characteristics of the patients are given in Table 1. With a 1:2 random allocation to treatment groups, group A was smaller compared to group B or C. BMD was slightly but non significantly higher in group A. Patients in group A were a little younger than in group B ($P = 0.3$) and C ($P = 0.06$). No further differences in baseline characteristics were observed.

BMD of the spine, in-group change

In group A, BMD of the spine increased continually during the 1.0, 2.25 and 3.5-year study period (Table 2, Figure 2). In group B, lumbar BMD increased during the 1.0 and 2.25-year period, and in the third year, a further but non significant increase was observed (Table 2, Figure 2). In group C, BMD of the spine increased continually during the 1.0, 2.25 and 3.5-year period, again with the greatest increases in the first and second year (Table 2, Figure 2).

BMD of the spine, compared between-groups

Comparing the increase in lumbar spine BMD of the groups A, B and C at 1.0, 2.25 and 3.5 years, no group revealed superior results. There was no difference for group B receiving added sodium fluoride or for group C receiving added ibandronate in comparison with group A receiving only cholecalciferol and calcium citrate nor was there a significant difference in the comparison of groups B and C at any time in the 3.5-year study period (Table 2, Figure 2).

Table 1 Baseline characteristics (mean \pm SD) n (%)

	Group A calcium/ vitamin D	Group B _{0/1} + sodium fluoride	Group C _{0/1} + ibandronate
No. of patients	32	62	54
Male/female	14/18	29/33	27/27
Age (yr)	33.8 \pm 9.76	35.7 \pm 12.8	36.8 \pm 13.1
Duration of disease (yr)	7.4 \pm 1.7	9.4 \pm 2.1	8.1 \pm 1.9
Smoking	13 (40.6)	23 (37.1)	19 (35.2)
Postmenopausal	0	2	1
Extent of disease			
Ileal disease	11 (34.4)	20 (32.3)	21 (38.9)
Colonic disease	5 (15.6)	8 (12.9)	7 (13)
Ileocolonic disease	16 (50.0)	34 (54.8)	26 (48.1)
Bowel resection			
No bowel resection	18 (56.2)	39 (62.9)	29 (53.7)
Ileal resection	8 (25.0)	12 (19.4)	14 (25.9)
Colonic resection	2 (6.2)	5 (8.1)	4 (7.4)
Ileocolonic resection	4 (12.6)	6 (9.7)	7 (13)
Patients with bowel resection during study	4	6	5
Use of corticosteroids			
No previous use	3 (9.4)	6 (9.7)	6 (11.1)
Cumulative dose < 10 g	22 (68.8)	36 (58.1)	34 (63)
Cumulative dose > 10 g	7 (21.8)	28 (32.2)	14 (25.9)
Body weight (kg)	69.4 \pm 15.51	63.71 \pm 11.9	64.8 \pm 13.91
Body height (cm)	172 \pm 7.63	170 \pm 8.8	170 \pm 9.0
BMI (kg/m ²)	23.54 \pm 5.34	22.01 \pm 3.6	22.4 \pm 3.92
CDAI	141.5 \pm 100.96	145 \pm 95.5	135.9 \pm 85.03
T-score spine	-1.57 \pm 0.31	-1.82 \pm 0.75	-1.89 \pm 0.71
BMD spine (g/cm ³)	0.90 \pm 0.04	0.87 \pm 0.09	0.85 \pm 0.08
Pre-existing vertebral fractures ¹	6 (22.2) of patients with 10 fractures	9 (19.2) of patients with 18 fractures	14 (28.6) of patients with 28 fractures

¹Intention to treat (ITT) analysis. BMI: Body mass index; CDAI: Crohn's disease activity index; BMD: Bone mineral density.

BMD of the femur, in-group change and compared between-groups

There was no significant change in femur BMD in any of the 3 groups during the entire follow-up period, and no significant differences between groups A, B and C at 1.0, 2.25 and 3.5-year follow-up in the change in femur BMD (data not shown).

BMD of the spine (ITT)

A pre-planned ITT analysis was performed. As in PP analysis, comparing the increase in BMD in in- and between-group A, B and C analysis, cholecalciferol and calcium alone did not perform any worse than with additional sodium fluoride or ibandronate, and no group revealed superior results (Table 3, Figure 3) Mean observation time in the ITT analysis was 2.65 years.

Prevalence and incidence of vertebral fractures

For assessment of prevalent fractures and fracture incidence, the ITT population was analyzed, i.e. 123 (83.1%) patients who completed at least the first 12-mo follow-up. The duration of follow-up did not differ significantly for the treatment groups A, B and C. At baseline, a total of 56 vertebral fractures was seen in 29 (23.6%) of 123 patients,

Table 2 Bone mineral density of spine and femur, in- and between-group change

Lumbar spine		Baseline	First year	Second year	Third year
Group A calcium/vitamin D	<i>n</i> (%)	32	27 (84.4)	22 (68.6)	14 (43.8)
	T-score	-1.57 ± 0.31	-1.32 ± 0.42 ^b	-1.21 ± 0.49 ^b	-1.18 ± 0.36 ^b
	Δ T-score (95% CI)		+0.28 ± 0.35 (0.162–0.460)	+0.33 ± 0.49 (0.109–0.558)	+0.43 ± 0.47 (0.147–0.708)
	BMD	0.90 ± 0.04	0.92 ± 0.05	0.94 ± 0.05	0.94 ± 0.04
Group B + sodium fluoride	<i>n</i> (%)	62	47 (75.8)	36 (58.0)	30 (48.4)
	T-score	-1.82 ± 0.75 ^h	-1.60 ± 0.84 ^d	-1.40 ± 1.01 ^d	-1.37 ± 0.95 ^d
	Δ T-score (95% CI)		+0.22 ± 0.33 (0.125–0.321)	+0.47 ± 0.60 (0.262–0.676)	+0.51 ± 0.44 (0.338–0.682)
	BMD	0.87 ± 0.08	0.88 ± 0.09	0.90 ± 0.11	0.91 ± 0.11
Group C + ibandronate	<i>n</i> (%)	54	49 (90.1)	34 (63.0)	27 (50)
	T-score	-1.89 ± 0.71	-1.69 ± 0.78 ^e	-1.61 ± 0.83 ^e	-1.56 ± 0.78 ^f
	Δ T-score (95% CI)		+0.22 ± 0.38 (0.111–0.329)	+0.36 ± 0.53 (0.147–0.578)	+0.41 ± 0.48 (0.238–0.576)
	BMD	0.85 ± 0.08	0.87 ± 0.08	0.89 ± 0.08	0.90 ± 0.08
Total		148	123 (83.1)	92 (62.2)	71 (47.8)

In-treatment group change, baseline to first, second and third year: Group A, ^b*P* < 0.01; group B, ^d*P* < 0.01; group C, ^e*P* < 0.025, ^f*P* < 0.01; Between-treatment groups: ^h*P* < 0.01, group A *vs* group C. BMD: Bone mineral density.

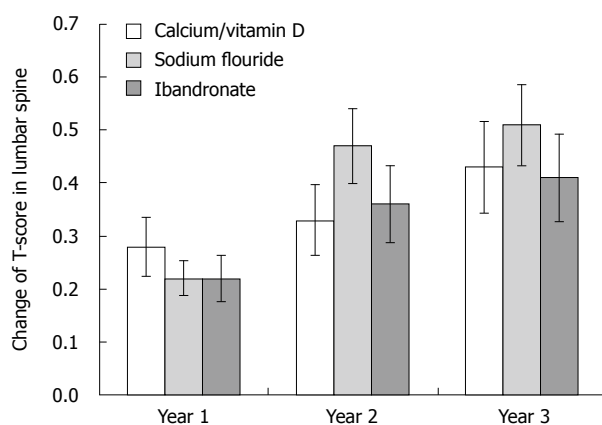


Figure 2 Change in T-score of the lumbar spine from baseline to first, second and third study year, during the 3.5-year long-term study in treatment groups A, B or C, in the per protocol population.

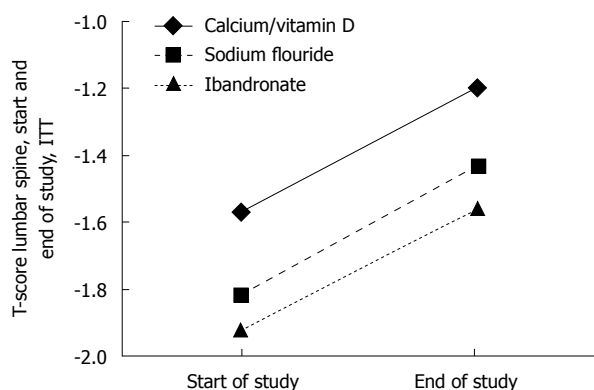


Figure 3 T-score of the lumbar spine from baseline to end of study depending in treatment groups A, B or C, in the intention to treat population. ITT: Intention-to-treat.

and one incident vertebral fracture in group B receiving sodium fluoride, but no other fractures, e.g. fractures of the hip or radius, was observed during the entire follow-up (Table 4).

Table 3 Bone mineral density of the lumbar spine, in- and between-group change (intention-to-treat)

Lumbar spine	Baseline	End of study	Δ
Group A (<i>n</i> = 27)			
T-score	-1.57 ± 0.31	-1.20 ± 0.46 ^b	+0.38 ± 0.46
BMD	0.9 ± 0.04	0.94 ± 0.06	+0.04 ± 0.05
Follow-up (yr)		2.58 ± 1.0	
Group B (<i>n</i> = 47)			
T-score	-1.82 ± 0.40	-1.43 ± 0.62 ^b	+0.37 ± 0.50
BMD	0.87 ± 0.05	0.91 ± 0.06	+0.04 ± 0.05
Follow-up (yr)		2.92 ± 0.89	
Group C (<i>n</i> = 49)			
T-score	-1.91 ± 0.40	-1.56 ± 0.56 ^b	+0.35 ± 0.49
BMD	0.86 ± 0.04	0.90 ± 0.66	+0.04 ± 0.05
Follow-up (yr)		2.44 ± 1.17	

^b*P* < 0.01. BMD: Bone mineral density.

Clinical course of the underlying CD and change in BMD

Seventy (57%) of the 123 patients who completed at least the first 12-mo study period were treated with systemic glucocorticoids at least once during the study, as reported in the standardized questionnaire completed at every follow-up examination at 3-mo intervals. Change in spine and femur BMD did not differ from the change observed in patients who had not received any systemic steroids (data not shown). While a slight increase in BMI and an improvement in CDAI was observed during the study period with no significant differences in and between the 3 treatment groups, the increase in BMI and the decrease in CDAI again did not correlate with the change in spine and femur BMD in all patients (data not shown).

Laboratory markers and change in BMD

No significant difference in inflammation parameters (leukocytes, platelets, ESR, CRP) were obtained in and between the groups A, B and C. Focusing on calcium-phosphate-homeostasis we investigated calcium and phosphate as well as the 25(OH)- and 1,25(OH)₂-vitamin-D₃ serum levels

Table 4 Prevalence and incidence of vertebral fractures

	All patients	Group A calcium/vitamin D	Group B + sodium fluoride	Group C + ibandronate
No. of patients	123	27	47	49
Patients with fractures, <i>n</i> (%)	29 (23.6)	6 (22.2)	9 (19.2)	14 (28.6)
No. of fractures	56	10	18	28
New fractures (<i>n</i>)	1	0	1	0
T-score lumbar spine	-1.80 ± 0.34	-1.57 ± 0.31	-1.82 ± 0.40	-1.91 ± 0.04
BMD lumbar spine (g/cm ²)	0.87 ± 0.05	0.90 ± 0.04	0.87 ± 0.05	-0.86 ± 0.04
Follow-up (yr)	2.65 ± 1.00	2.58 ± 1.00	2.92 ± 0.89	2.44 ± 1.17

BMD: Bone mineral density.

and parathyroid hormone. Only 25(OH)-vitamin-D₃ serum levels increased significantly in all 3 groups over time, but no change was seen in the other calcium phosphate homeostasis parameters investigated. No correlation of any serum levels of any parameter of calcium phosphate homeostasis with BMD or change in the BMD of the spine or femur could be observed (data not shown).

Adverse events

Adverse events (AEs) were reported in the standardized questionnaire used throughout the study at every 3-mo follow-up examination. AEs occurred in 35 patients (9 in group A; 14 in group B; 12 in group C). Most AEs were related to worsening of CD (28 patients), with 15 patients who had a bowel resection during study follow-up. One patient in the ibandronate group had to be withdrawn due to pregnancy (Figure 1). Study medication was generally well tolerated. Seven patients reported undigested calcium citrate and 2 undigested sodium fluoride pills in their feces. Six patients reported minor and completely reversible bone pain (< 2 h) or flu-like symptoms after intravenous infusion of ibandronate, manageable with acetaminophen if needed.

DISCUSSION

This is one of the most extended studies in the management of reduced BMD in CD. In our randomized study, we compared the effectiveness of cholecalciferol and calcium supplementation alone or along with additional sodium fluoride or additional ibandronate. More than 140 CD patients with reduced BMD (T-score < -1) were included in this study with a maximum follow-up of 3.5 years. In this young CD patient setting, increases in BMD were similar in all in- and between-treatment-group analyses, calcium and cholecalciferol supplementation not only prevented further bone loss but increased lumbar BMD and the effect was not increased further by addition of sodium fluoride or ibandronate. Regarding the prevention of fractures, the overall fracture rate in this study was too small to demonstrate between-group differences.

There were a number of limitations with the design of our study that could affect the interpretation of results. First, the study was not placebo-controlled nor blinded, and the dropout rate was high particularly after the first year. For ethical reasons we decided not to deny a basic

therapeutic regimen with cholecalciferol and calcium to any patient with reduced BMD. Unfortunately, it is a flaw of the study design that there was therefore no placebo or simple observation arm. Also, by using a blinded study comparing an oral *vs* iv administered study drug, a single tertiary outpatient clinic such as ours doing an investigator initiated trial as large as this would just be overworked. The dropout rate after the first year and only about 50% of patients completing the study reflects again the setting of our tertiary outpatient clinic where patients usually only show up if a primary or secondary health care center refer them for special reasons and problems. To manage this and to avoid misleading results we did the pre-planned ITT analysis, and found no difference in the results compared to PP analysis in in- and between-group analyses and with a mean observation time of 2.65 years, which was still longer than any follow-up in the CD patient setting before.

Oral treatment of osteoporosis with bisphosphonates relies on compliance and the absorption is low, probably especially in CD patients. When we planned this study, ibandronate was the only bisphosphonate to be administered safely as an iv bolus injection, and therefore offered an interesting alternative suitable for outpatient treatment^[42]. At that time, data of a study investigating 3-monthly iv injections of ibandronate in the treatment of postmenopausal osteoporosis were published, and treatment was reported to be safe and effective with a dose of 1 mg^[43]. This is why we had a 1 mg ibandronate 3-monthly iv intervention arm in our study. A recent meta-analysis pooled data from 4 phase III clinical trials to assess the relationship between ibandronate dose, changes in BMD, and rates of fractures. Lumbar spine BMD increased with increasing ibandronate dose and the incidence of fractures decreased as lumbar BMD increased. The pooled data pointed out the effectiveness of ibandronate to increase BMD and decrease fracture rate^[44]. In our predominantly young CD patient setting, the increase in lumbar BMD with 1 mg 3-monthly iv dosing equaled the efficacy of ibandronate for the treatment of postmenopausal osteoporosis, and the overall increase in BMD in our CD patient setting was as good as with higher doses in postmenopausal osteoporosis^[43,44].

When we planned this study, the discussion whether sodium fluoride can not only increase BMD but also prevent fractures was still open, and based on our pilot study we decided to have again a sodium fluoride intervention

arm. Here, the increase in lumbar BMD was somewhat less than in our pilot studies^[32,45]. In both, serum fluoride at 0, 6 and 12 mo was in the effective range of 0.095–0.19 mg/L^[46]. Nevertheless, the difference was most probably due to the lower sodium fluoride dose in the present study (50 mg *vs* 75 mg) which we chose based on an investigation using the same 50 mg dose and slow-release formula in postmenopausal women reporting an increase in BMD of 4%–5% per year^[27]. There remains little information available on sodium fluoride and fracture rate and therefore the efficacy of sodium fluoride in preventing fractures remains controversial^[27,28]. Nevertheless, Rubin has reported the efficacy of slow-release sodium fluoride in the prevention of vertebral fractures in postmenopausal osteoporosis^[29]. In our study, only one incident vertebral fracture was diagnosed in the sodium fluoride group. With the scientific interest focused on bisphosphonates, this question will be left open and up to now, sodium fluoride is not approved for the treatment of osteoporosis, if any, in most countries..

To this day, some other studies have evaluated the management of osteoporosis in CD, most using bisphosphonates. The primary end-point in all these studies was BMD and none reported the prevalence and incidence of fractures. Haderslev *et al*^[34] examined in a 12-mo double-blind, randomized, placebo-controlled trial the effect of 10 mg alendronate daily and reported a significant increase in lumbar BMD compared to placebo. Bartram *et al*^[33] reported an increase in BMD within 1 year with either a daily dose of 500 mg calcium and 400 IU vitamin D alone or with 3-monthly infusions of 30 mg pamidronate. The gain in BMD was a little more pronounced in the pamidronate group. Siffledeen *et al*^[35] reported a randomized trial of etidronate (400 mg orally) or not for 14 d and 500 mg calcium and 400 IU vitamin D for 76 d. This cycle was repeated 8 times. BMD significantly increased in both the etidronate- and the non-etidronate-treated groups.

Only a minority of recently diagnosed IBD patients had optimal serum 25-hydroxyvitamin-D₃ levels and serum 25-hydroxyvitamin-D₃ was positively correlated with baseline BMD of the lumbar spine, total hip, and total body, in a study by Leslie *et al*^[15]. Therefore, optimization of vitamin D may play an important role in preventing IBD-related bone disease^[13]. Vogelsang *et al*^[30] prevented BMD loss in CD patients by long-term vitamin D supplementation. Increases in BMD were especially prevalent among patients who had normal serum levels of 25-hydroxyvitamin-D₃ (68%), whereas increases occurred in only 18% of patients with low serum levels of 25-hydroxyvitamin-D₃.

Our study in CD patients with reduced BMD (T-score < -1, i.e. osteopenia according to WHO criteria as published in 1994^[36]) confirmed for the first time that the safe and well tolerated cholecalciferol and calcium supplementation alone not only prevented further bone loss but increased BMD of the lumbar spine for the better. Additional sodium fluoride or ibandronate had no benefit over cholecalciferol and calcium alone in managing reduced BMD. CD patients may take cholecalciferol and calcium first, and only add optional bisphosphonates, first and foremost in

patients with reduced BMD and prevalent fractures, taking into account all the data on bisphosphonates and fracture rate in postmenopausal osteoporosis which we still do not have for CD. Our results support the common clinical practice reported with the implementation of the American College of Gastroenterology and American Gastroenterology Association osteoporosis screening guidelines in inflammatory bowel disease^[47], with specific therapies based on DXA findings initiated in 69% of patients: oral calcium and vitamin D supplementation in 69% and bisphosphonates in 20%^[48].

COMMENTS

Background

Reduced bone mineral density (BMD) commonly affects patients with Crohn's disease (CD). Many facts link the 2 states together. With reduced BMD, the fracture risk increases.

Research frontiers

In postmenopausal women, therapy for reduced BMD is well established, but not in CD. In postmenopausal women, the standard of care is bisphosphonates. In CD, this question is still open. In this study, the authors test the effectiveness and safety of basic cholecalciferol and calcium supplementation alone or along with oral sodium fluoride or intravenous ibandronate to improve BMD compared to baseline.

Innovations and breakthroughs

In this study, sodium fluoride or ibandronate had no added benefit over basic cholecalciferol and calcium supplementation alone in increasing BMD in patients with CD and reduced BMD at baseline. One vertebral fracture in the sodium fluoride group was not sufficient to suggest a difference between groups. The study medication was safe and well tolerated.

Applications

In CD patients with reduced BMD, cholecalciferol and calcium supplementation is common clinical practice. Our data support this approach to improve bone BMD in CD patients.

Peer review

This is an interesting paper for readers.

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S- Editor Sun H L- Editor Cant MR E- Editor Lin YP

T-regulatory lymphocytes in peripheral blood of gastric and colorectal cancer patients

Antoni M Szczepanik, Maciej Siedlar, Marek Sierzega, Dominika Goroszeniuk, Karolina Bukowska-Strakova, Antoni Czupryna, Jan Kulig

Antoni M Szczepanik, Marek Sierzega, Antoni Czupryna, Jan Kulig, 1st Department of General and Gastrointestinal Surgery, Jagiellonian University Medical College, 31-501 Krakow, Poland

Maciej Siedlar, Karolina Bukowska-Strakova, Department of Clinical Immunology Polish-American Institute of Pediatrics, Jagiellonian University Medical College, 30-663 Krakow, Poland
Dominika Goroszeniuk, SHO Orthopaedics, Derriford Hospital, Plymouth NHS Trust, Derriford Road, Plymouth, PL6 8DH, United Kingdom

Author contributions: Szczepanik AM, Siedlar M and Sierzega M made substantial contributions to the conception, design, drafting and critical revision of the article; Goroszeniuk D, Czupryna A and Kulig J analyzed the data and contributed to drafting the paper; Bukowska-Strakova K contributed to lymphocyte subpopulation analysis, data acquisition and interpretation; all authors approved the published version of the paper.

Supported by Ministry of Science and Higher Education of Poland Grants 2P05C 001 29 and K/PBW/000421

Correspondence to: Antoni M Szczepanik, MD, 1st Department of General and Gastrointestinal Surgery, Jagiellonian University Medical College, 40 Kopernika street, 31-501 Krakow, Poland. msszczep@cyf-kr.edu.pl

Telephone: +48-12-4248007 Fax: +48-12-4248007

Received: July 12, 2010 Revised: August 27, 2010

Accepted: September 3, 2010

Published online: January 21, 2011

Abstract

AIM: To assess the absolute number of T-regulatory cells (Tregs; CD4⁺CD25⁺Foxp3⁺) in the peripheral blood of gastric and colorectal cancer patients.

METHODS: We enrolled 70 cancer patients (33 gastric cancer, 37 colorectal cancer) and 17 healthy volunteers. The CD3⁺CD4⁺ lymphocytes and CD4⁺CD25⁺Foxp3⁺ Tregs in the peripheral blood were analyzed with flow cytometry. The absolute numbers of Tregs were calculated based on the CD4⁺CD25⁺Foxp3⁺ cells percent-

age of CD3⁺CD4⁺ cells and the absolute numbers of CD3⁺CD4⁺ cells per microliter.

RESULTS: The mean number of CD4⁺CD25⁺Foxp3⁺ cells per microliter in colorectal cancer patients was 15.7 (SD: 21.8), for gastric cancer patients 12.2 (SD: 14.3), and for controls 17.5 (SD: 11.4). The absolute number of Tregs was significantly lower in gastric cancer patients than in controls ($P = 0.026$). There was no statistically significant difference for gastric *vs* colorectal cancer or colorectal cancer *vs* controls. The absolute number of Tregs was also significantly depressed in N⁺ *vs* N⁻ cancer patients [22.0 (27.7) *vs* 10.1 (9.0), $P = 0.013$], and in the subgroup of gastric cancer patients [30.3 (27.6) *vs* 9.6 (8.0), $P = 0.003$]. No statistical difference was observed in the proportion of Tregs in the CD4⁺ population between the groups.

CONCLUSION: The absolute number of Tregs in peripheral blood of gastric cancer but not colorectal cancer patients was significantly decreased in comparison with that in healthy controls.

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Key words: CD4⁺CD25⁺Foxp3⁺ cells; T regulatory cells; Peripheral blood; Gastric cancer; Colorectal cancer

Peer reviewer: Ki-Baik Hahm, MD, PhD, Professor, Gachon Graduate School of Medicine, Department of Gastroenterology, Lee Gil Ya Cancer and Diabetes Institute, Lab of Translational Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon, 406-840, South Korea

Szczepanik AM, Siedlar M, Sierzega M, Goroszeniuk D, Bukowska-Strakova K, Czupryna A, Kulig J. T-regulatory lymphocytes in peripheral blood of gastric and colorectal cancer patients. *World J Gastroenterol* 2011; 17(3): 343-348 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/343.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.343>

INTRODUCTION

There is evidence that regulatory T lymphocytes Tregs might be important for immunotolerance to self- and allo-antigens^[1]. Activity of these cells is one of the mechanisms of immune evasion by tumors, which inhibits the antitumor activity of effector cells. They also suppress the antigen-presenting function of dendritic cells and the activity of natural killer cells^[2,3]. Tregs are the subset of CD4⁺ cells that express high levels of the interleukin-2 receptor α chain CD25. Therefore, Tregs are described as CD4⁺CD25⁺ cells. However, the value of CD25 as a specific marker is limited, because it is also expressed on activated CD4⁺ cells. Recent studies have shown that Foxp3, a member of the transcription factor family, represents a specific marker for Tregs^[4]. However, Foxp3 is a nuclear protein and it is impossible to use it for isolation of cells. Many previous studies on Tregs in neoplastic diseases that were performed before the discovery of Foxp3 used CD4⁺CD25⁺ subpopulation as an equivalent to Tregs. Recently, flow cytometric detection of CD4⁺CD25⁺Foxp3⁺ in human cancer studies has been described^[5]. The main outcome from studies in human cancer is the increase in the proportion of Tregs/CD4⁺ cells among tumor-infiltrating lymphocytes (TILs), metastatic lymph nodes and peripheral blood^[6]. The increase in Tregs was in some studies a prognostic factor of poor survival. However, these data are not uniform for all types and locations of tumors^[7-9].

The aim of our study was to assess the absolute number of Tregs (CD4⁺CD25⁺Foxp3⁺) in the peripheral blood of gastric and colorectal cancer patients.

MATERIALS AND METHODS

The study consisted of 70 patients (33 gastric cancer and 37 colorectal cancer) treated in a single institution between 2006 and 2009. All these patients had histologically confirmed disease. The median age was 68 years (range: 32-82 years). There were 42 male and 28 female patients. All these patients underwent laparotomy. The details of clinicopathological characteristics are summarized in Table 1.

None of the patients received chemotherapy, radiotherapy, immunotherapy or other form of therapy that influenced the immune system. Patients had no history of autoimmune disease or recent infection.

The blood of 17 healthy volunteers was tested as controls. The control group consisted of 10 men and seven women with a mean age of 42 years (range: 25-52 years).

The study was approved by the Ethical Committee of Jagiellonian University.

Blood samples were collected prior to any interventional procedure in sterile EDTA vacutainers. Peripheral blood samples (100 μ L) obtained from cancer patients were incubated in TruCount tubes (BD Biosciences, San Jose, CA, USA) with a monoclonal antibody cocktail: FITC-conjugated anti-CD3 and PE-conjugated anti-CD4 (5 μ L; BD Biosciences) for 30 min at 4°C. The samples were treated with 400 μ L FACS Lysing Solution (BD Biosciences), and after erythrocyte lysis, 10000 CD3⁺CD4⁺

Table 1 Clinicopathological characteristics

	No. of cases
Sex	
Male	42
Female	28
Age (yr)	
≤ 65	32
> 65	38
Tumor grade	
1	9
2 or 3	61
Lymph node metastases	
No	24
Yes	46
Distant metastases	
No	41
Yes	29
Stage (AJCC 2002)	
I	13
II	9
III	13
IV	35

cells along with beads were acquired on a FACSCanto flow cytometer and analyzed with FACSDiva Software (BD Biosciences). The absolute numbers of CD3⁺CD4⁺ lymphocytes in samples were calculated on a basis of bead and lymphocyte counts. Tregs (CD4⁺CD25⁺Foxp3⁺) were stained in 200 μ L EDTA peripheral blood samples using the Human Regulatory T Cell Staining Kit (eBiosciences, UK), according to manufacturer's instructions, and acquired on the flow cytometer. The absolute numbers of Tregs were calculated based on the CD4⁺CD25⁺Foxp3⁺ cells percentage of CD3⁺CD4⁺ cells and the absolute numbers of CD3⁺CD4⁺ cells per microliter.

Statistical analysis

All quantitative variables were described as mean (SD). The Mann-Whitney *U* test and the χ^2 test were used when appropriate to compare distribution of individual variables between groups. *P* < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 14 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Absolute number of CD4⁺CD25⁺Foxp3⁺ cells in peripheral blood

The mean number of CD4⁺CD25⁺Foxp3⁺ cells per microliter in colorectal cancer patients was 15.7 (21.8), for gastric cancer patients 12.2 (14.3) and for controls 17.5 (11.4) (Figure 1).

The difference between colorectal cancer patients and the control group was not significant (*P* = 0.079). There was a significant difference between the gastric cancer patients and the control group (*P* = 0.026). The difference between the gastric cancer and colorectal cancer patients was not significant.

The absolute number of CD4⁺CD25⁺Foxp3⁺ cells did not differ according to sex in either the colorectal or gastric

Table 2 Mean (SD) count of CD4⁺CD25⁺Foxp3⁺ cells

	Colorectal cancer	<i>P</i> ¹	Gastric cancer	<i>P</i> ¹	Overall	<i>P</i> ¹
Overall	15.7 (21.8)		12.2 (14.3)	0.449		
Sex		0.779		0.912		0.801
Male	16.9 (25.2)		11.3 (11.9)		14.4 (20.3)	
Female	13.5 (14.6)		13.4 (17.5)		13.5 (15.9)	
Age (yr)		0.849		0.080		0.309
≤ 65	18.7 (28.5)		10.3 (16.9)		15.0 (24.1)	
> 65	12.7 (12.2)		13.7 (12.4)		13.2 (12.2)	
Tumor grade		0.501	NA	1.000		0.865
1	16.8 (15.5)				14.9 (15.5)	
2 or 3	15.9 (24.8)				15.2 (21.9)	
Lymph node metastases		0.210		0.003		0.013
No	19.9 (28.1)		30.3 (27.6)		22.0 (27.7)	
Yes	11.0 (10.4)		9.6 (8.0)		10.1 (9.0)	
Distant metastases		0.596		0.882		0.447
No	17.6 (24.6)		15.8 (20.4)		17.0 (23.1)	
Yes	10.0 (7.8)		9.9 (8.3)		9.9 (8.0)	
Stage		0.267		0.195		0.088
I or II	19.9 (28.9)		33.4 (30.9)		22.4 (29.0)	
III or IV	10.9 (10.4)		9.4 (7.8)		9.9 (8.9)	

¹Mann-Whitney *U* test. NA: Not applicable.

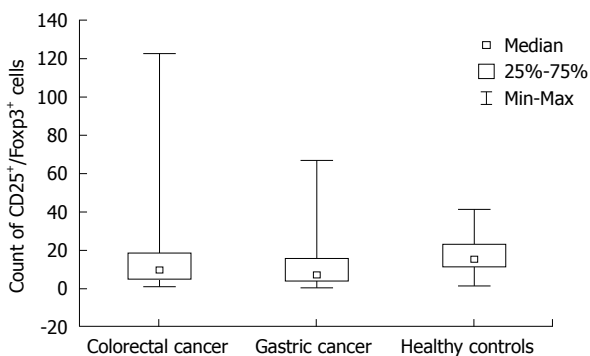


Figure 1 The absolute number of CD4⁺CD25⁺Foxp3⁺ cells in peripheral blood of colorectal and gastric cancer patients and controls.

cancer patients. Patients aged ≤ 65 years and > 65 years had similar results for both cancer types (Table 2). The analysis of TNM stage revealed that, for more advanced-stage cancer, Treg count was lower but the difference was not significant.

In colorectal cancer patients, Treg absolute count was not related to tumor grade, lymph node status or distant metastases. There was also no difference between gastric cancer subgroups according to Lauren's histological classification. In gastric cancer patients, the number of CD4⁺CD25⁺Foxp3⁺ cells was significantly lower in peripheral blood of N⁺ patients (*P* = 0.003). For the pooled group of patients (gastric and colorectal cancer), this difference was also significant (*P* = 0.013). There was no difference between M⁻ and M⁺ gastric cancer patients for absolute number of Tregs, or in the entire group of cancer patients.

Ratio of CD4⁺CD25⁺Foxp3⁺ cells/CD4⁺ cells in peripheral blood

The CD4⁺CD25⁺Foxp3⁺/CD4⁺ lymphocyte ratio did not show any differences between N⁺ and N⁻ and M⁺ and M⁻

patients for both types of cancer. This proportion was not related to sex or age (Table 3). There was no difference between stages I / II and III / IV for either type of cancer, nor in the pooled group of cancer patients.

DISCUSSION

The prevalence of Tregs in various compartments in patients with tumors has been described as a potential prognostic factor. Tregs have been found in TILs (primary and metastatic tumors), in metastatic lymph nodes, malignant ascites, pleural effusion, and peripheral blood^[6,10]. The prognostic significance of these findings is not uniform. Moreover, the impact of Tregs has been reported as their density in tumor stroma, proportion of TILs, Tregs/CD4 ratio, Tregs/CD3 ratio, or Tregs/CD8 ratio^[11-14]. Mostly, the proportion of CD25⁺Foxp3⁺ cells among CD4⁺ TILs has been analyzed.

Most of these studies were retrospective and based on the immunohistochemical examination of paraffin-embedded, previously collected specimens. On the other hand, peripheral blood is easily accessible and enables repeated measurements prior to surgery or in the follow-up period. Therefore, it is important to establish the pattern of Tregs in peripheral blood of patients with various tumors. However, the number of Tregs in peripheral blood changes less markedly than in TILs^[11].

The present study was a pilot study, therefore, assessment of the number of cells was performed preoperatively only. The results of the assessment in the early post-operative period would probably be influenced by the pro-inflammatory and anti-inflammatory post-injury reactions. This might change the lymphocyte subpopulations. Our present results can form a background for subsequent studies on Tregs in the preoperative period and follow-up of cancer patients.

Table 3 Mean (SD) proportion of CD4⁺CD25⁺Foxp3⁺ cells in the CD4⁺ population (percentage)

	Colorectal cancer	<i>P</i> ¹	Gastric cancer	<i>P</i> ¹	Overall	<i>P</i> ¹
Overall	2.18 (1.79)		2.01 (1.56)	0.760		
Sex		0.332		0.455		0.820
Male	2.31 (1.68)		1.89 (1.62)		2.12 (1.65)	
Female	1.98 (2.01)		2.18 (1.51)		2.08 (1.75)	
Age (yr)		0.456		0.610		0.777
≤ 65	2.32 (1.58)		1.88 (1.63)		2.13 (1.59)	
> 65	2.05 (2.00)		2.11 (1.54)		2.08 (1.76)	
Tumor grade		0.477		1.000		0.758
1	2.46 (1.72)		0		2.19 (1.80)	
2 or 3	2.11 (1.90)		2.10 (1.36)		2.09 (1.80)	
Lymph node metastases		0.316		0.388		0.172
No	2.60 (2.13)		2.73 (2.04)		2.63 (2.07)	
Yes	1.73 (1.25)		1.94 (1.53)		1.85 (1.41)	
Distant metastases		0.818		0.854		0.798
No	2.23 (1.98)		1.95 (1.72)		2.14 (1.88)	
Yes	2.03 (1.09)		2.05 (1.49)		2.04 (1.36)	
Stage		0.254		0.423		0.138
I or II	2.56 (2.22)		2.75 (2.35)		2.59 (2.18)	
III or IV	1.66 (1.33)		1.66 (1.32)		1.66 (1.31)	

¹Mann-Whitney *U* test. The Tregs/CD4⁺ ratio in healthy volunteers group was 2.1%, and this was equal to that observed in gastric cancer patients.

In our study, the absolute number of CD25⁺Foxp3⁺ cells in peripheral blood did not differ between gastric and colorectal cancer patients. There was no difference between sex and age groups. Some authors have described increased prevalence of Tregs among T-lymphocyte populations in elderly patients^[15]. Others have not observed this tendency^[16]. There is also no clear evidence in the literature that the Tregs population is sex-related.

The absolute number of Tregs was significantly lower in gastric cancer patients than in controls. This is probably in contrast with other studies in gastric cancer patients, however, it is impossible to compare these results directly. The main problem is the difference in description of Tregs. Some early studies have analyzed the population of CD4⁺CD25⁺ cells, others CD4⁺CD25^{high} cells, and more recently, CD4⁺CD25⁺Foxp3⁺ lymphocytes^[12,13,17-20]. Most probably this is not the last word in the identification of functionally active Tregs. Even if these populations have a common core, they are not identical. Moreover, the technical process of identification can vary and bias the final result.

In colorectal cancer patients, the absolute number of Tregs in peripheral blood did not differ from that in healthy controls. There are not sufficient data to compare this result with other studies, because the main colorectal cancer studies have concentrated on prevalence of Tregs among TILs^[12,13,21]. The pattern of Tregs localization and its correlation with tumor stage and prognosis also differs between gastric and colorectal cancer. The high prevalence of Tregs in colorectal cancer has been reported as a positive factor, contrary to gastric cancer for which it has been reported as a negative factor^[7,8,11,13,22]. The reason for these differences might also be related to the different results observed in gastric and colorectal cancer patients in our study. However, the regulatory mechanisms of Treg maturation, activation and distribution are not fully under-

stood. Therefore, we could not clarify the observed differences between the results in gastric and colorectal cancer.

We found that N⁺ gastric cancer patients had significantly lower absolute counts of Tregs in peripheral blood than had N⁻ patients.

Our study included 25 node-positive patients and this probably influenced the mean Treg count in the whole group. Several studies have revealed an increase in Tregs in metastatic lymph nodes in gastric and esophageal cancer^[23]. Our study might have demonstrated Treg migration to the metastatic lymph nodes and accumulation in the peritumoral infiltrate in more advanced tumors. There is some evidence that, in gastric cancer, Tregs might migrate to the tumor microenvironment *via* a chemokine-mediated mechanism^[24].

The increase in Tregs among TILs has also been observed in colorectal cancer patients^[25], but we did not observe a significant drop in peripheral blood Tregs in node-positive colorectal cancer patients. However, for the pooled N⁺ *vs* N⁻ group, the difference was significant. The lack of difference between M⁻ and M⁺ patients supports the hypothesis that the peripheral Tregs population does not change significantly during metastasis formation. The increase in CD4⁺CD25^{high} to CD4⁺ cell ratio in comparison to that in healthy donors has been described in metastatic cancer^[26]. However, the absolute numbers of Tregs in these populations have not been reported.

In our study, the number of patients was < 40 for each cancer type. These relatively small numbers preclude statistical analysis of Tregs counts at single TNM stages. Therefore, we performed our analysis on I / II *vs* III / IV stages. The absolute number of Tregs was lower in the more advanced group, but the difference did not reach statistical significance.

The proportion of Tregs to CD4⁺ cells was 2.18% for colorectal cancer and 2.01% for gastric cancer patients.

This value is located between that reported in the literature of 4%-6% for gastrointestinal cancer and 1%-3% for the healthy population^[27]. However, the calculated percentages of Tregs (of CD4 or of CD3) or proportions of Tregs/CD8 is influenced by at least two variables. It should be considered that subpopulations of lymphocytes can change during tumor progression^[28]. Therefore, the ratio and absolute numbers should be included.

In conclusion, our study was focused on the peripheral blood Tregs as a potential marker of disease, which was relatively easy to measure during the pretreatment and follow-up periods. The absolute number of Tregs in the peripheral blood of gastric cancer patients was significantly decreased in comparison to that in the healthy controls. This phenomenon was even strongly expressed in patients with lymph node metastasis. This was not observed in colorectal cancer patients. Our findings suggest that the population of Tregs in peripheral blood does not simply mimic stromal Tregs. Further studies on larger groups of patients are necessary to evaluate the Treg population in cancer patients.

COMMENTS

Background

The prevalence of T regulatory lymphocytes (Tregs) in various cancers has been described as a prognostic factor. The assessment of the absolute number of Tregs (CD4⁺CD25⁺Foxp3⁺) in the peripheral blood of gastric and colorectal cancer patients can be used to monitor disease during treatment.

Research frontiers

Our study was focused on peripheral blood Tregs as a potential disease marker, which was relatively easy to measure during pretreatment and follow-up periods. The prevalence of Tregs in various compartments in patients with tumors has been described as a potential prognostic factor. Tregs have been found in tumor-infiltrated tissues and fluids, but the prognostic significance of these findings is not uniform. The impact of Tregs has been reported as their density in tumor stroma or the proportion of Tregs among tumor-infiltrating lymphocytes (TILs), Tregs/CD4 ratio, Tregs/CD3 ratio, or Tregs/CD8 ratio. However, overall, the proportion of CD25⁺Foxp3⁺ cells amongst CD4⁺ TILs is analyzed.

Innovations and breakthroughs

Many studies of Tregs as a prognostic factor have been retrospective and based on immunohistochemical examination of paraffin-embedded, previously collected specimens. On the other hand, peripheral blood is easily accessible and enables repeated measurements prior to surgery or in the follow-up period. Therefore, it is important to establish the pattern of Tregs in peripheral blood of patients with various tumors.

Applications

In utilizing peripheral blood Tregs as a potential disease marker, this study demonstrates a way of improving the assessment and management of patients with gastric cancer.

Peer review

The authors assessed the absolute number of Tregs (CD4⁺CD25⁺Foxp3⁺) in the peripheral blood of gastric and colorectal cancer patients. The absolute numbers of Tregs were calculated based on the CD4⁺CD25⁺Foxp3⁺ cells percentage of CD3⁺CD4⁺ cells and the absolute numbers of CD3⁺CD4⁺ cells per microliter. The absolute number of Tregs in the peripheral blood of gastric cancer patients was significantly decreased in comparison to the healthy controls. This phenomenon was even strongly expressed in patients with lymph node metastasis, but not observed in colorectal cancer patients. The findings suggest that the population of Tregs in peripheral blood does not simply mimic stromal Tregs. Further studies on larger groups of patients are necessary to evaluate the Treg population in the blood of cancer patients.

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S- Editor Sun H L- Editor Kerr C E- Editor Zheng XM

Detection of *Helicobacter pylori*: A faster urease test can save resources

Andriani Koumi, Theodoros Filippidis, Vassilia Leontara, Loukia Makri, Marios Zenon Panos

Andriani Koumi, Marios Zenon Panos, Department of Gastroenterology, Euroclinic of Athens, Athanassiadou 9, 11521 Athens, Greece

Andriani Koumi, Department of Gastroenterology, NIMTS Hospital, 11521 Athens, Greece

Theodoros Filippidis, Vassilia Leontara, Department of Histopathology, Euroclinic of Athens, Athanassiadou 9, 11521 Athens, Greece

Loukia Makri, Department of Statistics, Ministry of Finance, 1439 Nicosia, Cyprus

Author contributions: Koumi A and Panos MZ designed and performed the study and wrote the paper; Filippidis T and Leontara V performed the histology/immunohistochemistry; Makri L performed the statistical analyses.

Correspondence to: Dr. Marios Zenon Panos, MD, FRCP, AGAF, Department of Gastroenterology, Euroclinic of Athens, Athanassiadou 9, 11521 Athens, Greece. mpanos@euroclinic.gr
 Telephone: +30-21-6416772 Fax: +30-21-6416530

Received: June 18, 2010 Revised: August 8, 2010

Accepted: August 15, 2010

Published online: January 21, 2011

Abstract

AIM: To investigate whether differences in the rapidity of a positive result for *Helicobacter pylori* can save resources, by comparing two commercially available urease kits.

METHODS: One hundred and eighty-five adults (130 outpatients, 55 inpatients) undergoing gastroscopy were entered prospectively. Patients were divided into two groups: Group 1 (if they were not on PPIs, antibiotics, H₂A, bismuth or sucralfate for up to 14 d prior to the endoscopy) and Group 2 (if they were on, or had been on, any of the above medication in the previous 14 d). At endoscopy two sets of biopsies, taken in random order, were placed in the wells of the *Campylobacter*-like organism (CLO) test (Kimberly-Clark, Utah, USA) and the Quick test (Biohit Plc, Helsinki, Finland). Five additional gastric biopsies were taken for histology/Giemsa and immunohistochemical study. The two urease test slides

were read at 2 min, 30 min, 2 h and 24 h. Sensitivity and specificity at 24 h were determined.

RESULTS: At 24 h, for all patients, there was no difference in sensitivity (100% vs 97.5%), specificity (99.3%), positive (97.5%) and negative predictive values (100% vs 99.3%) between the CLO and Quick tests, respectively. There was a positive result at 30 min in 17/41 (41.5%) CLO tests, and in 28/40 (70%) Quick tests, $P = 0.05$. Quick test enabled the prescription of eradication therapy before discharge in all 28/40 patients. Only 12 (30%) follow-up appointments were needed. If the CLO test had been used alone, only 17 (41.5%) prescriptions would have been possible prior to discharge and 24 (58%) follow-up appointments would be needed ($P = 0.001$). Of 2000 gastroscopies performed annually at our unit, a saving of 123 follow-up appointments (total: 8856 Euros or 11808 USD) would be achieved if we switched to the Quick test.

CONCLUSION: Direct comparison of locally available urease test kits is worthwhile, since the appropriate choice results in a significant saving of resources. Local costs and follow-up protocols will determine the magnitude of these savings.

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Key words: *Campylobacter*-like organism test; Diagnosis; *Helicobacter pylori*; Quick test; Urease test kits

Peer reviewer: Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Koumi A, Filippidis T, Leontara V, Makri L, Panos MZ. Detection of *Helicobacter pylori*: A faster urease test can save resources. *World J Gastroenterol* 2011; 17(3): 349-353 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/349.htm>
 DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.349>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped gram-negative bacterium which was identified in 1979^[1]. It produces urease in abundance, the activity of which, through the production of ammonia, together with the bacterium's motility and ability to adhere to the gastric mucosa, enables its survival in the acid environment of the stomach. *H. pylori* is a causative agent for chronic active gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma^[2]. It has also been shown to be associated with extragastric diseases, such as iron deficiency anemia and idiopathic thrombocytopenic purpura^[3-5].

Non-invasive methods of *H. pylori* detection include serum antibody detection, fecal antigen tests^[6] and the urea breath test^[7]. Invasive methods of *H. pylori* detection require endoscopy in order to obtain gastric tissue for histologic determination, bacterial culture or for use in urease detection kits.

Urease detection kits are inexpensive and easy to use. Biopsies from the gastric mucosa are placed in a well containing a yellow colored agar gel which contains urea and a pH indicator. Urease cleaves urea liberating ammonia, which is alkaline turning the agar color red, so indicating the presence of a urea-producing organism. The test enables the determination of the *H. pylori* status of the patient within 24 h, with a substantial proportion giving a positive result within a few hours^[8]. This represents a clear advantage over the costly and labor-intensive method of histological examination with special stain.

The aims of our study were to: (1) evaluate the sensitivity and specificity of two commercially available urease detection kits; (2) compare the time interval required for each kit to give a positive result; and (3) determine whether any differences would expedite patient management and save resources, by enabling treatment to be prescribed before patients are discharged from the endoscopy unit, thus avoiding a follow-up appointment.

MATERIALS AND METHODS

The study protocol was approved by the Hospital Ethics Committee. Patients over the age of 18 years referred for upper gastrointestinal endoscopy, in whom *H. pylori* detection was indicated, were enrolled prospectively, after written informed consent was obtained. Before gastroscopy, patients were asked whether they were, or had been in the previous 14 d, on treatment with proton pump inhibitors (PPIs), histamine type 2 receptor antagonists (H₂A), antibiotics, bismuth, or sucralfate. Patients not on PPIs, antibiotics, H₂A, bismuth or sucralfate for up to 14 d prior to the endoscopy, for the purpose of analysis were subsequently assigned to Group 1 and patients who were on, or had been on any of the above medication in the previous 14 d were assigned to Group 2. Patients on anticoagulants or with known prolonged international normalized ratio (INR), activated partial thromboplastin time (aPTT), or platelet count below 100 000/mL were excluded. Gastroscopy was performed routinely under light intravenous

sedation and local anesthetic spray to the oropharynx.

The two urease detection kits used for comparison in this study were (1) the *campylobacter*-like organism (CLO) test Rapid Urease Test (Kimberly-Clark, Utah, USA), the gel of which contains urea United States Pharmacopeia (29 mg/mL), phenol red (a pH indicator), buffers and a bacteriostatic agent to prevent the growth of contaminating urease-positive organisms and (2) the *H. pylori* Quick test (Biohit Plc, Helsinki, Finland).

Both kits were kept at room temperature for at least 10 min prior to endoscopy. At endoscopy, two biopsy specimens, one from the antrum and one from the body (mid greater curve) of the stomach^[7] were obtained for each urease test, each pair \leq 1 cm apart. Each tissue pair was embedded in the same gel-containing well of the kits under investigation. Samples for the two urease tests were taken in a random order (sealed envelope). In each instance, following the biopsies for the urease tests, three biopsies from the antrum and two from the body of the stomach were obtained (within 1 cm of the previous biopsies) for histology/Giemsa and immunohistochemical staining. For each set of biopsies a new disposable spiked forceps with fenestrated cup was used (cup diameter 2.5 mm, Wilson Cook Medical Inc., Winston-Salem, NC, USA). The exact time of the placement of the biopsies in the urease test wells was recorded and the wells inspected for color change at 2 min, 30 min, 2 h and 24 h. The test was assigned positive when there was a color change of at least 2 mm radius of red cloud around the biopsy specimen, or complete color change of the yellow well to red or magenta.

Patients were discharged after 30-45 min post-endoscopy. Where a positive result was obtained, *H. pylori* eradication therapy was prescribed prior to discharge. The number of prescriptions issued before discharge was recorded. Patients not issued a prescription prior to discharge were given follow-up appointments for the result of the urease test and prescription of eradication therapy, where indicated. The financial burden of these extra appointments was calculated from data supplied by the accounts department of our hospital, comprising estimated administrative costs and cost of medical time.

Histology

The gastric mucosa tissue was fixed by a routine fixation system and was embedded in paraffin blocks. A series of three to four thick sections of each block were used for routine stains (hematoxylin/eosin-Giemsa) and immunohistochemistry. Immunohistochemical evaluation was performed as follows: de-paraffined sections of all blocks were pretreated in citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 min. The primary antibody was then added (polyclonal rabbit anti-*H. pylori* serum at 1:250 dilution; Thermo Fischer Scientific, Runcorn, Cheshire, UK) and incubated for 30 min at room temperature. To detect antibody, a visualization system with diaminobenzene was used. Giemsa and immunostained slides were examined independently by two experienced histopathologists (Filippidis T and Leontara V)

Table 1 Sensitivity, specificity and predictive values for *Campylobacter*-like organism test and Quick test (all patients)

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	40	1	41
Negative	0	144	144
Quick test			
Positive	39	1	40
Negative	1	144	145

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 91.24-100], specificity 99.3% (95% CI: 96.2-99.88), positive predictive value (PPV) 97.6% (95% CI: 87.4-99.57), negative predictive value (NPV) 100% (95% CI: 97.4-100); Quick test: sensitivity 97.5% (95% CI: 87.12-99.56), specificity 99.3% (95% CI: 96.2-99.88), PPV 97.5% (95% CI: 87.12-99.56), NPV 99.3% (95% CI: 96.2-99.88).

who were blind to the urease test results, using light microscopy; first separately and then their results were compared. Any differences were resolved by discussion between the two histopathologists. A true positive test was determined when any two of the four tests (CLO test, Quick test, Giemsa stain, immunohistochemical stain) were positive.

Statistical analysis

The sensitivity, specificity, positive and negative predictive values of the urease tests were determined for the overall number of the patients and separately for the group of patients not on PPIs, antibiotics, H₂A, bismuth or sucralfate for up to 14 d prior to the endoscopy (Group 1) and for the group of patients who were on, or had been on any of the above medication in the previous 14 d (Group 2).

Statistical comparison of the two urease tests was by the student *t*-test for two dependent proportions, χ^2 test and the McNemar test. A statistically significant difference in the comparison of the two kits was considered when *P* value was ≤ 0.05 . Confidence intervals (CI) were determined at the 95% level.

RESULTS

Sensitivity and specificity of CLO test and Quick test at 24 h - all patients

Between April and October 2007, 185 adult patients (101 male, 84 female); age range 18-82, mean 49 years, were entered into the study. One hundred and thirty were outpatients (70%) and 55 (30%) inpatients. The overall results were as follows.

CLO test was positive at 24 h in 41 cases (22%) and negative in 144 cases (78%). Quick test was positive at 24 h in 40 cases (22%) and negative in 145 cases (78%). Histology/Giemsa/immunohistochemistry was positive for *H. pylori* in 44 cases (23.8%).

For all 185 patients, the sensitivity, specificity, positive (PPV) and negative predictive value (NPV) for the CLO test and Quick test were similar (Table 1). The concordance of the CLO test and Quick test for a positive result was 95% and for a negative result was 98%.

Table 2 Sensitivity, specificity and predictive values of the *Campylobacter*-like organism and Quick tests in Group 1

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	26	0	26
Negative	0	79	79
Quick test			
Positive	25	0	25
Negative	1	79	80

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 87.13-100], specificity 100% (95% CI: 95.36-100), positive predictive value (PPV) 100% (95% CI: 87.13-100), negative predictive value (NPV) 100% (95% CI: 95.36-100); Quick test: sensitivity 96.1% (95% CI: 81.11-99.32), specificity 100% (95% CI: 95.36-100), PPV 100% (95% CI: 86.68-100), NPV 98.7% (95% CI: 93.25-99.78).

Table 3 Sensitivity, specificity and predictive values of the *Campylobacter*-like organism and Quick tests in Group 2

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	14	1	15
Negative	0	65	65
Quick test			
Positive	14	1	15
Negative	0	65	65

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 78.47-100], specificity 98.5% (95% CI: 91.9-99.73), positive predictive value (PPV) 93.3% (95% CI: 70.18-98.81), negative predictive value (NPV) 100% (95% CI: 94.42-100); Quick test: sensitivity 100% (95% CI: 78.47-100), specificity 98.5% (95% CI: 91.9-99.73), PPV 93.3% (95% CI: 70.18-98.81), NPV 100% (95% CI: 94.42-100).

Comparison of CLO and Quick test for patients on or off antisecretory drugs or antibiotics

Of the total 185 patients, Group 1 comprised 105 patients of whom 31 (29%) were inpatients. Group 2 comprised 80 patients of whom 24 (30%) were inpatients. None had been on bismuth or sucralfate. At 24 h, sensitivity, specificity, PPV and NPV was the same for the two kits, both for Group 1 and Group 2 (Tables 2 and 3).

Table 4 displays separately the results of the two urease test kits for Group 1 and 2. At 30 min, taking the CLO test and Quick test together, a total of 33 out of 51 tests were positive in Group 1, as compared to only 12 out of 30 in Group 2 (*P* = 0.03). At 2 h, there was no statistically significant difference between Group 1 and Group 2 (*P* = 0.11). In Group 1, 13 out of 26 CLO tests and 20 out of 25 Quick tests were positive at 30 min (*P* = 0.02), with no difference at 2 h. There was no statistically significant difference in the rapidity of the two urease tests at 30 min and 2 h in Group 2 (*P* = 0.13, *P* = 0.14, respectively).

Comparison of rapidity of a positive result for the CLO and Quick tests

The number of positive CLO and Quick tests for all patients at 2 min, 30 min, 2 h and 24 h is shown in Table 5. Of a total of 40 positive Quick tests at 24 h, only 12 re-

Table 4 Number of patients with a positive *Campylobacter*-like organism and Quick test at 2 min, 30 min, 2 h, 24 h for Groups 1 and 2

Time	Group 1		Group 2	
	CLO test	Quick test	CLO test	Quick test
2 min	2	5	0	3
30 min	13 ^a	20 ^a	4	8
2 h	23	25	11	14
24 h	26	25	15	15

^a*P* = 0.02. CLO test: *Campylobacter*-like organism test.**Table 5** Number of patients with a positive *Campylobacter*-like organism and Quick test at 2 min, 30 min, 2 h, 24 h (all patients)

Time	CLO test	Quick test	<i>P</i> -value
2 min	2	8	0.03
30 min	17	28	0.05
2 h	34	39	0.28
24 h	41	40	0.45

CLO test: *Campylobacter*-like organism test.

mained negative at 30 min (30%), whereas 24 of a total of 41 positive CLO tests at 24 h (58%) remained negative at 30 min (*P* = 0.001). This enabled the prescription of *H. pylori* eradication therapy before departure from the endoscopy unit for 28/40 patients with a positive Quick test at 30 min and only 12 (30%) follow-up appointments were given.

Estimation of differences in financial costs and resources

Based on the above results if the CLO test had been used alone, only 17 (41.5%) prescriptions would have been possible (*P* = 0.05) prior to discharge and 24 (58%) follow-up appointments would be needed (*P* = 0.001). The additional financial cost of each of the additional 12 follow-up appointments at our hospital, for consultation and the prescription of eradication therapy, would be 17 Euros in administrative costs and 55 Euros in medical time (total: 72 Euros or 96 USD).

At our unit, just over 2000 gastroscopies are performed annually. Given our observed overall prevalence of *H. pylori* colonization of 41/185 (22%), we can expect 440 *H. pylori*-positive cases each year. Extrapolating from the data we present here on a difference of 28% in negative results at 30 min (58% CLO negative at 30 min *vs* 30% Quick negative), if the Quick test was used in preference to the CLO test, a saving of 123 follow-up appointments (total: 8856 Euros or 11 808 USD) would be achieved at our unit each year.

DISCUSSION

The diagnosis of *H. pylori* infection relies on various testing methods, with the gold standard being histology/

staining^[9]. Urease testing can provide rapid testing in the endoscopy suite, or in the hours following, but does not provide a gold standard assessment of infection.

We selected the CLO test and the Quick test for comparison because they were available locally. We considered that comparison of a greater number of urease test kits would not be justified due to the excessive number of gastric biopsies that this would entail.

Our results indicate, by using two biopsies placed in the same well, that there is no difference in the overall performance of the CLO test and Quick test at 24 h, with sensitivity at 100% and 97.5%, and specificity at 99%, respectively. There was, however, a significant difference in the rapidity of a positive test, in favor of the Quick test, which resulted in a significantly greater number of prescriptions issued prior to discharge at 30-45 min than would have been the case if the CLO test had been used alone.

Previous studies using similar methods also reported the sensitivity of the urease detection kits to be over 90%^[10-12]. Goh *et al*^[11] compared the HUITAI rapid urease test to histology and culture for *H. pylori* detection. Two biopsy specimens were used (antrum and body of stomach), as in our study. The sensitivity and specificity of the kits were 98.2% and 99%, respectively. In another study by Wong *et al*^[12], the PyloriTek kit was evaluated using as gold standard histology and an in-house rapid urease test. In this study, only one biopsy from the antrum was used yielding 96.3% sensitivity and 97.9% specificity, and the benefit of the addition of a corpus biopsy was found to be marginal^[12].

The results from the comparison of the reaction time of Groups 1 and 2 (Table 4) indicate that in patients with recent intake of antisecretory drugs or antibiotics the positivity of both urease tests is delayed at 30 min, although the final result at 24 h is not influenced. These findings are in agreement with those of van Keeken *et al*^[10]. On the other hand, a decrease in sensitivity, in addition to delayed positivity, was reported by Prince *et al*^[13], whilst Midolo *et al*^[14] reported that false positive tests when acid suppression therapy is in use occur only after 24 h of incubation. The mechanism by which these medications interfere with the results is thought to be either by directly inhibiting *H. pylori* urease, or by changing the *H. pylori* colonization pattern^[13].

There have been a number of previous comparisons of the speed of urease test kits: van Keeken *et al*^[10] compared the accuracy and reaction time of a new dry rapid urease test, the GUT test, with the CLO test, culture and histology. The urease test was found reliable to read 60-120 min after endoscopy. Said *et al*^[15] compared the accuracy and reaction time of a urease test, the Pronto Dry, with the CLO test and histology. A positive reaction time was achieved at 30 min, similar to the present study. In the study by Goh *et al*^[11], the rapidity of the HUITAI rapid urease test was also examined. The median positive reaction time was 1.0 min (25%-75% inter-quartile range: 1.0-3.0 min); more rapid than that observed in the present study^[11]. However, no data were given concerning the rapidity of the HUITAI test and its possible impact on resources. Caution should be exercised when

comparing studies of urease test reaction times in different populations, such as the European and Far Eastern. A crucial determinant of the rapidity of a positive urease test is the bacterial load present in the gastric biopsies. This may be higher in the Far East^[16].

In our study, there was a significant difference in the rapidity of a positive urease result at 2 and 30 min after placement of the biopsies in the test wells (Table 5), in favor of the Quick test. As a result, we were able to prescribe *H. pylori* eradication therapy before discharge from the endoscopy unit in a significantly higher number of patients (so obviating the need for follow-up visit for the prescription of eradication therapy) than would have been the case if the CLO test had been used alone. The prevalence of *H. pylori* infection of 22% observed in our study is consistent with the 19% reported in a recent seroepidemiological study of Hellenic Navy recruits^[17]. This rate is much lower than that reported in studies of the previous decade and is thought to be due to an improvement in lifestyle and socioeconomic status, in line with observations in other developed countries^[16-18].

On the basis of our results we calculated that there would be a substantial annual saving in medical and administrative time as well as financial cost, if we adopted the Quick test in preference to the CLO test. In busier endoscopy units or areas of higher *H. pylori* prevalence, the benefit would be higher. Precise financial savings for each endoscopy unit would need to be calculated according to the outpatient follow-up protocol and to local costs. These vary widely between countries and institutions. Where the practice of endoscopy units is to delegate the reading of the urease test and prescription of eradication therapy to other providers, the cost saving would be transferred to the latter.

We conclude that in selecting from locally available urease test kits, direct comparison of the rapidity of a positive result is worthwhile because the appropriate choice of kit would result in a significant saving of resources.

COMMENTS

Background

Helicobacter pylori (*H. pylori*), is a urease (enzyme) producing organism responsible for chronic active gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma. One method of rapid detection is by the use of urease detection kits which are inexpensive and easy to use. These kits consist of a well containing a yellow colored agar gel; during gastroscopy, biopsies from the gastric mucosa are placed in the well. The presence of *H. pylori* will turn the agar color red, so indicating the presence of *H. pylori*. The test enables the determination of the *H. pylori* status of the patient within 24 h, and therefore the prescription of eradication therapy.

Research frontiers

According to the literature many urease detection kits have been studied for their sensitivity and specificity as well as for their rapidity. In this article, the authors emphasize the impact of the rapidity of the test on the financial and administrative costs.

Innovations and breakthroughs

The preferential use of a rapid urease test kit results in substantial annual savings in medical and administrative time as well as in financial cost.

Applications

Direct comparison of locally available commercial urease tests is worthwhile because it may lead to saving of resources.

Peer review

The diagnosis of *H. pylori* infection relies on various testing methods, with the gold standard being histology/staining. Urease testing can provide rapid testing in the endoscopy suite, or in the hours following, but does not provide a gold standard assessment of infection.

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Dietary zinc and metallothionein on small intestinal disaccharidases activity in mice

Cuong D Tran, Johanna Cool, Cory J Xian

Cuong D Tran, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia

Cuong D Tran, Discipline of Physiology, School of Medical Sciences, The University of Adelaide, Adelaide, SA 5000, Australia

Johanna Cool, Discipline of Agricultural and Animal Science, School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA 5000, Australia

Cory J Xian, Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia

Author contributions: Tran CD, Cool J and Xian CJ performed the majority of the experiments; Tran CD and Xian CJ were involved in editing the manuscript in addition to providing financial support for this work; Tran CD designed the study and wrote the manuscript.

Supported by (in part) the MS McLeod Post-Doctoral Fellowship obtained from the Women's and Children's Hospital Foundation (to Tran CD)

Correspondence to: Dr. Cuong D Tran, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006,

Australia. cuong.tran@health.sa.gov.au

Telephone: +61-8-81616991 Fax: +61-8-81616088

Received: May 1, 2010 Revised: June 16, 2010

Accepted: June 23, 2010

Published online: January 21, 2011

Abstract

AIM: To examine the effect of increasing dietary zinc (Zn) intake and the lack of metallothionein (MT) expression on activity of small intestinal disaccharidases.

METHODS: MT- I and II knockout (MT^{-/-}) and wild-type (MT^{+/+}) female mice at 3.5 wk of age were randomly fed with a diet containing 2 (2 Zn), 15 (15 Zn) or 50 (50 Zn) mg Zn/kg ($n = 8/\text{group/genotype}$) for 5 wk. Small intestinal segments (duodenum, jejunum and ileum) were collected and either fixed in 10% formalin for histological analysis or snap frozen in liquid nitrogen for sucrase, lactase and maltase activity analyses.

RESULTS: Plasma Zn was significantly ($P < 0.05$) lower (33%) in MT^{-/-} compared with MT^{+/+} mice fed the 2 Zn diet. Villus height and crypt depth were increased by approximately 15% in MT^{+/+} mice compared with MT^{-/-} mice. Duodenal disaccharidase activities were significantly higher in MT^{+/+} compared with MT^{-/-} mice particularly in those fed the 2 Zn diet. For the 50 Zn diet, jejunal sucrase and lactase activities were significantly higher in MT^{-/-} (13313 ± 2314 ; 4107 ± 364 $\mu\text{mol glucose/well/min/g tissue}$, respectively) compared with MT^{+/+} mice (7054 ± 608 ; 1818 ± 174). Similarly, ileal lactase activities were higher in MT^{-/-} (1480 ± 192) compared with MT^{+/+} (629 ± 353) mice particularly those fed the 2 Zn diet.

CONCLUSION: Increasing dietary Zn has little effect on disaccharidases activity in MT wild-type mice. The presence of MT may enhance morphological and functional development of the gut.

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Key words: Lactase; Maltase; Sucrase; Metallothionein; Diet; Zinc

Peer reviewers: Dr. Stefan Wirth, Professor, Children's Hospital, Heusnerstt, 40, Wuppertal 42349, Germany; Dr. Devinder Kumar Dhawan, Professor, Department of Biophysics and Coordinator, Nuclear Medicine, Panjab University, Chandigarh 160014, India

Tran CD, Cool J, Xian CJ. Dietary zinc and metallothionein on small intestinal disaccharidases activity in mice. *World J Gastroenterol* 2011; 17(3): 354-360 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/354.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.354>

INTRODUCTION

Gastrointestinal function is characterized by growth, structural and functional changes of the crypts and villi, macro-

molecular absorption capacity and alteration in the activity of the small intestinal brush-border disaccharidases^[1]. It is well documented that weaning is associated with marked changes in the histology and biochemistry of the small intestine, and such changes include increased cell proliferation and differentiation^[2] and altered activity of the brush border disaccharidases, lactase and sucrase^[1]. These changes are often used as indicators of small intestinal maturity and development following the consumption of a solid diet.

The brush border disaccharidases, lactase and sucrase, are considered accurate markers of enterocyte maturity and functional capacity^[3]. It is well documented that dietary modification is correlated with significant changes in the histology and biochemistry of the small intestine^[4]. Furthermore, pigs fed an inorganic zinc (Zn) diet and weaned pigs from sows fed an inorganic Zn diet compared with those fed the control diet had improved gut morphology^[5,6]. In addition, Zn deficiency has been shown to reduce villous dimensions and increase villous density in the small intestine; however after a short period of Zn supplementation, villous density basal width and the maximum height of individual villi returned to normal^[7]. Duff and Ettarh^[8] demonstrated that Zn-fed mice produced more crypt cells in the distal part of small intestine compared with controls. Furthermore, it has been shown that weanling rats fed a semi-purified Zn-deficient diet have significantly lower levels of sucrase, maltase, lactase, leucine aminopeptidase, and alkaline phosphatase compared with rats fed a control or high Zn diet^[9,10].

Zn is an essential nutrient required for cell growth, differentiation, and survival, and its deficiency causes growth retardation, immunodeficiency, and other health problems^[11]. Therefore, Zn homeostasis must be tightly controlled in individual cells. The transcellular uptake of Zn occurs in the distal duodenum and proximal jejunum^[12-15] from the brush border membrane. The mechanisms of exogenous zinc uptake have not yet been entirely elucidated, although both saturable and nonsaturable processes are involved^[16]. Zn has been shown to be a potent inducer of its endogenous binding protein, metallothionein (MT)^[17,18], a low molecular weight, intracellular cysteine-rich, metal-binding protein that consists of 4 isoforms (MT-1 to MT-4), with MT-1 and MT-2 being the most widely expressed isoforms^[19]. MT is found mainly in the liver, kidneys, intestine and pancreas^[17]. MT synthesis is induced by a number of metals, cytokines and stress hormones as well as by a wide range of chemicals, many of which act indirectly *via* a stress or inflammatory response^[20,21].

Metal regulation of MT genes has been covered in several recent reviews^[22,23]. Briefly, the binding of Zn to the metal transcription factor-1 allows the protein to bind to metal response elements in the promoter region which, in turn, initiates MT-gene transcription. Functions of MT include protection from cell apoptosis, promotion of cell proliferation and differentiation, regulation of Zn pools in circulation and in cells, scavenging of free radicals and protection against toxicity of heavy metals^[17-19]. We hy-

pothesized that an increase in dietary Zn and a lack of MT-1 and MT-2 expression will alter small intestinal disaccharidases activity. Thus the aims of the present study were to investigate the effects of various concentrations of dietary Zn intake (2, 15, or 50 mg Zn/kg diet) and the lack of MT-1 and MT-2 expression on small intestinal morphology and activity of disaccharidases in mice. This is applicable to the young infant starting complementary feeding whether dietary Zn may aid in nutrient absorption.

MATERIALS AND METHODS

Animals

Twenty four MT wild-type (MT+/+) C57BL/6 mice were obtained from the University of Adelaide (Adelaide, South Australia) and 24 MT-1 and 2 null (MT-/-) mice were obtained from a breeding colony at the Children, Youth and Women's Health Service Animal Care Facility (North Adelaide, South Australia). MT-/- (mixed genetic background of OLA129 and C57BL6 strains) mice were F3 derivatives of the interbreeding of normal C57BL6 mice^[24].

At 3 wk old, mice were randomly allocated to be fed with either a 2, 15 or 50 mg Zn/kg diet (2 Zn, 15 Zn and 50 Zn) for 5 wk ($n = 8/\text{group/genotype}$) and body weight was recorded weekly. Mice were fed a casein-based diet^[25] supplemented with ZnSO₄ to 2, 15 or 50 mg Zn/kg. The casein-based diet contained (g/kg): cornflour starch, 514; casein, 180; sucrose, 152; wheat bran, 50; peanut oil, 50; D,L-methionine, 2.5; choline chloride, 1 and codliver oil, 4.4. The mineral profile (g/kg diet) was: KH₂PO₄, 17.155; CaCO₃, 14.645; NaCl, 12.530; MgSO₄ · 7H₂O, 4.99; FeC₆H₅O₇ · 5H₂O, 0.296; CaPO₄, 0.170; MnSO₄ · 4H₂O, 0.080; CuSO₄, 0.123; KI, 0.00025; (NH₄)₆Mo₇ · O₂₄ · 4H₂O, 0.00125; Na₂SeO₃, 0.00005. The vitamin profile (mg/kg diet) was: thiamine HCl, 70; riboflavin, 30; niacin (nicotinic acid), 50; pantothenic acid, 150; pyridoxal HCl, 15; hydroxycobalamin, 0.02; inositol, 400; p-aminobenzoic acid, 50; folic acid, 10; biotin, 0.4 and glucose, 225. For the purpose of this study we chose the 15 mg Zn/kg diet as the normal (control) diet and the 2 and 50 mg Zn/kg diet as the low and high Zn diet respectively. The Zn diets were given to the animals *ad libitum* and the Zn content of the diets was validated by atomic absorption spectrophotometry using a Perkin-Elmer 3030 (Überlingen, Germany).

Tissue collection

At the end of the experimental period, all mice were CO₂ asphyxiated and blood was withdrawn by cardiac puncture. The mice were then killed by cervical dislocation. The liver and gut were excised and the pancreas and mesentery removed. The gut was separated into stomach, small intestine, cecum and colon. The contents of the small intestine were flushed thoroughly with saline. The small intestine was then divided into the duodenum, from the gastro-duodenal junction to the ligament of Treitz, and into two segments of equal length comprising the jejunum, and ileum. A 4 cm segment of the duodenum, jejunum and ileum were excised for histological assess-

ment and disaccharidase activity analysis, respectively. The protocol adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approval was obtained from the Animal Care and Ethics Committee of the Women's and Children's Hospital (South Australia).

Plasma Zn

Blood samples were placed in lithium heparin tubes to obtain plasma, which was stored at -20°C until analysis. Plasma samples were diluted (1:2) in 10% trichloroacetic acid (Sigma-Aldrich, Sydney Australia) and were analyzed for Zn concentration ($\mu\text{mol/L}$) by atomic absorption spectrophotometry using a Perkin-Elmer 3030 (Perkin-Elmer Pty Ltd, Uberlingen, Germany).

Histological assessment

Small intestinal segments of 2 cm were removed and fixed in 10% formalin overnight, processed and embedded in paraffin wax (cross section), from which 4 μm -thick small intestinal sections were cut using the RM2235 microtome (Leica, Germany) and mounted onto glass slides. Sections were dewaxed and stained with Lillie-Mayer's hematoxylin and eosin and coverslipped. On each section, approximately 40 crypt depths and villi heights (expressed as μm) were measured using the Eclipse50i light microscope (Nikon, Japan) and Image ProPlus 5.0 package (Media Cybernetics, USA).

Disaccharidase assays

Disaccharidase activities in intestinal segments were assessed using a microplate modification of the Dahlqvist^[26] assay in duplicates. Gut segments were homogenized in 10 mmol/L phosphate-buffered saline by Ultra-TurraxT25 homogenizer (Janke and Kunkel, Germany). Homogenates were centrifuged at 3500 rpm at 4°C for 10 min. The supernatant was then aliquoted and snap-frozen at -80°C for subsequent sucrase, maltase and lactase activity assays. Glucose standards (0, 1.25, 2.5, 5, 10, 20, 30 and 40 nmol/L) were prepared. Samples were diluted 1:20, 1:50 and 1:100 with 50 mmol/L phosphate buffer and then pipetted onto a 96-well plate. A solution of 0.2 mol/L sucrose, lactose or 0.004 mol/L maltose was added (50 μL /well) and the plate incubated at 37°C for 30 min. Tris-glucose oxidase was added to all wells and incubated at 37°C for another 30 min. Absorbance was determined using a Tecan spectrophotometer (Sunrise, Austria) set at 490 nm wavelength. Disaccharidase activities were analyzed using Table-Curve (Systat Software, USA) and results were expressed as μmol glucose/well/min/g of tissue.

Statistical analysis

Histological analysis data were not normally distributed therefore data were log transformed and are presented as geometric mean \pm SE of the mean. Disaccharidase activity data are expressed as mean \pm SE of the mean. All data were analyzed using two-way analysis of variance followed by Tukey's *post-hoc* test (SigmaStats3.0). The significance level was determined as $P < 0.05$.

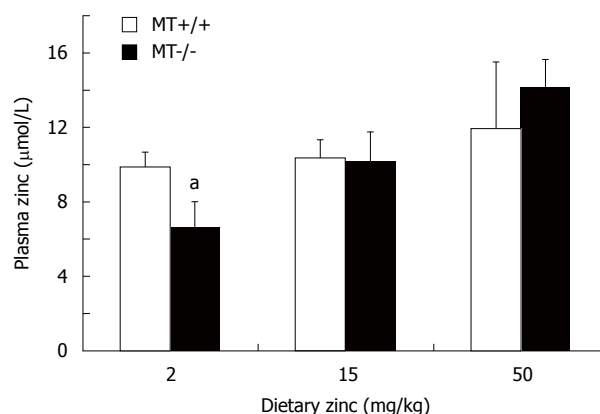


Figure 1 Comparison of plasma zinc (mean \pm SE of the mean) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk ($n = 8/\text{group/genotype}$). ^a $P < 0.05$ vs MT+/+ mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

RESULTS

Body weight and plasma Zn

There were no differences in body weights of both MT+/+ and MT-/- mice between the dietary Zn groups (data not shown). There were no differences in plasma Zn levels between MT+/+ and MT-/- mice when fed the 15 Zn or 50 Zn diet. However, there was a significant ($P < 0.05$) decrease (33%) in plasma Zn in MT-/- mice fed the 2 Zn diet compared with MT+/+ counterparts (Figure 1). There was an apparent trend of an increase in plasma Zn levels as the dietary Zn levels increased in MT-/- mice. Whereas in MT+/+ mice this was more tightly controlled with no difference in plasma Zn with increasing dietary Zn.

Small intestinal weight and length

An increase in dietary Zn did not change small intestinal weight between MT+/+ and MT-/- mice. However, there was a significant ($P < 0.05$) difference in small intestinal weight in MT-/- mice fed 2 Zn (0.74 ± 0.04 g) and 50 Zn (0.70 ± 0.03 g) compared with those fed the 15 Zn (0.59 ± 0.02 g). Interestingly, MT-/- mice had a significantly ($P < 0.05$) longer small intestine compared with MT+/+ mice, in particular in mice with the 2 Zn (29.8 ± 0.8 cm *vs* 25.7 ± 0.9 cm, respectively) and 50 Zn diets (29.1 ± 0.6 cm *vs* 26.8 ± 0.6 cm, respectively).

Villus height and crypt depth

Histological analysis (Tables 1 and 2) of the small intestine in MT+/+ and MT-/- mice showed that increasing dietary Zn concentrations did not alter villus height and crypt depth ($P > 0.05$). Differences in ileal villus height were insignificant irrespective of genotypes and dietary groups (Table 1). However, shorter villi were observed in the duodenum and jejunum of MT-/- mice fed with 15 Zn and/or 50 Zn diet(s) compared with MT+/+ mice (Table 1). Crypt depth was also significantly shorter in the duodenum (all dietary groups), jejunum (15 Zn and 50 Zn) and ileum (2 Zn) in MT-/- mice ($P < 0.05$) (Table 2).

Disaccharidase activities

MT-/- mice fed the 2 Zn diet had a significantly reduced

Table 1 Villus height (μm) in MT+/+ and MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet for 5 wk

Intestinal segments	Dietary Zn intake (mg/kg)	Genotype	
		MT+/+	MT-/-
Duodenum	2	0.47 ± 0.03	0.37 ± 0.05
	15	0.42 ± 0.03	0.42 ± 0.03
	50	0.50 ± 0.03^a	0.34 ± 0.03
Jejunum	2	0.42 ± 0.03	0.45 ± 0.03
	15	0.44 ± 0.03^a	0.34 ± 0.04
	50	0.48 ± 0.03^a	0.38 ± 0.04
Ileum	2	0.21 ± 0.01	0.22 ± 0.02
	15	0.20 ± 0.01	0.24 ± 0.02
	50	0.22 ± 0.04	0.21 ± 0.02

^a $P < 0.05$ vs MT-/- mice. Data are expressed as mean \pm SE of the mean ($n = 8$ /diet/genotype). MT: Metallothionein.

Table 2 Crypt depth (μm) in MT+/+ and MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet for 5 wk

Intestinal segments	Dietary Zn intake (mg/kg)	Genotype	
		MT+/+	MT-/-
Duodenum	2	0.099 ± 0.006^a	0.069 ± 0.004
	15	0.182 ± 0.014^a	0.069 ± 0.005
	50	0.104 ± 0.004^a	0.085 ± 0.009
Jejunum	2	0.086 ± 0.004	0.080 ± 0.003
	15	0.084 ± 0.003^a	0.066 ± 0.004
	50	0.093 ± 0.003^a	0.077 ± 0.003
Ileum	2	0.091 ± 0.004^a	0.077 ± 0.005
	15	0.087 ± 0.006	0.075 ± 0.004
	50	0.085 ± 0.004	0.075 ± 0.004

^a $P < 0.05$ vs MT-/- mice. Data are expressed as mean \pm SE of the mean ($n = 8$ /diet/genotype). MT: Metallothionein.

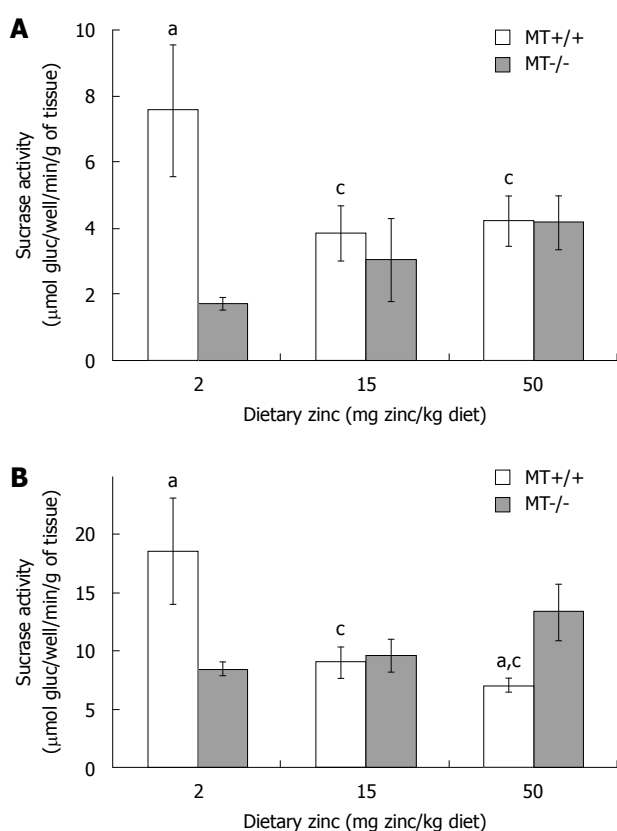


Figure 2 Comparison of sucrase activity in the duodenum (A) and jejunum (B) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk ($n = 8$ /group/genotype). ^a $P < 0.05$ vs MT-/- mice fed the 2 or 50 mg/kg Zn diet, respectively; ^c $P < 0.05$ vs MT+/+ mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

duodenal and jejunal sucrase activity compared with MT+/+ mice (Figure 2). Interestingly, sucrase levels were significantly lowered in the jejunum of MT+/+ mice receiving 15 Zn and 50 Zn (by 12%) compared with 2 Zn (Figure 2B). MT+/+ mice fed with 50 Zn also showed a lower activity in the jejunum compared with MT-/- mice. Results in both figures showed a trend of a decrease in sucrase activity in MT+/+ mice and an increase in MT-/- mice as dietary Zn concentration increased ($P < 0.05$). No significant differences were obtained for the ileum regardless of dietary Zn and genotypes ($P > 0.05$).

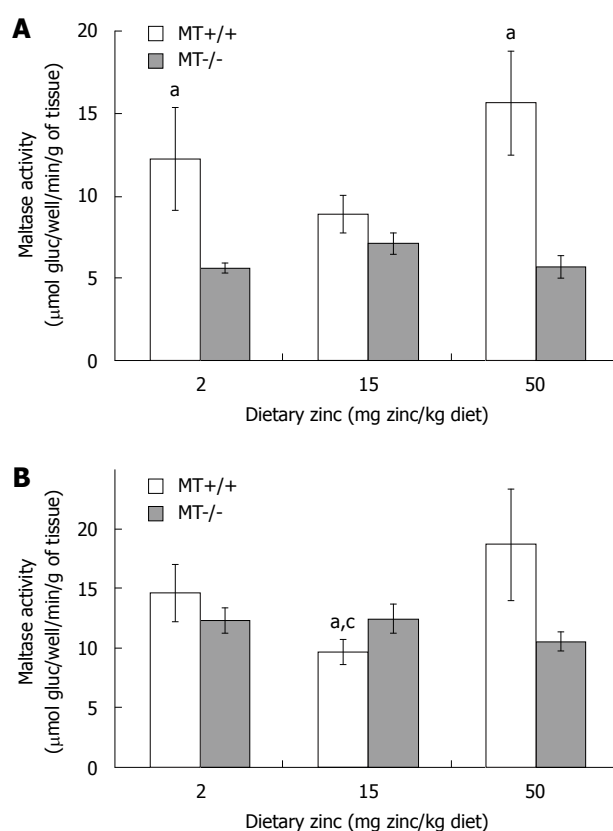


Figure 3 Comparison of maltase activity in the duodenum (A) and jejunum (B) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk ($n = 8$ /group/genotype). ^a $P < 0.05$ vs MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet, respectively; ^c $P < 0.05$ vs MT+/+ mice fed the 50 mg/kg Zn diet. MT: Metallothionein.

Duodenal maltase activity of MT+/+ mice fed with 2 Zn and 50 Zn was 37% and 47% higher, respectively, than that of MT-/- mice receiving same levels of dietary Zn (Figure 3A). When dietary Zn intake was increased from 15 Zn to 50 Zn, MT+/+ mice exhibited a marked increase in jejunal maltase activity (Figure 3B). Significant differences were also observed between MT+/+ and MT-/- mice receiving 15 Zn ($P < 0.05$). No differences were seen in ileal maltase activity regardless of dietary Zn and genotypes ($P > 0.05$).

In MT+/+ mice, there was a trend of a decrease in

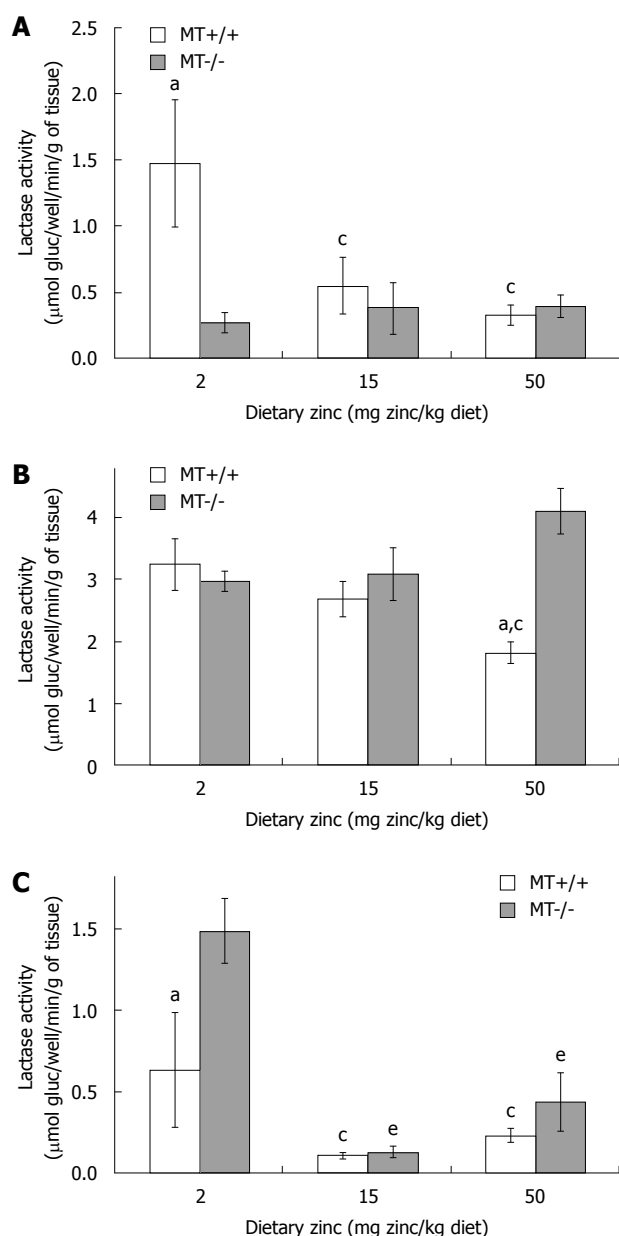


Figure 4 Comparison of lactase activity in the duodenum (A), jejunum (B) and ileum (C) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk ($n = 8/\text{group/genotype}$). ^a $P < 0.05$ vs MT-/- mice fed the 2 or 50 mg/kg Zn diet; ^c $P < 0.05$ vs MT+/+ mice fed the 2 mg/kg Zn diet; ^e $P < 0.05$ vs MT-/- mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

lactase activity when the dietary Zn levels increased in different regions of the small intestine (Figure 4A-C). For example, MT+/+ mice receiving 50 Zn showed 28% reduction ($P < 0.05$) in jejunal lactase activity compared with the 2 Zn group (Figure 4B). Interestingly, in MT-/- mice, while the increase in dietary Zn levels did not appear to alter the lactase activity in the duodenum (Figure 4A) and in the jejunum (Figure 4B), the ileal activity was significantly lower in the 15 Zn and 50 Zn groups by 84% and 55%, respectively, compared with MT-/- mice fed with 2 Zn (Figure 4C). Genotype comparisons showed that, in MT-/- mice, the lactase activity in the duodenum was significantly lower when fed the low Zn diet (2 Zn, Figure 4A), but was significantly higher ($P < 0.05$) in the jejunum

when fed the high Zn diet (50 Zn, Figure 4B) and in the ileum when fed the low Zn diet (2 Zn, Figure 4C), compared with their MT+/+ counterparts.

DISCUSSION

The development profile of disaccharidase activity in rodents seems to be well correlated with the transition from milk to solid food consumption^[1]. Although it has been reported that dietary modification is associated with altered activity of the brush border disaccharidases^[6,8,27], the influence of dietary Zn levels and the interaction of dietary Zn with its endogenous binding protein MT on small intestinal maturation has been unclear. Thus, in the present study, we examined the effect of increasing dietary Zn concentration and the role of MT-1 and 2 gene knockout on morphological changes and disaccharidase levels in weanling mice. To our knowledge, this is the first study to assess Zn supplementation on small intestinal morphology in wild-type and MT-1 and 2 null mice.

The measurements of villus height and crypt depth provide an indication of the maturity and functional capacity of small intestinal enterocytes. In the present study, although we have shown that increasing dietary Zn intake did not appear to significantly increase growth of the small intestine morphologically, as demonstrated in the histological analysis of villus height and crypt depth, the MT+/+ mice fed the high Zn diet had a higher villus height in most of the regions of the small bowel. This is consistent with a previous study^[6], showing villus height in the jejunum of sows fed Zn was greater than those fed the control diet. Similar trends were observed in other studies showing that, although not significant, pigs supplemented with Zn tend to have an increased villus height: crypt depth ratio in the jejunum and higher goblet cell counts in the ileum^[5,28]. It has been speculated that the differences in the intestinal morphology of pigs are being masked because of deterioration in the mucosa immediately after weaning, a common phenomenon in weaning pigs. However, growth performance was improved by the addition of ZnO which responded linearly to incremental doses of Zn^[28]. In our current study, we further showed that the presence of MT markedly increases villus height and crypt depth in the small intestine, in particular in mice fed a high dietary Zn, and that crypt depth is generally greater in the wild-type mice than the null mice, suggesting the importance of MT- I and II in supporting optimal intestinal growth in response to dietary Zn supply.

Considering the role of Zn as a coenzyme for more than 300 enzymes particularly in RNA-DNA synthesis and cell proliferation^[11], these improvements in intestinal morphology may be explained by the beneficial effects of Zn on cell proliferation, differentiation, and protein synthesis^[29]. This is supported by the trend of increase in plasma Zn with increasing dietary Zn in the present study and is consistent with our previous study^[30]. In contrast, Zn deficiency has been shown to cause villus atrophy, elevated levels of mucosal cell apoptosis, ulceration, inflammation as well as reduction in crypts proliferation^[8].

Furthermore, it has been reported that Zn deficiency may impair carbohydrate digestion, as reflected in decreased disaccharidase activities, and may contribute to poor nutrition^[10]. The results of these findings suggest that dietary addition of Zn in the normal diet of mice is vital for small intestinal mucosal integrity as well as improved small intestinal morphology.

In general, intestinal disaccharidase activities are lower in MT^{-/-} compared with MT^{+/+} mice particularly at the low dietary Zn concentration (except for jejunal sucrase and lactase activity in the ileum), indicating that the combination of low dietary Zn and the lack of MT expression have a negative impact on the activity of disaccharidases in the small intestine. MT is a binding protein that regulates the quantity of Zn absorbed by binding Zn within the mucosal cells, thereby regulating its transfer across the basolateral membrane into the circulation and deposition in the liver^[17,31]. We have shown that MT^{-/-} mice accumulate less Zn^[25], whereas transgenic mice accumulate more^[19]. This is consistent with our data where MT^{-/-} mice have lowered plasma Zn compared with MT^{+/+} mice, in particular with low dietary Zn intake, suggesting that the presence of MT- I and II plays a role in maintaining Zn homeostasis at low dietary Zn intake. It is possible that the low Zn status may be a contributing factor to decreased levels of disaccharidase activities in MT^{-/-} mice. This is consistent with other studies^[7,9,10,32] which reported that Zn deficiency causes marked reductions in intestinal mucosal protein content and disaccharidase activity. Furthermore, MT has been shown to have metallo-regulatory functions in cellular growth and differentiation^[33], and the lack of MT- I and MT- II expression may also be another contributing factor to the lower levels of disaccharidase activities in MT^{-/-} mice.

Interestingly, increasing dietary Zn in the diet did not have a significant increase on intestinal disaccharidase activity in the wild-type mice. This is consistent with previous studies^[7,34] where Zn supplementation has little or no effect on brush border disaccharidases. We have shown that increasing the dietary Zn concentration to 50 mg Zn/kg diet increases maltase activity only. It is possible that the highest dietary Zn concentration in the present study may be too low to induce disaccharidase activity.

MT has long been implicated in the regulation of absorption and excretion of Zn by the intestine^[31,35] and the presence of MT has been shown to restrict Zn absorption at high Zn concentrations^[12]. It has been argued that MT limits Zn absorption by sequestering it in the intestinal wall, thereby transiently reducing its absorption and favoring Zn transfer back into the gut lumen^[12,14]. Furthermore, it has been shown that at high Zn concentration, MT^{-/-} mice absorb Zn more readily and retain more Zn in the intestinal wall compared to MT-transgenic mice^[36]. The high Zn intakes may explain why MT^{-/-} mice have significantly greater jejunal sucrase and lactase activities than MT^{+/+} mice by inducing small intestinal disaccharidase activities. These adaptive mechanisms play a central role in nutrient processing and absorption which maintain normal homeostasis in these mice. This is also consistent with

MT^{-/-} mice having a heavier and longer small intestine, an adaptive mechanism to absorb more Zn to maintain Zn homeostasis when dietary Zn is high. These changes in intestinal disaccharidase activity in response to the interaction of dietary Zn and endogenous MT remain to be investigated. It is possible that intestinal disaccharidase activity and MT expression may not be related. To our knowledge this is the first study to report intestinal brush border enzyme activities in MT^{-/-} mice. Thus, the role of MT in the expression of disaccharidase activities, particularly in MT^{-/-} mice, warrants further investigation.

In conclusion, limiting the level of dietary Zn in the diet does not affect the activity of small intestinal brush border disaccharidases in wild-type mice. However, the presence of MT enhances the morphological and functional development of the gastrointestinal tract. Thus, simple Zn supplementation may be insufficient to drive growth and development of the small intestine. In light of the present findings, future studies investigating the close interaction between Zn and MT on gut maturation and development are warranted.

ACKNOWLEDGMENTS

The authors would like to thank Ms Rosa Katsikeros for proofreading this manuscript.

COMMENTS

Background

The development of brush border enzymes and gut maturation is associated with complementary feeding. However, it is unclear whether the activity of the brush border disaccharidases is influenced by dietary zinc levels and the presence of the endogenous binding protein metallothionein.

Research frontiers

The role of metallothionein on gut maturation and development, in particular, in small intestinal morphology and brush border enzymes is an important area of research in the future.

Innovations and breakthroughs

The present study demonstrated that the presence of metallothionein, a zinc binding protein, may have a positive impact on villous morphology and digestive and absorptive function of the small intestine.

Applications

Future studies investigating the role of different levels of metallothionein in the gut on small intestinal morphology and the activities of brush border enzymes which may elucidate the underlying mechanism of metallothionein in altering intestinal physiology, villous architecture, and enzyme activities.

Terminology

Disaccharidases - enzymes located on the brush border and which are essential for digestion and absorption.

Peer review

Tran *et al* report on a basic research study in metallothionein wild-type and knockout mice testing the influence of different amounts of alimentary zinc on small intestinal disaccharidases. The paper is well written and the point is clearly made.

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S- Editor Tian L L- Editor Cant MR E- Editor Lin YP

Intrahepatic biliary cystic neoplasms: Surgical results of 9 patients and literature review

Ali Emre, Kürşat Rahmi Serin, İlgin Özden, Yaman Tekant, Orhan Bilge, Aydın Alper, Mine Güllüoğlu, Koray Güven

Ali Emre, Kürşat Rahmi Serin, İlgin Özden, Yaman Tekant, Orhan Bilge, Aydın Alper, Department of General Surgery, Hepatopancreatobiliary Surgery Unit, Istanbul Faculty of Medicine, Istanbul University, Istanbul 34093, Turkey

Mine Güllüoğlu, Department of Pathology, Istanbul Faculty of Medicine, Istanbul University, Istanbul 34093, Turkey

Koray Güven, Department of Radiology, Istanbul Faculty of Medicine, Istanbul University, Istanbul 34093, Turkey

Author contributions: Emre A and Serin KR contributed equally to this work; Özden İ, Tekant Y, Bilge O and Alper A designed research, reviewed the literature and assisted in writing the paper; Güllüoğlu M analyzed the pathology specimens; Güven K reanalyzed the radiologic findings.

Correspondence to: Dr. Kürşat Rahmi Serin, Department of General Surgery, Hepatopancreatobiliary Surgery Unit, Istanbul Faculty of Medicine, Istanbul University, Millet Cad., Fatih, Istanbul 34093, Turkey. dr_krserin@yahoo.com

Telephone: +90-212-6211200 Fax: +90-212-6353082

Received: August 12, 2010 Revised: September 18, 2010

Accepted: September 26, 2010

Published online: January 21, 2011

biliary cystadenoma in 8 patients and cystadenocarcinoma in 1 patient. All symptoms resolved after surgery. There has been no recurrence during a median (range) 31 (7-72) mo of follow up.

CONCLUSION: In spite of the improvement in imaging modalities and increasing recognition of biliary cystadenoma and cystadenocarcinoma, accurate preoperative diagnosis may be difficult. Complete surgical removal (liver resection or enucleation) of these lesions yields satisfying long-term results.

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Key words: Biliary cystadenoma; Cystadenocarcinoma; Enucleation; Hepatic resection

Peer reviewer: Perry Shen, MD, Associate Professor, Department of General Surgery, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157-1095, United States

Emre A, Serin KR, Özden İ, Tekant Y, Bilge O, Alper A, Güllüoğlu M, Güven K. Intrahepatic biliary cystic neoplasms: Surgical results of 9 patients and literature review. *World J Gastroenterol* 2011; 17(3): 361-365 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/361.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.361>

Abstract

AIM: To investigate the eligible management of the cystic neoplasms of the liver.

METHODS: The charts of 9 patients who underwent surgery for intrahepatic biliary cystic liver neoplasms between 2003 and 2008 were reviewed retrospectively. Informed consent was obtained from the patients and approval was obtained from the designated review board of the institution.

RESULTS: All patients were female with a median (range) age of 49 (27-60 years). The most frequent symptom was abdominal pain in 6 of the patients. Four patients had undergone previous laparotomy (with other diagnoses) which resulted in incomplete surgery or recurrences. Liver resection ($n = 6$) or enucleation ($n = 3$) was performed. The final diagnosis was intrahepatic

INTRODUCTION

The first account of an intrahepatic biliary cystadenoma (IHBCA) was published in 1887 and the first resection was performed in 1892^[1]. The tumor was redefined by Edmondson in 1958 as a multilocular lesion with an ovarian-like stroma^[2]. However, in subsequent years, unilocular cystadenomas as well as cystadenomas without an ovarian-like stroma have been reported. Only 38 cases could be included in an extensive review in 1977^[3]. With

the widespread availability of modern imaging techniques and developments in safe liver surgery, the number of reported cases increased to approximately 150 by 1994 (approximately 100 patients in the 1994 review by Devaney *et al.*^[2], and other earlier papers^[4-6] not included in the review).

Biliary cystadenocarcinoma was first described in 1943^[7]; a review published in 1998 included 113 patients^[8]. Devaney *et al.*^[2] proposed three subsets of cystadenocarcinoma based on the pathology material submitted to their institutional laboratories for primary diagnosis or consultation: (1) cystadenocarcinoma originating from a benign cystadenoma with ovarian-like stroma (occurs exclusively in women); (2) *de novo* cystadenocarcinoma occurring almost only in men; and (3) cystadenocarcinoma that occurs in women but does not contain an ovarian-like stroma.

The long list of possibilities in the differential diagnosis includes simple cysts, parasitic cysts, degenerated metastatic tumors, mucin-producing metastatic tumors, congenital cystic dilation, cystic hemangioma, lymphangioma, hepatic foregut cyst, mesenchymal hamartoma and teratoma^[2,9-11]. Imaging techniques are the primary diagnostic tools. However, the relative scarcity of the cystadenomas and cystadenocarcinomas diagnosed by different techniques and reported over a longer period than a century renders making definite statements on pathognomonic findings difficult. Also, the high frequency of simple cysts in patients older than 40 years of age (14%-24% depending on age) greatly complicates the problem in patients with unilocular cystadenomas^[12]. It is possible that some IHBCAs are misdiagnosed as simple liver cysts.

IHBCA is a premalignant lesion; intrahepatic biliary cystadenocarcinoma (IHBCAC) cannot be reliably differentiated from IHBCA by imaging or preoperative aspiration cytology. Therefore both types of lesion should be excised^[4,10,11,13-17].

In this article, we communicate our institutional experience on IHBCA and IHBCAC and review the related surgical literature.

MATERIALS AND METHODS

The charts of patients examined for cystic liver lesions between 2003 and 2008 were studied retrospectively.

The diagnosis of IHBCA was made by radiologic criteria (ultrasonography with computed tomography or magnetic resonance imaging). Important radiologic features were^[18-23]: (1) Presence of a multilocular or unilocular mass with a well-defined capsule; and (2) Presence of one or more of the following structures exhibiting contrast enhancement: papillary projections, internal septations with nodular areas, wall thickness irregularities and mural nodules.

Because the necessity and utility of performing cyst fluid aspiration for tumor marker [carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9)] measurements and cytologic examination have been controversial issues until recently, the decisions in individual patients were left to the discretion of the attending surgeon.

Surgical intervention was performed if radiologic findings suggested an IHBCA or IHBCAC. All specimens were sent for histopathological examination. Frozen section was performed after enucleation procedures without any diagnosis of malignancy.

All patients were followed by computed tomography for possible recurrences every 6 mo in the first two post-operative years and then annually.

RESULTS

In the study period, 210 patients with cystic liver lesions were examined at our unit; 168 patients had parasitic cysts; 33 of the 42 nonparasitic cystic lesions were simple hepatic cysts. The final diagnoses in the remaining patients were IHBCA ($n = 8$) and IHBCAC ($n = 1$). These nine patients were all female with a median (range) age of 49 (27-60) years.

The most common symptom was abdominal pain observed in 6 patients. Three patients were asymptomatic; cystic liver masses had been discovered incidentally during radiological examinations for other purposes.

Four patients had undergone previous laparotomy (with other diagnoses) which resulted in incomplete surgery or recurrences. Two of these patients were operated on with the preoperative diagnosis of cystic echinococcal disease (one at our hospital). At surgery, the cystic lesions were misdiagnosed as simple liver cysts and unroofing was performed. However, histopathologic examination showed IHBCA in one and IHBCAC in the other. The remnant tumors in both patients were resected with appropriate surgical margins. Another patient was operated on with the diagnosis of echinococcal cyst at another hospital in the third month of her pregnancy. Operative findings did not confirm the preoperative diagnosis; a partial resection was performed and the histopathological diagnosis was IHBCA. In the course of the pregnancy, the size of the remnant cystic lesion increased from 12 to 27 cm in diameter. After a successful delivery, she was referred to our institution for hepatic surgery. A 58 year-old woman was operated on for cholecystolithiasis at another hospital; however, there was a suspicion of a malignant cystic lesion in segment V of the liver, the operation was stopped and she was referred to our hospital.

None of the patients had clinical or biochemical findings of cholestasis. Serum CEA levels were within normal range in all patients; serum CA 19-9 levels were within the normal range in 7 patients (including the single patient with IHBCAC) and were increased in 2 other patients (99 and 77 U/mL respectively; range 0-34 U/mL).

Preoperative percutaneous cyst fluid aspiration was performed in 4 patients. CA 19-9 levels were markedly increased in all samples (above 10000 U/mL; normal range for serum: 0-34 U/mL) and CEA levels were increased in 2 (15 and 18 ng/mL, respectively; normal range for serum: 0-4 ng/mL). Cyst fluid samples for postoperative examination were obtained intraoperatively in 4 other patients; both CA 19-9 (10000 U/mL and 379 U/mL) and CEA (27 U/mL and 651 U/mL respectively) were increased in 2 patients and within normal range in the other 2.

CEA and CA 19-9 measurement was not performed in the patient with cystadenocarcinoma.

Cytologic examination results were nondiagnostic, including the single patient with cystadenocarcinoma.

Preoperative evaluation of the period is the same as hepatobiliary operation's. The operative technique was determined according to the location of tumor in the liver and proximity to major vascular structures. Six patients were treated by hepatic resection: 4 by major hepatectomies (1 by right hepatic lobectomy, 1 by left hepatectomy, 1 by left lateral sectionectomy, and 1 by central bisegmentectomy) and 2 by nonanatomic resections. In 3 patients, the tumor was removed by enucleation. Enucleation was performed as in hemangiomas as described by Alper *et al*^[24]. Frozen section was performed routinely after enucleation procedure and no invasive malignancy was diagnosed in these 3 patients. Therefore, no additional hepatic resection was performed.

Perioperative findings, length of the operation time and blood loss were uneventful in 9 patients.

There was no major complication and mortality.

Histopathologic examination revealed IHBCA in 8 patients and IHBCAC in 1. An ovarian-like mesenchymal stroma was observed in 8 patients including the patient with IHBCAC.

All patients were followed up for median (range) 31 (7-72) mo without recurrence.

DISCUSSION

Although the incidence of IHBCA and IHBCAC has been reported to be less than 5% of all hepatic cystic lesions^[25], this figure, which is quoted in other papers^[11,26] should be interpreted with caution since the frequency of simple cysts in patients older than 40 years of age varies between 14% and 24%^[12]. The true incidences of both lesions are probably much lower since the largest surgical series reported includes 34 IHBCAs^[13] and 6 IHBCACs^[6]. The controversy in the literature stems from the lack of established criteria for preoperative diagnosis especially in the case of unilocular IHBCAs^[4,10,27].

In spite of the improvements in imaging techniques, the differential diagnosis of simple hepatic cysts and IHBCAs is still problematic. In a Cleveland Clinic series, 10 of 18 patients underwent incorrect and unnecessary procedures such as percutaneous aspiration, ethanol injection, unroofing and omentoplasty^[16]. In 1 of the patients in the present series, a patient with right upper abdominal quadrant pain was diagnosed as having cholecystolithiasis and a simple hepatic cyst in segment V of the liver. However, during surgery, the surgeon suspected the possibility of a cystic tumor and terminated the operation. Although radiologic features such as papillary projections, internal septations with nodular areas, wall thickness irregularities and mural nodules suggest the possibility of a IHBCA^[28,29], all of these except papillary projections may be observed in simple cysts as well albeit at a lower frequency^[29].

Liver echinococcal cysts pose another diagnostic problem in endemic countries^[30]. In our series, 3 cases under-

went inappropriate initial procedures with the misdiagnosis of hydatid disease. Although that absence of a germinative membrane and daughter cysts may have alerted the surgeons intraoperatively, their lack of experience precluded further interventions in the first operation. In 1 of these patients, the incidental observation of the natural history of an IHBCA under the hormonal milieu of pregnancy is interesting. The patient underwent unroofing of a 12 cm cyst at the 3rd month of pregnancy; the lesion size increased to 27 cm in a matter of 6 mo. This is in accordance with the female hormone-dependency of these lesions, previous observations in pregnant patients^[11,25,31-34] and possible association with oral contraceptive use^[34].

Although serum levels of CA19-9 and CEA may be increased in some patients^[26,27,35-37], this is not a universal finding^[10]. In the present series serum CA 19-9 levels were high in 2 patients (the single patient with IHBCAC not among them); all serum CEA levels were within the normal range.

Levels of cystic fluid CA 19-9 have been proposed "as a diagnostic help in liver cysts of unknown nature"^[38] and some centers incorporated cyst fluid tumor marker (CA19-9 and CEA) measurements into their management algorithm^[13]. However, definite diagnostic criteria for CA19-9 and CEA levels have not been established because the published data were largely limited to the reports on increased levels in small numbers of IHBCA patients without statistically robust comparison with levels in simple cysts. Consequently, the same problem occurred in the differentiation of IHBCAs and IHBCACs^[26,35,36,38-40].

In the widely cited important contribution by Koffron *et al*^[13], the cyst fluid CEA and CA 19-9 levels of 22 IHBCA patients were compared with the levels in 4 patients with simple cysts and 4 patients with polycystic liver disease. All 8 control cases had normal levels; in contrast CA19-9 was markedly increased in all IHBCA patients; there were mild to marked increases in CEA levels as well^[13]. This paper was given serious consideration by some of our attending physicians who experienced dilemmas in some patients. For example, a 75-year-old woman underwent complete aspiration of two hepatic cysts in the right lobe; the CA 19-9 levels were above 10000 U/mL whereas CEA levels were within the normal range. The presumptive diagnosis at that time was an IHBCA; surgery was not offered due to the comorbid illnesses. That she has not had a recurrence for 2.5 years suggests that the lesions might be simple cysts rather than cystadenomas and an operation would have been unnecessary.

Two important papers published in 2009 shed more light to this issue. Waanders *et al*^[41] conducted cyst fluid CA 19-9 measurements in 109 polycystic liver disease patients and 24 simple cyst patients and detected "extremely high" levels in both groups. Although the absence of pathologic confirmation is a potential weakness in interpretation (i.e. some of the patients may have had unilocular cystadenomas), the universally increased levels in all 24 patients are strong evidence for increased levels in simple cysts. Although the number of patients in the other paper^[29] is smaller (14 patients with hepatic simple cysts), a major

strength is that all patients had pathologically confirmed diagnoses. Both normal and dramatically increased CA 19-9 and CEA levels were detected in simple cyst patients; there were no significant differences between the simple cyst patients ($n = 14$) and IHBCA patients ($n = 17$). These recent data suggest that cyst fluid tumor marker levels do not provide additional information in patients with suspected IHBCA.

Cyst fluid cytology has not been found to be useful in the differentiation of IHBCA and IHBCAC^[33,42] because demonstration of malignant cells is rare, i.e. a negative cytology result will give a false sense of security. Needle biopsy of papillary projections or mural nodules may be more useful for this purpose^[13]; however this is generally unnecessary since there is a surgical indication for IHBCA and definite preoperative diagnosis of IHBCAC is not strictly required^[8]. Since there is a risk of tumor cell implantation due to the aspiration procedure^[42], routine aspiration of hepatic cystic lesions should be avoided.

There is a general consensus that an IHBCA should be removed completely either by enucleation or liver resection because lesser procedures are associated with recurrence rates as high as 90%^[4,40,43,44]. Satisfactory results with enucleation using the dissection plane between tumor and liver tissue have been reported^[4,13,45]. Enucleation, which allows maximum preservation of hepatic parenchyma, is an appropriate procedure for benign lesions. One concern is that the IHBCA may harbor a malignancy which may be missed by preoperative imaging. In such instances, enucleation would be inappropriate even in patients with noninvasive carcinoma^[2]; therefore hepatectomy with negative surgical margins is preferred. Although frozen section examination may sometimes yield a false-negative result for cancer^[42], it is still wise to perform it on samples from solid parts of enucleated tumors^[13] because resection of the adjacent parenchyma may be conducted in patients with carcinoma. Some groups advocate routine resection for these lesions^[27]. Left hepatectomy was performed in this series for the only patient with IHBCAC in whom the tumor was located at median and lateral sections. Two patients with lesions in lateral and 1 patient in posterior sections were treated by enucleation. Frozen section was performed after enucleations and no invasive malignancy was detected. Major hepatectomies had to be performed in 3 IHBCA patients with lesions very close to vascular structures. Nonanatomic resections were carried out in 2 cases.

In conclusion, with the improvement and widespread availability of radiologic modalities, cystic biliary liver neoplasms are being detected more frequently. However, the differential diagnosis from simple cysts and in endemic countries, from echinococcal cysts, is still challenging. Although there are no pathognomonic findings except for papillary projections (not present in many cases), radiological imaging finding such solid parts, papillary projections and septation or mural nodules in cystic lesion are the basis of preoperative diagnosis. Cyst fluid examination with cytology and CEA and CA 19-9 level measurement do not provide additional information. Partial resections are inappropriate. The treatment of choice is total exci-

sion either enucleation of IHBCAs and formal resection for IHBCACs and suspicious lesions.

COMMENTS

Background

Biliary cystadenomas and cystadenocarcinomas are both rare neoplasms of the biliary system. They may be easily misdiagnosed and operated on as simple cysts or hydatid cysts. Inappropriate drainage and unroofing operations result in recurrences. Reliable preoperative differentiation of the premalignant form-cystadenoma- and the malignant form cystadenocarcinoma is difficult except in obviously invasive lesions.

Research frontiers

Contrary to the previous popular opinion, recent data suggest that cyst fluid tumor marker levels do not provide additional information in patients with suspected intrahepatic biliary cystadenoma. Also, cyst fluid cytology has not been found to be useful in the differentiation of intrahepatic biliary cystadenoma and intrahepatic biliary cystadenocarcinoma, because demonstration of malignant cells is rare, i.e. a negative cytology result will give a false sense of security. Reliable techniques should be developed for reliable preoperative differential diagnosis of simple hepatic cysts, biliary cystadenomas and cystadenocarcinomas.

Innovations and breakthroughs

Surgical removal of the whole cyst with negative resection margins is recommended by many authors in order to avoid recurrences. In some cases, this is impossible because of the proximity to the vascular structures and importantly, aggressive surgery is unnecessary for a benign lesion. In three patients, the authors performed enucleation due to proximity to the vascular structures; frozen section revealed no malignancy. These patients have experienced no recurrence.

Applications

The treatment of choice is total excision; either enucleation of intrahepatic biliary cystadenomas and formal resection for intrahepatic biliary cystadenocarcinomas and suspicious lesions. Frozen section should be routine after enucleation.

Peer review

It's an interesting review of a very rare neoplasm of the biliary system.

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S- Editor Sun H L- Editor O'Neill M E- Editor Lin YP

Analysis of the delayed approach to the management of infected pancreatic necrosis

Nilesh Doctor, Sujith Philip, Vidhyachandra Gandhi, Maharra Hussain, Savio G Barreto

Nilesh Doctor, Sujith Philip, Vidhyachandra Gandhi, Department of Gastrointestinal Surgery, Jaslok Hospital and Research Center, Mumbai, 400026, India

Maharra Hussain, Department of Surgery, Bhatia Hospital, Mumbai, 400026, India

Savio G Barreto, Department of General and Digestive Surgery, Flinders Medical Center, Adelaide, 5042, South Australia
 Author contributions: Philip S and Gandhi V were involved in collecting and analyzing the data; Hussain M and Barreto SG were also involved in editing the manuscript; Doctor N designed and wrote the manuscript.

Correspondence to: Dr. Nilesh Doctor, Department of Gastrointestinal Surgery, Jaslok Hospital and Research Center, Mumbai, 400026, India. dmileshbela@gmail.com

Telephone: +91-22-23696031 Fax: +91-22-23801815

Received: July 20, 2010 Revised: September 2, 2010

Accepted: September 9, 2010

Published online: January 21, 2011

CONCLUSION: This series supports the concept of delayed single-stage open pancreatic necrosectomy for IPN. Advances in critical care, antibiotics and interventional radiology have played complementary role in improving the outcomes.

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Key words: Necrosectomy; Infected necrosis; Pancreas; Severe acute pancreatitis; Inflammation

Peer reviewer: Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Rome - Sapienza, Viale del Policlinico 155, Rome 00161, Italy

Doctor N, Philip S, Gandhi V, Hussain M, Barreto SG. Analysis of the delayed approach to the management of infected pancreatic necrosis. *World J Gastroenterol* 2011; 17(3): 366-371 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/366.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.366>

Abstract

AIM: To analyze outcomes of delayed single-stage necrosectomy after early conservative management of patients with infected pancreatic necrosis (IPN) associated with severe acute pancreatitis (SAP).

METHODS: Between January 1998 and December 2009, data from patients with SAP who developed IPN and were managed by pancreatic necrosectomy were analyzed.

RESULTS: Fifty-nine of 61 pancreatic necrosectomies were performed by open surgery and 2 laparoscopically. In 55 patients, single-stage necrosectomy could be performed (90.2%). Patients underwent surgery at a median of 29 d (range 13-46 d) after diagnosis of acute pancreatitis. Sepsis and multiple organ failure accounted for the 9.8% mortality rate. Pancreatic fistulae (50.8%) predominantly accounted for the morbidity. The median hospital stay was 23 d, and the median interval for return to regular activities was 110 d.

INTRODUCTION

Severe acute pancreatitis (SAP) is a disease with high morbidity and mortality^[1,2]. In the absence of specific effective therapy, management revolves around supportive care^[3,4].

While the overall reported mortality of acute pancreatitis (AP) varies between 5% and 12%^[2,5], SAP, which comprises around 10%-20% of AP, continues to have a high mortality rate of around 25%^[6,7] due to organ failure and sepsis arising from infected pancreatic necrosis (IPN).

Management of IPN has been widely studied over the last few decades^[6,8-20]. Data on indications, timing and technique of debridement for IPN are varied. However, while recent reports reflect the common theme of delayed surgery in IPN^[21], the ideal debridement technique continues to be debated^[6,12,15].

Reports regarding minimally invasive surgery for IPN are now being published^[22-25]. Thus, if we are to develop

evidence-based guidelines for the management of IPN, rather than comparing outcomes with the relatively higher mortality encountered in some reports published a few decades ago, a more balanced comparison should particularly include results from larger series, and include some of the more recent series in which surgery for IPN has been complemented by advances in critical care, interventional radiology and broader spectrum antibiotics.

We have been performing open necrosectomy in a uniform manner for the last 10 years with conventional abdominal drainage without post-operative peritoneal lavage.

The purpose of our study was to analyze the feasibility and outcome of performing open necrosectomy for IPN in a delayed fashion.

MATERIALS AND METHODS

During the time period between January 1998 and December 2009, patients with SAP who developed IPN and were referred to the authors' center for surgical management were analyzed for this report.

At admission, patients were scored for severity, based on the APACHE II scoring system^[1,26], and were managed with resuscitation and intensive (supportive) care strategies. SAP was defined clinically by the presence of associated organ failure and/or local complications such as necrosis, abscess, or pseudocyst^[27]. In addition, the patients were also defined as having SAP if the APACHE II score was ≥ 9 .

At admission, all the patients classified as having SAP were admitted to the intensive care unit (ICU) where resuscitation was commenced. The patients were started on antibiotics, which were usually fluoroquinolones and metronidazole during the initial few years. However, the choice of antibiotic was changed to carbapenems (meropenem 500 mg 6 hourly, for 7 to 14 d) thereafter, owing to the sensitivity of the local microbiological flora and based on the reports of the ability of carbapenems to penetrate the necrosus^[28]. Antifungals were commenced if the duration of antimicrobial therapy went beyond 7 d.

In patients with SAP, contrast-enhanced computed tomography (CECT) scan was performed for assessing the local severity by the Balthazar computed tomography (CT) severity index^[29]. A CT severity index of ≥ 7 was considered indicative of SAP. Nutrition was maintained by nasojejunal intubation and feeding. Percutaneous interventions were performed when clinically indicated, particularly in unstable patients as a temporizing measure or a bridge to surgery. The aim was to try and delay any intervention beyond the first 21 d.

Indications for surgery^[23,30,31]: (1) Sepsis syndrome - clinical deterioration that is progressive with or without organ system failure and accompanied by fever and leucocytosis; (2) IPN - confirmed by fine needle aspiration (FNA) cytology and microbiological examination; (3) CECT showing extensive pancreatic necrosis with air pockets diagnostic of IPN; and (4) "Persisting unwellness" - in the form of abdominal pain, malaise, inability to tolerate a diet, general lack of well being, and continuing weight loss.



Figure 1 Contrast-enhanced computed tomography of the abdomen showing a large hypodense collection with air pockets in the location of the pancreatic body and tail (white arrow) indicative of an infected pancreatic necrosis.

The necrosectomy was planned as a single stage. Surgery was defined as delayed if it was performed at least 21 d after the onset of pain, which was considered as Day 0 of the attack. Fresh imaging in the form of CECT was obtained just prior to the exploration. The areas of necrosis and fluid collection were carefully mapped. The patient underwent a laparotomy through a transverse upper abdominal incision. Free fluid was aspirated, and the lesser sac was exposed either through the transgastrocolic or transmesocolic route. All the pus and fluid were removed and sent for microbiological examination. The necrotic debris was also removed carefully with blunt finger dissection and sponge-holders, with an attempt not to damage any of the normal tissue. Particular care was taken not to divide bands across the cavity, especially in areas where known vessels could cross, e.g. middle colic artery. Copious lavage with warm normal saline was performed, which also helped to separate the necrotic tissue from the normal tissue. Bleeding was controlled with temporary packing, after which specific vessels were underrun with non-absorbable sutures. Other areas were explored, depending on the CT interpretation. These included the right and left paracolic gutters, head of the pancreas, gastrophatic omentum, pelvis, small bowel mesentery and the splenic hilum. Two 28 Fr tube drains were placed in the lesser sac and necrotic cavity. Loop ileostomy was performed selectively in the presence of extensive pericolic necrosis. Figure 1 is an abdominal CECT showing IPN. Figure 2 shows necrotic pancreas post-necrosectomy.

Post-operatively the patients were managed in the ICU. There was no attempt to perform post-operative lavage or flushing of the drains and the drains were removed once the output became minimal.

RESULTS

The 61 patients who required a necrosectomy for IPN included 49 male and 12 female patients. The mean age was 43 years (range 18-73 years). The predominant etiology for AP was gallstones (25 patients). Other etiologies included alcohol-induced (14 patients), idiopathic (13 pa-



Figure 2 Post-operative photograph demonstrating a complete necrotic pancreas.

tients), traumatic (3 patients), post-endoscopic retrograde cholangio-pancreatography (3 patients), and metabolic (3 patients).

The median time of patient transfer to our institute, which is a tertiary care center, was 9 d (range 4-40 d). No patient had surgical intervention prior to transfer, but 4 patients had already undergone percutaneous ($n = 2$) or endoscopic ($n = 2$) drainage for fluid collections prior to transfer.

Fifty-nine patients underwent an open necrosectomy while 2 patients had a laparoscopic necrosectomy. Patients underwent surgery at a median of 29 d (range 13-46 d) from the onset of symptoms. In only one patient, the necrosectomy had to be performed on day 13 for unresponsive multiple organ dysfunction syndrome (MODS). Delayed necrosectomy could be performed in the other 60 patients (98.3%). Re-exploration was required in five (5/59, 8%) patients for ongoing necrosis. In these patients further exploration was required on an average 2.4 occasions (range 2-3 occasions). The rate of re-exploration was 8%. Two patients required subsequent percutaneous drainage for residual intra-abdominal collections. Overall, a single-staged open necrosectomy was successful in 55 (90%) patients.

The microbiological cultures obtained from the necrotic tissue showed evidence of organism growth in 51 patients (83.6%). Mixed gram-positive and -negative organisms were encountered in 9 cases. Of the organisms isolated, 46 cultures were positive for gram-negative organisms, predominantly *E. coli*, *Klebsiella*, *Acinetobacter* and *Pseudomonas* and 11 grew gram-positive organisms. Fungi were isolated in 9 cases, all of which were in bacterial-positive cultures.

The various complications encountered have been listed in Table 1. The most common complication encountered was pancreatic fistula. Other complications were bowel fistulae, bleeding, recurrent sepsis, wound infection and secondary fungal infection.

The diagnosis of pancreatic fistula was based on amylase estimation of the drain fluid, which ranged from 9000 to 104000 U/mL. The drainage tube was maintained *in situ* and the patient was managed on an outpatient basis. Complete healing was achieved in 20 patients after an average

Table 1 Complications encountered in the 61 patients and their management

Complication	n (%)	Management
Pancreatic fistula	31 (50.8)	Tube drainage-20 Stenting-11 Fistulojejunostomy-1 Distal pancreatectomy-1
Enteric fistula	11 (18.0)	
Small bowel	2 (3.2)	Tube drainage-2
Large bowel	9 (14.7)	Defunctioning ileostomy-9 Spontaneous healing-5 Segmental colectomy-4
Bleeding	4 (6.5)	
Pseudo-aneurysm	3 (5)	Angioembolization-3
DIC	1 (1.6)	Platelets, factor VII
Secondary fungal infection	9 (14.7)	Antifungals
Wound infection	18 (29.5)	Wound drainage and dressings
Intestinal obstruction	3 (5)	Conservative-2 Laparotomy-1
Pseudocyst	2 (3.2)	Cystojejunostomy-1 Open drainage-1
Pelvic abscess	1 (1.6)	Pig tail drainage-1

DIC: Disseminated intravascular coagulation.

of 2 mo. Endoscopic stenting of the pancreatic duct was performed in 11 patients in whom the leak persisted for > 2 mo. Two patients required re-surgery in the form of a fistulojejunostomy and a distal pancreatectomy, as stent placement could not be achieved across the leak. Of the 11 patients who developed enteric fistulae in the post-operative period, 4 had undergone prophylactic ileostomy creation during the primary surgery due to the presence of extensive pericolic necrosis. Five patients required a loop ileostomy later. In 4 patients the colonic fistula healed without any sequelae, while 5 patients required segmental colectomy for colonic stricture or persistent leak for more than 6 mo. One patient died after colonic resection due to sepsis. The two patients with small bowel fistulae were managed conservatively with tube drainage. In 3 of the 4 patients who had post-operative hemorrhage, the source could be localized on angiography to pseudoaneurysms (splenic artery: 2, middle colic artery: 1) and this was managed by angioembolization. Another patient died due to coagulopathy and acidosis. Fifteen patients (24.5%) required readmission. The reasons for readmission included persistent pancreatic fistula (5 patients), colonic stricture (4 patients), intestinal obstruction (3 patients), pseudocyst formation (2 patients), and pelvic abscess formation (1 patient).

There were 6 deaths in the perioperative period with a mortality rate of 10%. These included 3 (of the five) patients who underwent re-explorations; 1 patient with post-operative hemorrhage, 1 patient with a colonic fistula and the patient who required an early necrosectomy. The cause of death was sepsis and MODS in all cases except the patient with hemorrhage. The median post-operative ICU stay was 7 d (range 3-30 d) and the median duration for which the patient required ventilatory support was 3 d (range 2-7 d). The median duration of hospital stay

following surgery was 23 d (range 11-88 d). The time to return to daily activity (defined as ability to perform daily personal activities, including feeding oneself and combing hair) was 16 d (10-20 d). The average time to return to regular activity was 110 d (60-140 d).

DISCUSSION

The ideal timing for a necrosectomy for IPN is a matter of debate. In our patients, we carried out a conservative management regimen with supportive care, antibiotics, early enteral feeding, and care of the patient in the ICU.

Using this management strategy we were able to perform a delayed necrosectomy, i.e. after 21 d, with potential benefits as follows: (1) Separation of viable from non-viable tissues making the operation technically easier; (2) Operating on a more hemodynamically stable patient; (3) Reduced bleeding as only non-viable tissue is removed^[12]; (4) Removal of less normal pancreas resulting in reduced long-term morbidity^[9]; and (5) Reduced local complications such as erosion into blood vessels/small bowel that could lead to post-operative hemorrhage or fistulae.

Mier *et al.*^[15] had previously put forward this principle of delayed surgery for IPN. The success of the approach was subsequently confirmed by other studies^[6,13,21].

With this strategy of delaying surgery, in our series necrosectomy was performed as a single stage in all but 6 patients. In patients where the initially severe clinical course improved and the patient developed signs of sepsis in the third week, CT scan was repeated to map the extent of necrosis. At this time the pancreatic and peripancreatic necrosis tended to be localized with a resolution of the changes during the acute attack, such as acute fluid collections, stranding of the mesentery, *etc.*

The mortality rate in our study following the performance of a delayed single-staged necrosectomy was 9.8%. This compares favorably with the mortality rate of 11%-38% reported for open, as well as minimally invasive, necrosectomy for IPN over the last few years^[8,10-12,17,18,23,32,33]. The indication for intervention in our patients was not solely based on an FNA as has been described previously^[12]. We feel that FNA plays a role in the early period after SAP where it helps to differentiate systemic inflammatory response syndrome (SIRS) from infection. However, since we did not operate on the patients in this period, we did not find the need to apply the use of FNA as routine. Our decision to intervene was based on clinical parameters that included features such as persistent "unwellness", persistent pain in the abdomen, leucocytosis, appearance of a new fever especially after the second week when SIRS would not be a cause of raised temperature and infection of the pancreatic necrosis would be the only likely possibility, and the CT scan appearance of pancreatic necrosis^[23,30,31].

The concept of delayed surgery has definitely been facilitated by improvements in critical care, fluid resuscitation and organ support that have contributed to the fall in the early mortality associated with SAP^[34,35]. These have contributed by targeting one of the most important de-

terminants of poor outcome in SAP, i.e. the early development and persistence of organ dysfunction^[36].

We have used carbapenems, in particular meropenem, based on the proven efficacy of the drug for prophylaxis in patients with SAP^[37]. The rationale for using antibiotics was that mortality for IPN is higher than that for sterile necrosis and antibiotic usage decreases the risk of infection^[38,39]. The use of antibiotics indiscriminately, however, can lead to a 12%-35%^[40,41] risk of opportunistic fungal infections, e.g. *Candida albicans* and *Aspergillus fumigatus*^[42], which further increase the mortality rate^[43,44]. The accepted indications for antibiotics in AP are: newly developed sepsis or SIRS, failure of two or more organ systems, proven infection, or an increase in serum C reactive protein in combination with other evidence supporting the presence of infection, e.g. CT scan^[45]. We isolated fungal cultures in only 9 patients, i.e. 15% of cases, which was quite similar to the findings of Grewe *et al.*^[46] who reported similar fungal superinfections after using a four-drug regimen for a mean of 23 d.

The incidence of enteric fistulae in our study (18%) was comparable to that reported by Howard *et al.*^[47]. The incidence of developing a colonic fistula is high in patients with pericolic spread of necrosis into the left paracolic gutter, as seen in our patients, and we strongly advocate the use of prophylactic loop ileostomy in these patients. These results, along with those for pancreatic fistulae (50.8%), however, fall within the range of studies reporting post-necrosectomy gastrointestinal and pancreatic fistula rates of 1%-43% and 3%-72%, respectively^[47]. The use of minimally invasive surgery has also been associated with enteric fistulae. In their series of 5 patients who underwent minimally invasive retroperitoneal necrosectomy, Lakshmanan *et al.*^[24] reported a 40% pancreatic fistula rate, while Connor *et al.*^[23] reported a 17% pancreatic fistula rate in their 24 patients. These results support the idea that such complications could largely be dependent on the nature of the disease rather than the procedure employed to treat it (open *vs* laparoscopy).

In our study we found that as a result of delaying the procedure beyond the first 3 wk, we were able to perform only a single, but effective, exploration in the vast majority of patients. Our re-operation rate was 8.2%, unlike that reported in other studies (22%-79%) where semi-open and open techniques of debridement, as well as early surgery, was practised^[8,17,48-50].

The ideal time for intervening, as well as the number of interventions, has been shown to play a significant role on the mortality rate^[6,21], as was seen in our study. Previous studies have stressed the significance of delayed necrosectomy. However, the best time for intervention continues to be controversial, though most studies have set the ideal time to be after 2-3 wk^[6,13,15,51-56].

The value of a single-staged procedure is that it helps to avoid the risk of bleeding and fistula formation, as seen in patients undergoing open packing or re-operations^[11,50,57]. The incidence of systemic complications is greater in patients who undergo re-operations^[49].

Finally, benefit was also seen when we compared the duration of hospital stay with other studies (23 d *vs* 30-93 d)^[8,17,23,32,40,49].

Our series provides further evidence to support the role of delayed open necrosectomy for IPN. The results are comparable, if not better, than reported smaller series using minimally invasive techniques. The results indicate that a multi-pronged conservative strategy aimed at supporting the patient, with timely intervention, may actually reduce the need for further interventions, reducing not only morbidity but also mortality in these patients.

In conclusion, this series provides further support to the concept of delayed single-stage open pancreatic necrosectomy for IPN. Advances in critical care, effective antibiotic therapy with carbapenems, the availability of interventional radiology and good supportive care have played a complementary role to surgery in improving outcomes in IPN. Prophylactic ileostomy may be considered in patients with necrosis extending into the paracolic gutters.

ACKNOWLEDGMENTS

We wish to thank Professor John Windsor for healthy discussion.

COMMENTS

Background

Infected pancreatic necrosis (IPN) continues to have a high morbidity and mortality. Delayed necrosectomy has been shown to have reduced mortality. This paper illustrates a method of delayed necrosectomy performed in a single stage.

Research frontiers

The ideal technique of necrosectomy continues to be debated (open, endoscopic, laparoscopic). Reports regarding minimally invasive surgery for IPN are now being published. Thus, if we are to develop evidence-based guidelines for the management of IPN, rather than comparing outcomes with the relatively higher mortality encountered in some reports published a few decades ago, a more balanced comparison should particularly include results from larger series, and include some of the more recent series in which surgery for IPN has been complemented by advances in critical care, interventional radiology and broader spectrum antibiotics.

Innovations and breakthroughs

Previously described methods of necrosectomy have employed open packing, closed packing or post-operative lavage, which have high morbidity, cost and prolonged hospital stay. This single-stage delayed necrosectomy attempts to treat the patients in a single-stage approach, helping to reduce the hospital stay and morbidity.

Applications

This procedure helps to treat patients who have IPN with a high success rate, in addition to acceptable morbidity and low mortality. As it is carried out in delayed fashion, the necrotic debris has separated out and is well organized, leading to less intraoperative bleeding. This helps to pave the way for minimally invasive methods to further improve results.

Peer review

This is a well conducted retrospective study in agreement with the standard surgical approach to necrotic pancreatitis.

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S- Editor Sun H L- Editor Logan S E- Editor Zheng XM

Liver function alterations after laparoscopy-assisted gastrectomy for gastric cancer and its clinical significance

Gui-Ae Jeong, Gyu-Seok Cho, Eung-Jin Shin, Moon-Soo Lee, Hyung-Chul Kim, Ok-Pyung Song

Gui-Ae Jeong, Gyu-Seok Cho, Eung-Jin Shin, Moon-Soo Lee, Hyung-Chul Kim, Ok-Pyung Song, Department of Surgery, Soonchunhyang University College of Medicine, Bucheon-si, 420-767, Gyeonggi-do, South Korea

Author contributions: Jeong GA and Cho GS performed the majority of this study; Cho GS and Shin EJ designed the study; Jeong GA wrote the manuscript; Lee MS, Kim HC and Song OP revised this manuscript; Cho GS and Kim HC edited the manuscript.

Correspondence to: Gyu-Seok Cho, MD, PhD, Associate Professor, Department of Surgery, Soonchunhyang University College of Medicine, Bucheon-si, 420-767, Gyeonggi-do, South Korea. gschogs@schbc.ac.kr

Telephone: +82-32-6215248 Fax: +82-32-6215016

Received: July 16, 2010 Revised: September 7, 2010

Accepted: September 14, 2010

Published online: January 21, 2011

Abstract

AIM: To evaluate the factors associated with liver function alterations after laparoscopy-assisted gastrectomy (LAG) for gastric cancer.

METHODS: We collected the data of gastrectomy patients with gastric cancer and divided them into 2 groups: open gastrectomy (OG) and LAG. We also collected the data of patients with colon cancer to evaluate the effect of liver manipulations during surgery on liver function alterations. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and alkaline phosphatase were measured on the pre-operative day and postoperative day 1 (POD1), POD3, POD5, and POD7.

RESULTS: No changes in liver function were observed after the operation in patients with colon cancer ($n = 121$). However, in gastric cancer patients ($n = 215$), AST and ALT levels increased until POD5 compared to those in colon cancer patients and these findings were observed both in the LAG and OG without a sig-

nificant difference except at POD1. The mean hepatic enzyme levels at POD1 in the LAG group were significantly higher than those in the OG group ($P = 0.047$ for AST and $P = 0.039$ for ALT). The factors associated with elevated ALT on POD1 in patients with gastric cancer were body mass index ($P < 0.001$), operation time ($P < 0.001$), intraoperative hepatic injury ($P = 0.048$), and ligation of an aberrant left hepatic artery ($P = 0.052$) but not type of operation (OG vs LAG, $P = 0.094$).

CONCLUSION: We conclude that the liver function alteration after LAG may have been caused by direct liver manipulation or aberrant hepatic artery ligation rather than the CO₂ pneumoperitoneum.

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Key words: Gastric cancer; Liver function; Pneumoperitoneum; Laparoscopy-assisted gastrectomy

Peer reviewer: Frank I Tovey, OBE, ChM, FRCS, Honorary Research Fellow, Department of Surgery, University College London, London, United Kingdom

Jeong GA, Cho GS, Shin EJ, Lee MS, Kim HC, Song OP. Liver function alterations after laparoscopy-assisted gastrectomy for gastric cancer and its clinical significance. *World J Gastroenterol* 2011; 17(3): 372-378 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/372.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.372>

INTRODUCTION

While laparoscopic surgery has some limitations, such as a longer operating time and a more difficult surgical procedure than open surgery, it also has several advantages, such as less postoperative pain, an earlier recovery, and esthetic merits. A major difference between laparoscopic and open surgery is that CO₂ is used to create a pneumo-

peritoneum during laparoscopic surgery, which may result in respiratory and hemodynamic changes^[1]. According to many studies, a laparoscopic cholecystectomy, a standard surgical procedure for gallbladder disease, results in an alteration of postoperative liver function more frequently than open cholecystectomy, and the cause of such a liver function alteration is believed to be the pneumoperitoneum created by the CO₂^[1-5]. Based on previous studies, laparoscopy-assisted gastrectomy (LAG) is expected to result in liver function alterations resulting from the CO₂ pneumoperitoneum; this occurs because the CO₂ exposure time is much longer in LAG because of the difficulty of the surgical technique and a longer operating time compared to laparoscopic cholecystectomy.

We investigated whether liver function alterations take place in patients who undergo LAG for gastric cancer and whether other factors affect liver function besides a CO₂ pneumoperitoneum. We also investigated the effect of the liver function alterations on the clinical results. To do this, the postoperative liver functions of patients who underwent open gastrectomy (OG) and of patients who underwent LAG were compared. In addition, the postoperative liver functions of patients who underwent open colectomy (OC) and of patients who underwent laparoscopy-assisted colectomy (LAC) for colon cancer were compared.

MATERIALS AND METHODS

Patients

We collected data from 237 consecutive patients who received a radical gastrectomy for gastric cancer at Soonchunhyang University Bucheon Hospital between January 2006 and December 2007. Of these 237 patients, 22 were excluded: 6 had hepatic cirrhosis, 5 had undergone hepatic resection simultaneously with gastrectomy for benign hepatic diseases or hepatic metastasis, 10 had received endoscopic treatment for biliary tract stones before the surgery or intraoperatively had undergone biliary system surgery, and one patient had intraoperatively received a biliary system iatrogenic injury. These patients were excluded because the surgical techniques may have affected their liver function during surgery. However, patients whose liver function was normal, including those who had hepatitis or fatty liver before the operation, and patients who underwent a cholecystectomy simultaneously, were not excluded.

Patients who underwent a colectomy without direct liver manipulation during the operation were the control group for the gastrectomy patients because we thought that traction or manipulation of the liver during the operation might affect postoperative liver function. Of the 133 patients who underwent a radical colectomy for colon cancer during the same period, those included in the study were selected according to the same exclusion criteria.

The patients were divided into 4 groups based on diagnosis and surgical technique: LAG group, OG group, LAC group, and OC group.

Methods

The patients' clinicopathologic characteristics, hepatic disease history, surgical method, surgical outcome, and liver function before and after surgery were retrospectively examined using their medical records. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (Bil), and alkaline phosphatase (ALP) were measured preoperatively and examined at postoperative day 1 (POD1), POD3, POD5, and POD7. The normal reference levels were AST 5-40 IU/L, ALT 0-40 IU/L, Bil 0.2-1.2 mg/dL, and ALP 35-115 IU/L. Patients who had hepatic enzyme levels higher than the normal reference level were defined as the "elevated" group, and patients with a normal reference level were defined as the 'normal' group.

Surgical methods

The operations were performed in the supine position for OG patients, in the reverse-Trendelenburg position for LAG patients, and in the Trendelenburg position for OC and LAC patients. For LAG and LAC, CO₂ was insufflated *via* a trocar to insert the laparoscope, and the pneumoperitoneal pressure was maintained at 12 mmHg or less. In patients undergoing LAG and OG, the range of the gastric resection or lymph node dissection depended on the tumor location or preoperative TNM stage; for gastric cancer invading the submucosal layer or deeper, a D2 lymph node dissection was performed according to the Japanese Gastric Cancer Guidelines. In the LAG and OG groups, to provide an operative field in the upper pancreatic border, the left lobe of the liver was lifted when the lesser curvature of the stomach was dissected. For the LAC and OC groups, the extent of colon resection was also determined based on tumor location and lesion stage before the surgery, and no difference was observed between the 2 groups.

Statistical analysis

All values are expressed as the mean \pm SD. The χ^2 -test, the independent *t*-test, and the paired *t*-test were conducted using SPSS software (version 15.0 for Windows; SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

RESULTS

Patient clinicopathologic characteristics

Two hundred and fifteen patients with gastric cancer participated in our study (124 patients in the OG group, 91 patients in the LAG group). The mean age of the patients was 58.6 years, with 164 males and 51 females. Of the patients with gastric cancer, 31 (14.4%) had underlying liver diseases: fatty liver in 15 and viral hepatitis in 16. A combined operation was performed in 46 cases (21.4%), most frequently a splenectomy, followed by a cholecystectomy, adrenalectomy, or distal pancreatectomy. Stage I was the most common TNM stage in both the OG and LAG groups (137 patients, 63.7%), but significantly more stage I patients were in the LAG group (92.3%) than in

Table 1 Patient characteristics *n* (%)

Variables	Stomach cancer (<i>n</i> = 215)			Colon cancer (<i>n</i> = 121)		
	LAG (<i>n</i> = 91)	OG (<i>n</i> = 124)	<i>P</i>	LAC (<i>n</i> = 43)	OC (<i>n</i> = 78)	<i>P</i>
Gender (M:F)	70:21	94:30	NS	22:21	43:35	NS
Age (yr, mean ± SD)	57.1 ± 13.2	59.7 ± 11.9	NS	58.9 ± 10.3	62.0 ± 13.5	NS
BMI (kg/m ² , mean ± SD)	23.8 ± 3.2	23.5 ± 3.4	NS	24.2 ± 3.6	24.7 ± 2.9	NS
Underlying liver disease			NS			NS
No	81 (89.0)	103 (83.1)		30 (69.8)	66 (84.6)	
Yes	10 (11.0)	21 (16.9)		13 (30.2)	12 (15.4)	
Fatty liver	6	9		11	8	
Hepatitis	4	12		2	4	
TNM stage			< 0.001			0.004
I	84 (92.3)	53 (42.7)		18 (41.9)	15 (19.2)	
II	4 (4.4)	22 (17.7)		8 (18.6)	35 (44.9)	
III	3 (3.3)	30 (24.3)		17 (39.5)	28 (35.9)	
IV	0	19 (15.3)		0	0	
Operation time (min, mean ± SD)	230.1 ± 77.6	165.2 ± 52.1	< 0.001	252.5 ± 77.2	184.1 ± 62.4	< 0.001
Combined operation			0.004			NS
No	80 (87.9)	89 (71.8)		42 (97.7)	74 (94.9)	
Yes	11 (12.1)	35 (28.2)		1 (2.3)	4 (5.1)	
Anesthetic agent			NS			NS
Sevoflurane	21 (23.1)	45 (36.3)		15 (34.9)	18 (23.1)	
Desflurane	70 (76.9)	79 (63.7)		28 (65.1)	60 (76.9)	
Postoperative morbidity	8 (8.8)	16 (12.9)	NS	1 (2.3)	5 (6.4)	NS
Postoperative mortality	1	1		1	0	
Hospital stay (d, mean ± SD)	10.7 ± 16.0	14.9 ± 12.6	NS	12.7 ± 3.8	17.9 ± 12.3	0.007

LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; LAC: Laparoscopy-assisted colectomy; OC: Open colectomy; BMI: Body mass index; NS: Not significant.

the OG group ($P < 0.001$). A combined operation was performed more frequently in the OG group than in the LAG group ($P = 0.004$), and the operating time was significantly longer in the LAG group (203 min) than in the OG group (165 min, $P < 0.001$). No significant differences between the 2 groups were observed for age, presence or absence of underlying liver disease, type of anesthetic agent used, or postoperative complications.

Among the patients with colon cancer, 121 were included in the study. Their mean age was 60.9 years, and they consisted of 65 males and 56 females. No differences in gender, age, presence or absence of an underlying liver disease, combined operation rate, anesthetic agent, or postoperative complications were observed between the LAC (43 patients) and the OC groups (78 patients), whereas significant differences were found between the 2 groups for operating time and hospital stay after the operation, operating time being significantly longer in the LAC group and length of stay significantly longer in the OC group (Table 1).

Morbidity and mortality after the operation

Postoperative complications occurred in 24 (11.2%) patients who underwent a gastrectomy: wound infection in 7, anastomotic leakage in 5, postoperative bleeding in 5, intra-abdominal fluid collection in 5, and other complications in 2. Six cases of complications (5%) occurred in patients who underwent a colectomy: postoperative ileus in 3, anastomotic leak in one, and wound infection in one.

Two patients who underwent a gastrectomy (one each in the OG and LAG groups) and one patient who under-

went a colectomy (LAC group) died after the operation. Thus, the postoperative mortality rate was 0.9% and 0.8% in patients who underwent a gastrectomy and colectomy, respectively. The causes of death were postoperative pneumonia (2 patients in the gastrectomy group) and sepsis (one patient in the colectomy group); no patient died of postoperative hepatic failure.

Postoperative changes in liver function

The postoperative AST and ALT levels were significantly higher than the preoperative levels in both the OG and LAG groups until POD5 (Table 2).

The mean hepatic enzyme levels at POD1 in the LAG group were significantly higher than those in the OG group ($P = 0.047$ for AST and $P = 0.039$ for ALT). No changes in preoperative or postoperative Bil and ALP levels were observed in either group.

No significant changes in the pre- and postoperative AST, ALT, Bil, and ALP levels were observed among patients who underwent a colectomy, and the changes were not significantly different between the LAC and OC groups (Table 2).

Approximately 65% of the patients in the LAG group and 59.7% in the OG group showed increased levels of AST at POD1, and 25.3% in the LAG group and 27.4% in the OG group showed increased levels of AST at POD3 ($P > 0.05$). Similar to the results with AST, 69.2% of the patients in the LAG group and 58.1% in the OG group showed increased levels of ALT at POD1, and 39.6% in the LAG group and 33.9% in the OG group

Table 2 Preoperative and postoperative liver enzymes (mean \pm SD)

Variables	Stomach cancer (<i>n</i> = 215)			Colon cancer (<i>n</i> = 121)		
	LAG (<i>n</i> = 91)	OG (<i>n</i> = 124)	<i>P</i> ¹	LAC (<i>n</i> = 43)	OC (<i>n</i> = 78)	<i>P</i> ²
AST (IU/L)						
Preoperative	22.1 \pm 7.6	21.8 \pm 7.3	NS	20.8 \pm 8.7	19.9 \pm 6.3	NS
POD1	73.8 \pm 67.2 ^a	57.6 \pm 45.9 ^a	0.047	23.2 \pm 11.8	22.2 \pm 11.5	NS
POD3	41.1 \pm 43.5 ^a	38.4 \pm 24.3 ^a	NS	22.9 \pm 9.5	22.4 \pm 8.4	NS
POD5	28.9 \pm 24.1 ^a	29.5 \pm 18.1 ^a	NS	25.3 \pm 10.8	22.3 \pm 10.8	NS
POD7	24.0 \pm 11.6	22.2 \pm 11.3	NS	28.9 \pm 26.2	24.7 \pm 13.4	NS
ALT (IU/L)						
Preoperative	23.9 \pm 13.5	23.8 \pm 15.8	NS	24.1 \pm 3.7	17.0 \pm 2.6	0.045
POD1	89.1 \pm 103.7 ^a	63.3 \pm 65.9 ^a	0.039	18.4 \pm 8.1	17.4 \pm 15.0	NS
POD3	62.2 \pm 97.3 ^a	51.5 \pm 57.6 ^a	NS	16.6 \pm 6.5	15.9 \pm 9.6	NS
POD5	43.2 \pm 49.1 ^a	39.4 \pm 40.6 ^a	NS	20.6 \pm 13.9	16.4 \pm 8.5	NS
POD7	33.7 \pm 25.4	26.9 \pm 17.9	0.033	32.1 \pm 43.8	21.0 \pm 25.4	NS
Total bilirubin (mg/dL)						
Preoperative	0.6 \pm 0.2	0.5 \pm 0.3	NS	0.5 \pm 0.2	0.5 \pm 0.3	NS
POD1	0.8 \pm 0.3	0.7 \pm 0.3	NS	0.7 \pm 0.4	0.6 \pm 0.3	NS
POD3	0.8 \pm 0.4	0.7 \pm 0.5	NS	0.4 \pm 0.2	0.4 \pm 0.2	NS
POD5	0.7 \pm 0.3	0.6 \pm 0.3	NS	0.5 \pm 0.2	0.4 \pm 0.2	NS
POD7	0.7 \pm 0.3	0.5 \pm 0.2	NS	0.5 \pm 0.2	0.4 \pm 0.2	NS
ALP (IU/L)						
Preoperative	65.6 \pm 17.6	65.8 \pm 22.1	NS	69.1 \pm 20.3	64.4 \pm 22.2	NS
POD1	46.7 \pm 11.9	47.8 \pm 14.6	NS	49.9 \pm 15.5	44.6 \pm 13.3	NS
POD3	44.2 \pm 10.5	45.8 \pm 14.5	NS	44.1 \pm 11.7	41.9 \pm 10.2	NS
POD5	50.6 \pm 23.5	48.6 \pm 15.6	NS	47.5 \pm 18.7	43.2 \pm 11.3	NS
POD7	57.9 \pm 38.3	54.7 \pm 22.6	NS	51.1 \pm 24.1	47.2 \pm 13.1	NS

¹*P* values represent the statistical difference between the laparoscopy-assisted gastrectomy (LAG) and open gastrectomy (OG) groups; ²*P* values represent the statistical difference between the laparoscopy-assisted colectomy (LAC) and open colectomy (OC) groups; ^a*P* < 0.05 vs preoperative liver enzymes using the paired *t*-test. POD: Postoperative day; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; NS: Not significant.

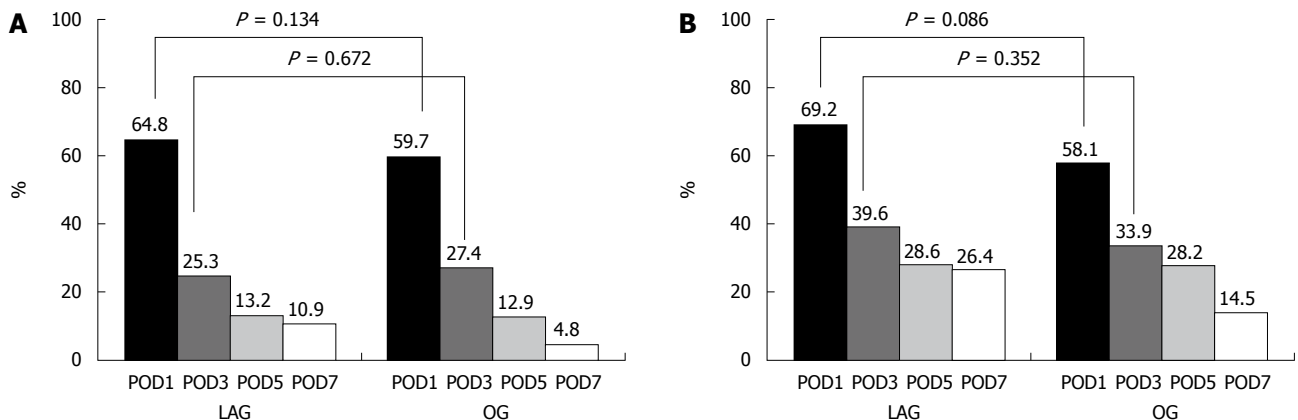


Figure 1 Frequency of patients who had elevated liver enzyme levels after a gastrectomy for gastric cancer. A: Aspartate aminotransferase; B: Alanine aminotransferase. LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; POD: Postoperative day.

showed increased levels at POD3 (*P* > 0.05, Figure 1).

Among the patients who underwent a gastrectomy, 13 showed more than a 5-fold increase in ALT levels from the normal level at POD1 (8 patients in the LAG group and 5 patients in the OG group). Of these 13 patients, 3 showed more than a 10-fold increase (2 patients in the LAG group and one patient in the OG group).

Factors that affected postoperative liver function after gastrectomy

Unlike LAG, no change in liver function was observed after LAC when using a CO₂ pneumoperitoneum. There-

fore, the characteristics of the patients who showed liver function alterations after LAG were examined to identify other factors that could cause changes in postoperative liver function. For this purpose, we investigated the ALT level at POD1, at which the change in postoperative liver function for those who underwent a gastrectomy was most prominent. The patients who underwent a gastrectomy were classified into 2 groups: a normal group that had a normal ALT range and an elevated group having higher ALT levels than the reference. We also examined their clinical features and surgical findings.

The changes in the ALT levels at POD1 for patients

Table 3 Comparison of alanine transaminase levels on the first postoperative day in patients who underwent a gastrectomy *n* (%)

Variables	Normal group (<i>n</i> = 80)	Elevated group (<i>n</i> = 135)	<i>P</i> value
Gender (M:F)	59:21	105:30	0.502
Age (yr, mean \pm SD)	58.6 \pm 13.4	58.6 \pm 12.0	0.981
BMI (kg/m ² , mean \pm SD)	22.3 \pm 3.3	24.4 \pm 3.0	< 0.001
Underlying liver disease			0.153
No	70 (87.5)	114 (84.4)	
Yes	10 (12.5)	21 (15.6)	
Fatty liver	2 (2.5)	13 (9.7)	
Hepatitis	8 (10.0)	8 (5.9)	
Operation time (min, mean \pm SD)	168.9 \pm 61.4	206.7 \pm 73.6	< 0.001
Types of operation			0.094
Open	52 (65.0)	72 (53.3)	
Laparoscopy-assisted	28 (35.0)	63 (46.7)	
Combined operation			0.093
No	58 (72.5)	111 (82.2)	
Yes	22 (27.5)	24 (17.8)	
Anesthetic agent			0.979
Sevoflurane	24 (30.0)	40 (29.6)	
Desflurane	56 (70.0)	95 (70.4)	
Intraoperative transfusion			0.894
No	71 (88.8)	119 (88.1)	
Yes	9 (11.3)	16 (11.9)	
Aberrant left hepatic artery			0.052
Absence or preservation	79 (98.8)	127 (94.1)	
Artery ligation	1 (1.3)	8 (5.9)	
Intraoperative hepatic injury			0.048
No	79 (98.8)	125 (92.6)	
Yes	1 (1.3)	10 (7.4)	
Mean ALT at POD1 (\pm SD)	28.2 \pm 8.1	101.4 \pm 97.1	< 0.001

BMI: Body mass index; POD: Postoperative day; ALT: Alanine aminotransferase.

who underwent a gastrectomy were not significantly affected by gender, age, underlying liver disease, presence or absence of postoperative complications, type of anesthetic agent, or intraoperative transfusion. The body mass index (BMI) was significantly higher in the elevated group than in the normal group, and the operating time in the elevated group was significantly longer than in the normal group. Furthermore, 7.4% of the patients who experienced intraoperative hepatic injury showed increased ALT levels, which was a significantly higher proportion than in the patients who did not experience intraoperative hepatic injury ($P = 0.048$). Of the patients with normal ALT levels, 1.3% of patients had ligation of an aberrant left hepatic artery during the operation, whereas in the elevated group, 5.9% of patients had ligation of this artery, although this result had no statistical significance ($P = 0.052$). Thirty-five percent of the patients in the normal group underwent LAG, whereas 46.7% of the patients in the elevated group underwent LAG, but no significant difference was found ($P = 0.094$) (Table 3).

DISCUSSION

Pneumoperitoneum, which is created by CO₂ insufflation during a laparoscopic operation, results in postoperative transient liver function abnormalities. Halevy *et al.*^[6] first

reported that aminotransferase levels increased significantly after laparoscopic cholecystectomy. They suggested that the increase in aminotransferase was due to increased intra-abdominal pressure, pressure against the liver with the lifting of the gall bladder, damage to the liver parenchyma by electrocautery, deformity of the extrahepatic biliary duct, the possibility of introducing a small stone into the biliary duct, or damage to the left hepatic artery during surgery. Other studies have reported that a laparoscopic cholecystectomy was more likely to result in liver function abnormalities than an open cholecystectomy and suggested that the cause of the abnormalities was reduced blood flow into the hepatic portal vein as a result of the increased intra-abdominal pressure caused by the pneumoperitoneum; the degree of pneumoperitoneal pressure is related to the degree of liver function abnormality^[2,7].

Animal studies have also shown that a pneumoperitoneum directly damages rat liver tissue, that the degree of damage is related to pneumoperitoneal pressure, and that the persistence of the pressure at 15 mmHg or more for 60 min or longer may result in irreversible hepatic damage^[8]. Contrary to these reports, a study on 1034 patients who underwent laparoscopic cholecystectomy reported that only 3.9% of the patients had a mild elevation in hepatic enzyme levels after the operation, and that the number of liver function abnormality cases after laparoscopic cholecystectomy was much lower than in other studies^[9].

Very few studies have reported liver function abnormalities after LAG for gastric cancer. Etoh *et al.*^[10] and Kim *et al.*^[11] reported that liver function abnormalities occurred in patients who underwent LAG but not in those who had OG. They asserted that liver function abnormalities may occur after LAG due to the CO₂ pneumoperitoneum, although objective evidence for their assertion was unavailable.

Because LAG is more difficult to perform than OG, the operating time can be longer, and consequently, the exposure time of the intra-abdominal organs to the pneumoperitoneum may also be longer than in OG. Given this, a pneumoperitoneum was expected to cause liver function abnormalities. Thus, this study sought to determine whether postoperative liver function abnormalities would occur in patients who underwent LAG.

The change in the absolute hepatic enzyme levels was more marked in the LAG group than in the OG group. Moreover, consistent with the results of previous studies, this change was most prominent on POD1, but the level returned to normal on POD5. When the patients were divided into an elevated enzyme group and a normal group, the frequency of change in the absolute hepatic enzyme levels in the elevated group did not differ between the LAG and OG groups (Figure 1). Furthermore, when the patients who underwent a colectomy, in which the hepatic parenchyma and the vessels around the liver were not excessively manipulated, were divided into LAC and OC groups, no liver function abnormalities were found in either group. Thus, we presumed that the major cause of liver function abnormalities after LAG may not be the pneumoperitoneum. Similar to this result, Nguyen *et al.*^[12]

also reported no difference in the increase in hepatic enzyme levels between patients who underwent laparoscopic and open gastric bypass. They performed laparoscopic and open Roux-en-Y gastric bypass on obese patients and found that the hepatic enzyme level increase was highest in the first 24 h after the operation, and that liver function returned to normal 72 h after the operation. They also reported no significant difference in the hepatic enzyme level increase between those who underwent a laparoscopic vs an open operation, and they asserted that a prolonged pneumoperitoneum in patients who underwent the laparoscopic operation would not have greatly affected the change in the postoperative hepatic enzyme levels. The statistical significance of the effect of prolonged operating time on the increase in hepatic enzyme level after gastrectomy in the present study can be attributed to the long exposure time to the pneumoperitoneum. However, it can also be attributed to the long ischemic time of the liver due to extended liver traction.

Thus, we divided our patients into a group with normal liver function and a group with elevated liver function, based on the ALT level at POD1, at which the liver function abnormality was most prominent, to determine whether liver function after LAG is influenced by factors other than a pneumoperitoneum. The change in liver function was based on the ALT level because ALT exists mostly in the liver, unlike AST, and is the gold-standard clinical chemistry marker for liver injury^[13,14]. The classification of the elevated group was based on the normal ALT reference range of 0-40 IU/L, which is used at the authors' hospital. As a result, the surgical technique (OG and LAG) did not affect the increase in the ALT levels after the operation, but the BMI in the elevated group was significantly higher than in the normal group. Also, the operating time was significantly longer in the elevated group than in the normal group. When the hepatic parenchyma was damaged or when the aberrant left hepatic artery was ligated, the ALT level increased.

Many studies on liver function abnormalities after a laparoscopic operation have reported that the cause of postoperative liver function abnormality was hepatic ischemia caused by reduced portal flow due to the pneumoperitoneum^[8]. However, we did not find any liver function abnormalities after LAC, whereas liver function abnormalities after an open gastrectomy were found when the hepatic parenchyma was directly damaged or when the aberrant hepatic artery was ligated. Given these conditions, the causes of liver function abnormalities after LAG can be attributed to excessive liver traction, direct damage to the hepatic parenchyma, ligation of an aberrant hepatic artery during the operation, or reduced portal flow due to the pneumoperitoneum.

Patients who underwent a gastrectomy showed significantly increased hepatic enzyme levels on POD1, regardless of the surgical technique, which returned to normal on POD5. This transient liver function abnormality was not clinically meaningful because it did not cause hepatic failure or clinical symptoms. However, as patients with hepatic cirrhosis or decreased liver function were not included in this study, further research is required to de-

termine whether LAG is safe for patients with decreased liver function. The assumption was made that LAG could be performed safely in patients with decreased liver function by taking the greatest possible care with ligation of the aberrant hepatic artery and not damaging the hepatic parenchyma, or if the major cause of the liver function abnormality after LAG was damage to the hepatic parenchyma due to excessive liver traction.

COMMENTS

Background

A major difference between laparoscopic and open surgery is that carbon dioxide (CO₂) is used to create a pneumoperitoneum during laparoscopic surgery, which may result in respiratory and hemodynamic changes. According to many studies, a laparoscopic cholecystectomy, a standard surgical procedure for gallbladder disease, results in an alteration of postoperative liver function more frequently than open cholecystectomy, and the cause of such liver function alteration is believed to be the pneumoperitoneum created by CO₂. Based on previous studies, laparoscopy-assisted gastrectomy (LAG) is expected to result in liver function alterations due to the CO₂ pneumoperitoneum. The paper investigated whether liver function alterations take place in patients who undergo LAG for gastric cancer and whether other factors affect liver function besides a CO₂ pneumoperitoneum.

Innovations and breakthroughs

In the study, patients who underwent a colectomy without direct liver manipulation during the operation were the control group for the gastrectomy patients because the authors thought that traction or manipulation of the liver during the operation might affect postoperative liver function. The change in the absolute hepatic enzyme levels was more remarkable in the LAG group than in the open gastrectomy (OG) group. Moreover, consistent with the results of previous studies, this change was most prominent on postoperative day 1 (POD1), but the level returned to normal on POD5. When the patients were divided into an elevated group and a normal group, the frequency of change in the absolute hepatic enzyme levels in the elevated group did not differ between the LAG and OG groups. Furthermore, when the patients who underwent a colectomy, in which the hepatic parenchyma and the vessels around the liver were not excessively manipulated, were divided into laparoscopy-assisted colectomy (LAC) and open colectomy groups, no liver function abnormalities were found in either group. Thus, authors presumed that the major cause of liver function abnormalities after LAG may not be the pneumoperitoneum.

Applications

Many studies on liver function abnormalities after a laparoscopic operation have reported that the cause of postoperative liver function abnormality was hepatic ischemia caused by reduced portal flow due to the pneumoperitoneum. However, authors did not find any liver function abnormalities after LAC, whereas liver function abnormalities after an open gastrectomy were found when the hepatic parenchyma was directly damaged or when the aberrant hepatic artery was ligated. Given these conditions, the causes of liver function abnormalities after LAG can be attributed to excessive liver traction, direct damage to the hepatic parenchyma, ligation of an aberrant hepatic artery during the operation, or reduced portal flow due to the pneumoperitoneum. The assumption was made that LAG could be performed safely on patients with decreased liver function by taking the greatest possible care with the aberrant hepatic artery ligation and not damaging the hepatic parenchyma, or doing so if the major cause of the liver function abnormality after LAG was damage to the hepatic parenchyma due to excessive liver traction.

Peer review

This is a well designed and researched paper. The positive findings show more marked changes in liver function after a laparoscopy-assisted gastrectomy for gastric cancer than after an open gastrectomy, lasting for up to 5 d, these differences are related to body mass index (BMI) and longer operation time. The authors could have suggested that these could be explained by the heavier retraction of the liver which would be required in patients with a greater BMI and the greater duration of the retraction in the laparoscopy group.

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S- Editor Shi ZF L- Editor Cant MR E- Editor Ma WH



Necrotic stercoral colitis: Importance of computed tomography findings

Cheng-Hsien Wu, Li-Jen Wang, Yon-Cheong Wong, Chen-Chih Huang, Chien-Cheng Chen, Chao-Jan Wang, Jen-Feng Fang, Chuen Hsueh

Cheng-Hsien Wu, Li-Jen Wang, Yon-Cheong Wong, Chen-Chih Huang, Chien-Cheng Chen, Chao-Jan Wang, Division of Emergency and Critical Care Radiology, Department of Medical Imaging and Intervention, Chang Gung Memorial Hospital, Chang Gung University, 333 Taoyuan, Taiwan, China

Jen-Feng Fang, Division of Trauma and Emergency, Department of Surgery, Chang Gung Memorial Hospital, Chang Gung University, 333 Taoyuan, Taiwan, China

Chuen Hsueh, Department of Pathology, Chang Gung Memorial Hospital, Chang Gung University, 333 Taoyuan, Taiwan, China

Author contributions: Fang JF was the surgical consultant; Hsueh C was the pathological consultant; Wu CH, Wong YC and Wang LJ were involved in editing the manuscript; Huang CC, Chen CC and Wang CJ performed the image interpretation; Wu CH and Wong YC designed the study and wrote the manuscript.

Correspondence to: Dr. Yon-Cheong Wong, Division of Emergency and Critical Care Radiology, Department of Medical Imaging and Intervention, Chang Gung Memorial Hospital, Chang Gung University, 5, Fu-Hsing Street, Gueishan, 333 Taoyuan, Taiwan, China. ycwong@adm.cgmh.org.tw

Telephone: +886-3-3281200 Fax: +886-3-3970074

Received: July 23, 2010 Revised: September 2, 2010

Accepted: September 9, 2010

Published online: January 21, 2011

Abstract

AIM: To study the computed tomography (CT) signs in facilitating early diagnosis of necrotic stercoral colitis (NSC).

METHODS: Ten patients with surgically and pathologically confirmed NSC were recruited from the Clinico-Pathologic-Radiologic conference at Chang Gung Memorial Hospital, Taoyuan, Taiwan. Their CT images and medical records were reviewed retrospectively to correlate CT findings with clinical presentation.

RESULTS: All these ten elderly patients with a mean age of 77.1 years presented with acute abdomen at our Emergency Room. Nine of them were with systemic med-

ical disease and 8 with chronic constipation. Seven were with leukocytosis, two with low-grade fever, two with peritoneal sign, and three with hypotensive shock. Only one patient was with radiographic detected abnormal gas. Except the crux of fecal impaction, the frequency of the CT signs of NSC were, proximal colon dilatation (20%), colon wall thickening (60%), dense mucosa (62.5%), mucosal sloughing (10%), perfusion defect (70%), pericolic stranding (80%), abnormal gas (50%) with pneumo-mesocolon (40%) in them, pericolic abscess (20%). The most sensitive signs in decreasing order were pericolic stranding, perfusion defect, dense mucosal, detecting about 80%, 70%, and 62.5% of the cases, respectively.

CONCLUSION: Awareness of NSC and familiarity with the CT diagnostic signs enable the differential diagnosis between NSC and benign stool impaction.

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Key words: Fecal impaction; Dense mucosa; Pericolic stranding; Stercoral colitis; Computed tomography

Peer reviewer: Dr. Giuseppe Chiarioni, Gastroenterological Rehabilitation Division of the University of Verona, Valeggio sul Mincio Hospital, Azienda Ospedale di Valeggio s/M, Valeggio s/M 37067, Italy

Wu CH, Wang LJ, Wong YC, Huang CC, Chen CC, Wang CJ, Fang JF, Hsueh C. Necrotic stercoral colitis: Importance of computed tomography findings. *World J Gastroenterol* 2011; 17(3): 379-384 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/379.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.379>

INTRODUCTION

Necrotic stercoral colitis is a necrotic process that occurs

in stercoral colitis (SC), caused by fecal impaction that results in pressure ulceration and regional necrosis. Perforation is rare, but has a mortality rate of 32%-57%^[1]. Early diagnosis with aggressive bowel cleansing and disimpaction may decrease the pressure and lessen the likelihood of ulceration of the colon^[2]. Fecal impaction frequently occurs in elderly patients, and those who are bed-ridden for a prolonged period of time.

Most patients present to the emergency room (ER) with an acute abdomen. Their physical examinations and laboratory data are often unreliable. Moreover, the peritoneal signs are often nonspecific and might be attributed to diverticulitis, which is more common in elderly patients^[3]. Computed tomography (CT) is readily available and is not operator-dependent; therefore, abdominal CT is often requested by emergency physicians to evaluate patients with acute abdominal conditions.

Very little has been published on NSC in the radiology literature^[3]. We reviewed the CT findings of 10 patients with NSC from our hospital, to call attention to this potentially fatal condition.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by Chang Gung Memorial Hospital (98-0044B).

Patients

Between November 2002 and August 2009, ten patients with surgically and pathologically confirmed NSC were recruited from the Clinico-Pathologic-Radiologic conference at Chang Gung Memorial Hospital, Taoyuan, Taiwan. We reviewed their abdominal radiographs, CT images, and medical records retrospectively.

CT protocol

All of these patients underwent CT examinations of the abdomen and pelvis before surgical exploration, while they stayed in the ER. CT examinations were performed by four-detector CT (LightSpeed QX/i Scanner, General Electric Medical Systems, Milwaukee, WI, USA). Helical CT images were acquired using either 7- or 5-mm slice collimation, reconstruction interval of 5 mm, pitch of 1.5-2, 120 kV, and 200-240 mA. One hundred milliliters of intravenous (IV) contrast agent was used routinely.

CT interpretation

Several CT findings of fecal impaction in the colon, thickening of the colon wall, and pericolic stranding indicated SC, whereas the presence of extraluminal gas bubbles or an abscess suggested that perforation had occurred^[2].

The CT examinations were retrospectively reviewed by two independent board-certified abdominal radiologists who were blinded to the CT official reports and the surgical and pathologic findings. They viewed the CT images

on a picture archiving and communication system (PACS) independently and discussed the findings until consensus was reached. If consensus could not be reached, a third abdominal radiologist was consulted. All abdominal radiographs were reviewed for abnormal gas. They were also requested to determine the presence or absence of the CT features of NSC, including location of fecal impaction, proximal colon dilatation, colon wall thickening, dense mucosa, mucosal sloughing, perfusion defect, pericolic stranding, pericolic abscess, and abnormal gas with or without pneumo-mesocolon. Vascular ischemic colitis was excluded based on patency of the inferior mesenteric artery and vein.

Definition of CT signs

The individual CT signs were defined as follows - Fecal impaction: distended colon with much feces or packing of dehydrated fecaloma in the colon; Proximal colon dilatation: a distended left-sided colon with a cylindrical shape and cross-sectional diameter > 6 cm; Colon wall thickening: regional wall thickness > 3 mm in the obstruction site; Dense mucosa: increased mucosal lining density on pre-contrast CT; Mucosal sloughing: mucosa dislodged into the lumen; Perfusion defect: discontinuity of the enhancement of colon mucosa or apparently decreased enhancement as compared with adjacent small bowel loops; Pericolic stranding: increased streaks of pericolic fat; Pericolic abscess: pericolic loculated fluid or mottled substance; Abnormal gas: gas migrating into or beyond the colon wall as pneumoperitoneum or pneumoretroperitoneum, i.e. pneumo-intestinalis coli; gas entrapped in the mural wall; pneumo-mesocolon: gas confined inside the mesocolon; and portal vein gas: air leakage into the portomesenteric vessels.

RESULTS

Demography and clinical information

Six men and four women aged 39-88 years (mean, 77.1 years) were studied (Table 1). All of the patients presented to our ER with acute abdomen. Chronic constipation and systemic medical disease were the common clinical problems in these patients. Abdominal discomfort was not greatly improved after local removal of impacted feces by digital evacuation or fleet enema. On arrival at the ER, two patients (20%) presented with a low grade fever (< 38.5°C), two (20%) presented with peritoneal signs, and seven (70%) presented with leukocytosis with one other at borderline criteria of leukocytosis. Three patients (30%) arrived at the ER with hypotensive shock (systemic blood pressure < 90 mmHg). Surgical intervention was indicated for all of the patients. Seven of the patients died; thus, the mortality rate was 70%. Among these seven patients, three died within 1 wk, highlighting the rapidly progressive course of the disease.

CT signs

The imaging findings of NSC are listed in Table 2. CT

Table 1 Clinical data of study patients with necrotizing stercoral colitis

No.	Age (yr)/sex	sBP	BT	Hx	Cor	PS	WBC	TI	Fe	Pe	Operation findings	Pathology	Outcome
1	76/M	183	38.4	+	DM, HTN Arrhythmia	-	22.2k/89	5'30"	RS	No	Ischemic change from sigmoid to rectum with necrotic mucosa	Ischemia necrosis with mucosal sloughing	Alive
2	86/M	130	34.4	+	CAD RF	-	45.6k/72	2'30"	S	S	Necrosis of descending and sigmoid colon with a 2-cm perforation	Perforating ulcer with transmural necrosis	Dead, 1 d after CT
3	79/F	147	36.1	+	DM	-	15.3k/76	7 d	D	D	Necrosis of nearly entire colon, with a 1.7-cm perforation	Mucosal ulcer with perforation	Dead, 19 d after CT
4	87/M	158	35.6	+	HTN	+	14.4k/90	4'40"	RS	S	A 2-cm perforator 2 cm proximal to the recto-sigmoid colon cancer	Transmural necrosis with a 2.1 cm perforator	Alive
5	80/F	120	33.6	+	HTN	-	3.6k/67	24'30"	S	S	Nearly entire colon necrosis with a 5-cm × 3-cm perforator at sigmoid colon	Ulcerative hole with transmural necrosis at sigmoid colon	Dead, 5 d after CT
6	70/F	145	36.4	+	DM	-	13.3k/80	15'40"	RS	No	Necrosis of distal ileum and entire colon	Transmural necrosis of bowel wall	Dead, 47 d after CT
7	88/F	81	35.0	+	-	-	4k/38	3'	RS	S	2 small perforators at proximal sigmoid colon	Gangrenous change with transmural necrosis of sigmoid colon	Dead, 8 d after CT
8	39/M	64	38.0	NA	ESRD	+	9.9k/79	26'	RS	No	Ischemic patches over sigmoid colon with impending perforation	Ischemic and gangrenous change of the sigmoid colon	Dead, 3 d after CT
9	83/M	158	37.0	NA	ARDS, HF HTN, COPD	-	17k/93	11'	RS	No	Ischemic change of small bowel and sigmoid colon	Transmural necrosis of sigmoid colon and mucosal necrosis of small bowel	Dead, 11 d after CT
10	83/M	64	35.3	+	CAD, HTN	-	46k/83	10'	RS	No	Patch necrosis of the T and D colon	Gangrenous change of the T and D colon	Alive

sBP: Systemic blood pressure (mmHg); BT: Body temperature (°C); Hx: History of constipation; Cor: Comorbidity; PS: Peritoneal signs at initial admission physical examination; WBC: White blood cell (number/percentage of segment) at admission; CT: Computed tomography; TI: Time interval between CT and surgery; Fe: Stool obstructive site; Pe: Perforator site; CAD: Coronary arterial disease; RF: Renal failure; HTN: Hypertension; HF: Heart failure; NA: Not applicable; -: Absent; +: Present; 5'30": 5 h and 30 min, *etc.*; RS: Recto-sigmoid colon; S: Sigmoid colon; T: Transverse colon; D: Descending colon; DM: Diabetes mellitus; ESRD: End-stage renal disease; ARDS: Acute respiratory distress syndrome; COPD: Chronic obstructive pulmonary disease.

Table 2 Imaging signs of necrotizing stercoral colitis

No.	Radiographic abnormal gas	CT signs										
		Fecal impaction	Obstructive site	Proximal dilatation	Wall thickening	Dense mucosa	Mucosal sloughing	Perfusion defect	Pericolic stranding	Abnormal gas	Pneumo-mesocolon	Abscess
1	N	Y	RS	N	N	NA	N	Y	Y	N	N	N
2	Y	Y	S	N	Y	NA	N	Y	N	Y	Y	N
3	N	Y	D	N	N	Y	N	N	Y	Y	N	N
4	N	Y	RS	N	Y	Y	N	Y	Y	Y	Y	Y
5	N	Y	S	N	Y	Y	N	Y	Y	Y	N	N
6	N	Y	RS	Y	Y	Y	N	Y	N	N	N	N
7	N	Y	RS	N	Y	Y	Y	Y	Y	Y	N	Y
8	N	Y	RS	Y	N	N	N	N	Y	N	N	N
9	N	Y	RS	N	Y	N	N	Y	Y	N	N	N
10	N	Y	RS	N	N	N	N	N	Y	N	N	N
Frequency	1/5	10/10	9/10	2/10	6/10	5/8	1/10	7/10	8/10	5/10	2/5	2/10
	(20%)	(100%)	(90%)	(20%)	(60%)	(62.5%)	(10%)	(70%)	(80%)	(50%)	(40%)	(20%)
κ-value	1	1	1	1	0.4	0.714	0.615	0.286	0.737	0.8	1	1

CT: Computed tomography; NA: Not applicable; N: No; Y: Yes; RS: Recto-sigmoid colon; S: Sigmoid colon; D: Descending colon.

examination revealed fecal impaction at the sigmoid colon in nine patients (90%) and at the distal descending colon in one (10%). Proximal colon dilatation was found in two patients (20%). Colon wall thickening (Figure 1) occurred in six patients (60%), dense mucosa (Figure 2A) in five (62.5%), mucosal sloughing (Figure 3A) in one (10%), and

colon mucosal perfusion defect (Figure 2B) was found in seven (70%) patients. Pericolic stranding (Figure 2C) was identified in eight patients (80%), and pericolic abscess formation (Figure 3B) was observed in two (20%) patients. Abnormal gas was present in five patients (50%): pneumo-mesocolon in two (40%, Figure 4), and one pa-

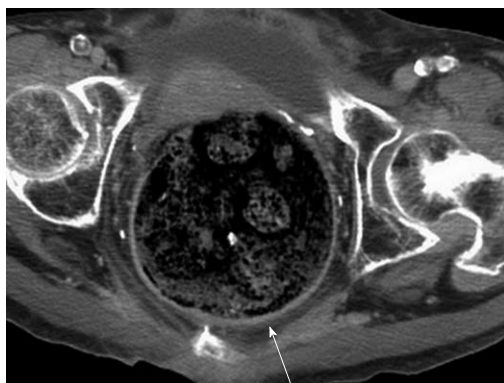


Figure 1 A 70-year-old woman (patient 6) with necrotic stercoral colitis. The computed tomography scan revealed stool impaction and distension of the rectosigmoid colon with asymmetrical wall thickening at the posterior aspect (arrow).

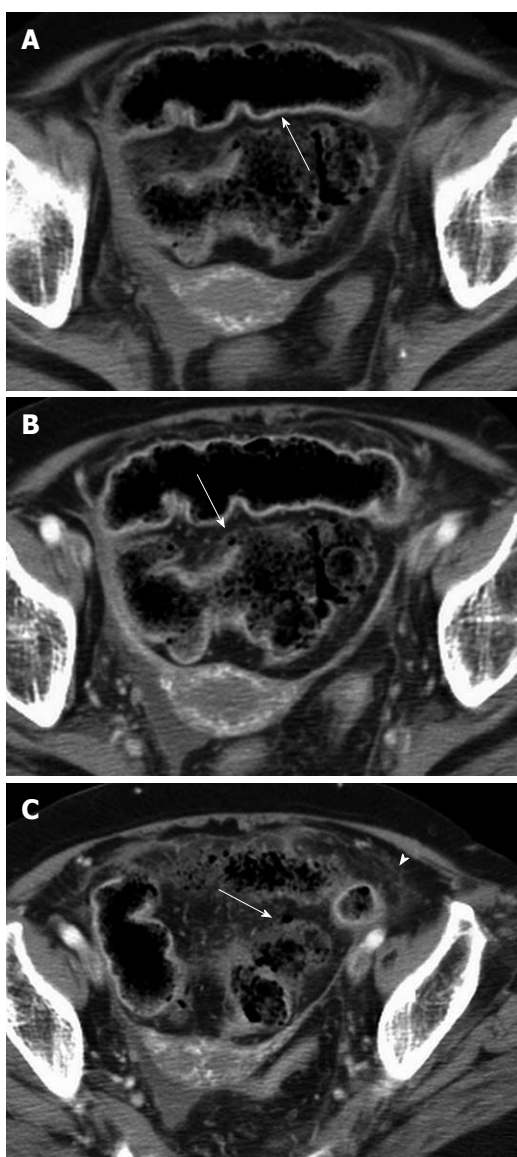


Figure 2 An 80-year-old woman (patient 5) with perforation of the necrotic stercoral colitis at the sigmoid colon. A: An unenhanced computed tomography (CT) scan reveals dense mucosa (arrow) conforming to the colon wall; B: An enhanced abdominal CT scan reveals discontinuation of the colonic mucosa (arrow) suggesting perfusion defect; C: A small air bubble abutting the damaged colon (arrow) and increased pericolic infiltration (arrowhead) can be seen.



Figure 3 An 88-year-old woman (patient 7) with perforation of the necrotic stercoral colitis at the sigmoid colon. A: An enhanced abdominal computed tomography scan reveals mucosal flap (arrow) slough into the lumen of the colon indicating mucosal sloughing; B: Air pockets (arrow) abutting the colonic wall and pericolic loculated fluid indicative of abscess formation (arrowhead).

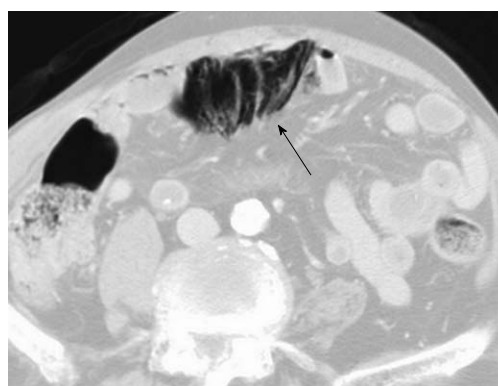


Figure 4 An 87-year-old man (patient 4) with perforated stercoral colitis at the proximal end of co-existing rectosigmoid colon cancer. An enhanced abdominal computed tomography scan at lung-window setting reveals air confined inside the mesocolon indicating pneumo-mesocolon (arrow).

tient (20%) with pneumoperitoneum was identified by radiograph.

Dense mucosa was evaluated with pre-contrast CT scanning in 8 of the patients who had undergone scanning of the lower abdomen. Dense mucosal lining conforming to the colon wall was differentiated from the fecalith which presented as clustered masses in the lumen with a calcified surface and gas in between.

Inter-observer agreement is shown in Table 2. Agreement was good to excellent for all signs except wall thick-

ening and perfusion defect. Disagreement occurred with respect to wall thickening in three patients, perfusion defect in four, dense mucosa in one, mucosal sloughing in one, pericolic stranding in one, and abnormal gas in one patient. Consensus was generally achieved following an open discussion and the opinion of a third radiologist when necessary.

DISCUSSION

Stercoral ulcer with perforation was first described by Berry in 1894, and to date, fewer than 150 cases have been reported^[4]. The incidence of perforated stercoral ulcer at autopsy ranges from 0.04% to 2.3%. Pre-mortem diagnosis is even less frequent, which suggests that the incidence of this condition is often underestimated^[3]. One study reported that stercoral perforation of the colon was found in 0.5% of all surgical colorectal procedures, 1.2% of all emergency colorectal procedures, and 3.2% of all colonic perforations^[5].

Fecal impaction and perforation occur most often in the sigmoid colon. The sigmoid colon is the narrowest region of the entire colon, and passage of stools with a more solid consistency can be difficult. In such cases, fecaloma exerts localized pressure on the walls of the sigmoid colon, the area with the most precarious vascular supply^[6], especially the vascular region known as Sudeck's point. Prolonged localized pressure and ischemia can give rise to pressure ulceration^[7,8].

Distention predisposes the colon to insufficient perfusion, leading to slight, moderate, or severe ischemic lesions^[9]. Ischemic colitis will occur when intraluminal pressure exceeds 35 cm H₂O for hours^[10]. Maurer *et al*^[5] have postulated that colonic dilatation and the presence of multiple fecalomas indicate additional stercoral ulceration and carry the risk of secondary perforation. This view was supported by Huang *et al*^[11] by visualization of stercoral ulceration during intraoperative colonoscopy.

Chronic constipation ($n = 8$) and systemic disease (90%, $n = 9$) were the common clinical problems of the patients in this study, some of them (50%, $n = 5$) presented with multiple necrotic foci involving long segmental bowel that spanned the territory of the superior and inferior mesenteric arteries. It is probable that long-term systemic disease weakens the colon, while stool impaction causes bowel dilatation and increases wall tension, which worsens perfusion insufficiency and leads eventually to necrosis and potentially to fatal perforation. Unfortunately, the early clinical signs such as fever (20%, $n = 2$), peritoneal signs (20%, $n = 2$), and leukocytosis (70%, $n = 7$) are insufficient to diagnose this severe condition in order to prompt appropriate intervention in these patients.

Obstructive colitis differs from colonic cancer with marginal ulceration at aspects of normal mucosa distal to cancer and, frequently, centimeters immediately proximal to the carcinoma are free of ulceration and inflammation^[12]. As an example of this, NSC was diagnosed in our case number 4.

Fecal impaction was present in all our patients and was

located mostly at the sigmoid colon (90%, $n = 9$), which was highly correlated with surgical findings and a result which agrees with other studies. Proximal dilatation was observed in two patients (20%), and was less frequent than we expected. It is possible that the colon could have ruptured prior to the CT scan, thus relieving the luminal pressure. This could also be due to the possible fulminant course which did not allow time for the colon to dilate. None of these two patients with proximal colon dilatation showed abnormal gas that would have indicated whether the colon had ruptured. Probably owing to absence of proximal colon dilatation in NSC, clinicians underestimate the stool impaction.

Colon wall thickening (60%, $n = 6$) is an indicator of stercoral colitis caused by edema or acute inflammation. Dense mucosa as a result of mucosal hemorrhage has been reported to be a sign of ischemic bowel^[13,14]. This was one of the most frequently observed signs of NSC and occurred in 62.5% (5 of 8) of our cases. Mucosal sloughing (10%, $n = 1$) and perfusion defect (70%, $n = 7$) indicated status of ischemia progressing to infarct of the colon. The radiologists' disagreement over wall thickening and perfusion defect may have been the result of subtle and localized changes. These findings indicate that the CT signs of NSC are not obvious, and that radiologists must be aware of the signs to make an early diagnosis. Pericolic fat stranding was the most frequent CT sign of NSC observed in our patients (8 of 10, 80%). Intraoperative findings indicated that pericolic fat stranding was the result of pericolic inflammation and edema. The pericolic reaction was most likely the cause of the intolerable abdominal pain experienced by these patients.

NSC with abnormal gas (50%, $n = 5$) often appears on CT scans as small gas bubbles in the proximity of the colon wall: pneumo-intestinalis coli or pneumo-mesocolon. This is usually undetected by radiography and differs from gastroduodenal perforation that usually presents massive pneumoperitoneum. Intraoperative findings indicate that the perforation can be temporarily plugged by a fecaloma. In our sample, a standing radiograph was not often obtained, partly because pneumoperitoneum was not suspected clinically, and the elderly patients were usually in a weakened state that impeded their assuming a standing posture. Pneumo-mesocolon was not always evident on the radiograph because it was obscured by the presence of a lot of fecal material in the abdomen. This explains why only one (20%) of five cases with abnormal gas was detected by radiography. Thus, abdominal CT, with meticulous searching for signs of abnormal gas, is required. Pericolic abscess formation was seen in two (20%) of our patients. When the NSC is perforated, the viscous nature of the fecal material causes it to further impede the peritoneum with soiling.

NSC differs from other colitis by absence of diarrhea clinically. It can be confirmed by intraoperative and histological findings^[5]. At surgery, stercoral ulcers and perforations are usually found on the anti-mesenteric side; ulcerations usually have sharp margins and measure 1-10 cm, and are occasionally multiple. Histological findings include

sharp demarcation without undermining at ulcer margins, and transmural necrosis at the perforated site. Treatment is usually resection of the affected bowel, colostomy, and Hartmann's procedure^[1,5].

Typically, only the more severe cases in this sample would have been discussed at the conference, and this resulted in a high mortality rate among our patients (70%; 7/10, which is higher than previously reported^[1]).

In the elderly and in nursing home patients, ascites associated with liver cirrhosis or malnutrition is often encountered. This could obscure the significance or specificity of pericolic fluid accumulation for colonic pathology. Thus, we did not investigate this factor for NSC.

This retrospective study consisted of a small population of patients with NSC; thus, the statistical significance and likelihood ratios of each CT sign for NSC could not be determined appropriately. Owing to the nature of the retrospective study, some important clinical data and imaging were unavailable. This study aimed to alert clinicians to the CT findings of NSC, a potentially fatal condition. A further study with a larger number of patients is needed to validate the accuracy of our CT findings.

In summary, elderly patients with a history of chronic constipation and systemic disease, presenting with fecal impaction and acute abdomen with indeterminate leukocytosis, are at risk of NSC. CT is justified to be suggested to investigate the possibility of NSC. Pericolic stranding, perfusion defect and dense mucosa were the most sensitive CT measures for NSC, detecting about 80%, 70%, and 62.5% of the cases, respectively. Awareness of NSC and familiarity with these CT signs enables us to make a differential diagnosis between this fatal condition and benign stool impaction.

COMMENTS

Background

In clinical practice, fecaloma-related necrotic stercoral colitis (NSC) is an infrequently and easily overlooked disease. It frequently occurs in patients who are elderly or have inactive status. High mortality is encountered when perforation takes place.

Research frontiers

In fact, most fecal impaction is usually relieved by non-invasive management, meaning that surgical and pathologic evidence for NSC has been unobtainable. In this era of liberal computed tomography (CT) use for patients with acute abdomen in emergency departments, few articles about CT of stercoral colitis have been published.

Innovations and breakthroughs

Over the last few years, the authors have collected ten patients with clinical,

surgical, pathologic and radiographic evidence of NSC. In practice, clinical clues alone are insufficient to exclude the disease. Characteristic CT presentations are useful to delineate this colon pathology, especially dense mucosa, pericolic stranding, perfusion defect, and mucosal sloughing of colon.

Peer review

The paper is an interesting one, but it needs major language refinement and some additional information to be provided. I then would suggest acceptance for publication, provided satisfactory revision requirements are met.

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S- Editor Sun H L- Editor Logan S E- Editor Zheng XM

Management of patients with sphincter of Oddi dysfunction based on a new classification

Jia-Qing Gong, Jian-Dong Ren, Fu-Zhou Tian, Rui Jiang, Li-Jun Tang, Yong Pang

Jia-Qing Gong, Jian-Dong Ren, Fu-Zhou Tian, Li-Jun Tang, Yong Pang, Department of General Surgery, the People's Liberation Army General Hospital of Chengdu Command, Chengdu 610083, Sicuan Province, China

Rui Jiang, Department of Radiology, General Hospital of Chengdu Command, Chengdu 610083, Sicuan Province, China

Author contributions: Gong JQ performed the research, analyzed the data and wrote the paper; Ren JD analyzed the data and wrote the paper; Tian FZ played a leading role in this research and designed the research; Jiang R, Tang LJ and Pang Y helped collect and analyze the data.

Supported by The Health and Medical Research Council of the People's Liberation Army, China, No. 08Z012

Correspondence to: Fu-Zhou Tian, Professor, Department of General Surgery, the People's Liberation Army General Hospital of Chengdu Command, Chengdu 610083, Sicuan Province, China. cdgj123@yahoo.com

Telephone: +86-28-86570621 Fax: +86-28-86570351

Received: August 30, 2010 Revised: September 26, 2010

Accepted: October 3, 2010

Published online: January 21, 2011

cases of double-duct-type SOD, which consisted of 7 (36.8%) patients who were cured endoscopically and 12 (63.2%) who were cured surgically. A total of 41 cases were diagnosed as biliary-pancreatic-reflux-type SOD. Twenty (48.8%) of them were treated endoscopically, 16 (39.0%) were treated by open abdominal surgery, and 5 (12.2%) were under observation.

CONCLUSION: The newly proposed SOD classification system introduced in this study better explains the clinical symptoms of SOD from the anatomical perspective and can guide clinical treatment of this disease.

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Key words: Sphincter of Oddi dysfunction; Classification; Diagnosis; Treatment

Peer reviewer: Dr. Abdul-Wahed Meshikhes, MD, FRCS, Chairman and Consultant Surgeon, Department of Surgery, King Fahad Specialist Hospital, Amir Bin Thabit St, Dammam, 31444, Eastern Province, Saudi Arabia

Abstract

AIM: To propose a new classification system for sphincter of Oddi dysfunction (SOD) based on clinical data of patients.

METHODS: The clinical data of 305 SOD patients documented over the past decade at our center were analyzed retrospectively, and typical cases were reported.

RESULTS: The new classification with two more types (double-duct, biliary-pancreatic reflux) were set up on the basis of the Milwaukee criteria. There were 229 cases of biliary-type SOD, including 192 (83.8%) cases cured endoscopically, and 29 (12.7%) cured by open abdominal surgery, and the remaining 8 (3.5%) cases observed with unstable outcomes. Eight (50%) patients with pancreatic-type SOD were cured by endoscopic treatment, and the remaining 8 patients were cured after open abdominal surgery. There were 19

Gong JQ, Ren JD, Tian FZ, Jiang R, Tang LJ, Pang Y. Management of patients with sphincter of Oddi dysfunction based on a new classification. *World J Gastroenterol* 2011; 17(3): 385-390 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/385.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.385>

INTRODUCTION

Sphincter of Oddi dysfunction (SOD) is characterized by a series of clinical pain symptoms caused by abnormalities in sphincter contractility^[1]. The sphincter of Oddi (SO), a fibromuscular sheath encircling the distal common bile duct (CBD), pancreatic duct and common channel, controls the flow of bile and pancreatic secretions into the duodenum and prevents reflux of duodenal contents into the pancreaticobiliary system^[2]. SO dyskinesia caused by injury or/and inflammation can result in a hypo- or

hypertonic sphincter with altered motility, causing an intermittent functional blockage of the sphincter. As it is often difficult to distinguish SO stenosis from dyskinesia, the term “sphincter of Oddi dysfunction” is used to cover both conditions^[3]. For SOD, the Milwaukee classification proposed by Hogan and Geenen has been widely accepted; it classifies SOD into two types: the biliary type and the pancreatic type^[4,6]. Biliary and pancreatic SOD are each sub-classified as type I, II and III on the basis of symptoms, laboratory tests and radiological imaging^[4,5]. This classification explains the clinical manifestations of SOD from the anatomical perspective and reveals the nature of SOD, thus providing a reliable anatomical basis for appropriate clinical treatment. In our long-term clinical practice, however, we have found this classification system somewhat flawed. For instance, the clinical manifestations in some patients with SOD are difficult to be satisfactorily defined using the Milwaukee criteria. Therefore, the existing system is insufficient in guiding the clinical treatment of all SOD patients. In this paper, we introduced a new classification of SOD based on the Milwaukee classification. In the new classification system, SOD is divided into four types according to anatomy, symptoms, endoscopic tests and radiological imaging. These types include not only those already established by the Milwaukee criteria (biliary and pancreatic types), but also two more new types (the double-duct and biliary-pancreatic reflux types) in an attempt to improve the clinical treatment of SOD.

MATERIALS AND METHODS

Patient data

A total of 1013 patients underwent endoscopic retrograde cholangiopancreatography (ERCP) from January 1999 to January 2009, and 305 (30%) patients who met the diagnostic criteria of SOD were included in this study. In the 305 SOD patients, there were 198 men and 107 women, aged from 36-79 years (mean age, 47.5 years) (Table 1). All the patients had to meet the following diagnostic criteria for SOD: (1) typical clinical symptoms, with or without abnormal liver function and amylase levels^[4,5]; and (2) baseline sphincter of Oddi manometry (SOM) pressure > 40 mmHg^[7], and/or paradoxical pressure response to cholecystokinin^[8], and/or delayed emptying of contrast medium in the common bile duct as indicated by ERCP^[9], and/or a diameter of common bile duct ≥ 10 mm without evidence of stones or tumors^[6]. Preliminary classification was performed based on the development of the disease, examinations and treatment, e.g. ERCP, magnetic resonance cholangiopancreatography (MRCP), endoscopic sphincterotomy (EST) and endoscopic pancreatic duct sphincterotomy (EPS). Some patients were ultimately classified according to the findings of surgical exploration. Intraoperative choledochography, choledochoscopy and pancreatoscopy were performed if necessary.

It may still take a long time to establish the diagnosis for double-duct type and biliary-pancreatic reflux type SOD. For SOD of the double-duct type, both the biliary and pancreatic ducts of the sphincter of Oddi are affected. So, the

Table 1 Clinical features of sphincter of Oddi dysfunction patients

Clinical features	n (%)
Abdominal pain	
Retrosternal pain	235 (77.05)
Epigastric pain	70 (22.95)
Radiating pain	45 (14.75)
Timing of onset	
After high-fat meals	241 (79.02)
After ordinary meals	21 (6.89)
Uncertain	43 (14.10)
Jaundice	
With	21 (6.89)
Without	284 (93.11)
Liver enzyme levels	
Abnormal	58 (19.02)
Normal	247 (80.98)
Serum amylase	
Abnormal	63 (20.66)
Normal	242 (79.34)
Cholangiectasis	
≥ 10 mm	289 (94.75)
< 10 mm	16 (5.25)
Pancreatic duct dilation	76 (24.92)

biliary pain and pancreatic pain may occur simultaneously or alternately. For SOD of the biliary-pancreatic reflux type, the patients may have a congenital abnormality in the convergence of the biliary and pancreatic systems. When the ampullary anti-reflux valve is dysfunctional, small amounts of bile may repeatedly reflux into the pancreatic duct, which often induces pancreatitis. That is why sometimes an over long common duct of the biliary and pancreatic duct is found in auxiliary examination and operation.

Therapeutic methods

The therapeutic methods and results in patients with different types of SOD are shown in Table 2.

Typical cases

Case 1: A 43-year-old man first suffered from pancreatitis in November 2004. Three months later, his pancreatitis recurred, and B-mode ultrasonography suggested cholecystolithiasis. The patient suffered from recurrent pains in the upper abdomen six months after cholecystectomy performed in March 2005 due to clinically diagnosed biliary pancreatitis. Computed tomography (CT) suggested swelling of the pancreatic head and pancreatic duct distension, accompanied by stones. Moreover, the common bile duct had a diameter of 8 mm. With the duodenum preserved, the patient underwent resection of the swollen head of the pancreas, which was assumed to be compressing the bile duct, resulting in bile duct distension before the surgery. However, three months after surgery, the patient began to experience biliary colic with a frequency of 2-3 times per month. Laboratory tests showed normal blood amylase levels and aggravated liver dysfunction with alkaline phosphatase increasing from 579 to 1858 IU/L and glutamyl transpeptidase from 658 to 2006 IU/L, accompanied by enlargement of the diameter of the common bile duct

Table 2 Detailed classification, treatment and outcomes associated with sphincter of Oddi dysfunction

	<i>n</i> (%)
SOD biliary type	229/305 (75.08)
EST preferred	213/229 (93.01)
Improved	202/213 (94.83)
Good	182/202 (90.10)
Moderate	10/202 (4.95)
Poor	10/202 (4.95)
Failed	11/213 (5.16)
Exploratory laparotomy	29/229 (12.66)
Choledochointestinal anastomosis	21/29 (72.41)
Sphincter of Oddi plasty	8/29 (27.59)
Conservative treatment	8/29 (27.59)
Pancreatic type SOD	16/305 (5.25)
EPS preferred	13/16 (81.25)
Improved	10/13 (76.92)
Good	8/10 (80.00)
Moderate	2/10 (20.00)
Failed	3/13 (23.08)
Pancreatojejunal anastomosis	8/16 (50.00)
Double-duct type SOD	19/305 (6.23)
EST preferred	11/19 (57.89)
Improved	7/11 (63.64)
Failed	4/11 (36.36)
Exploratory laparotomy	12/19 (63.16)
Choledocho-, pancreato-intestinal anastomosis	7/12 (58.33)
Pancreatic head resection, choledocho-intestinal anastomosis	1/12 (8.33)
Duodenopancreatectomy	1/12 (8.33)
Biliary sphincterotomy, pancreato-intestinal anastomosis	3/12 (25.00)
Biliary-pancreatic reflux type SOD	41/305 (13.44)
EST preferred	34/41 (82.93)
Improved	20/34 (58.82)
Failed	14/34 (41.18)
Laparotomic BPD	16/41 (39.02)
Conservative treatment	5/41 (12.16)

SOD: Sphincter of Oddi dysfunction; EST: Endoscopic sphincterotomy; EPS: Endoscopic pancreatic duct sphincterotomy; BPD: Biliopancreatic diversion.

from 8 to 14 mm. Endoscopic bile duct sphincterotomy was performed twice in three months but without satisfactory curative effect. Consequently, an open abdominal surgery was performed at our center for frequent biliary colic. The common bile duct was transected, and then a cholangiojejunostomy was performed. During the two-year follow-up, liver function returned to normal levels and no further abdominal pain was reported by the patient.

Case 2: A 41-year-old man was diagnosed as having acute pancreatitis in June 2001 due to pains in the upper abdomen and an increase in blood amylase levels. The patient was discharged one week later. In October 2001, the patient experienced abdominal pain again and was diagnosed as having pancreatitis from a swollen pancreas head according to CT examination. In addition, B-mode ultrasonography detected small stones in the gallbladder. Laparoscopic cholecystectomy was performed in February 2002. Pancreatitis recurred dozens of times during the period from Febru-

ary 2002 to August 2003. Accordingly, emergent EST was then carried out, with subsequent intraoperative findings including a swollen ampulla of Vater, duodenal hyperemia, and mucosal edema. No bile flow was observed within the ampulla of Vater after a 10-mm incision was cut. This suggested that EST had failed, and ERCP could not be performed. In November 2003, EST was performed again when duodenal hyperemia and edema subsided. ERCP was successful, which indicated a narrow and flexuous common duct of the bile and pancreatic ducts. After a 15-mm incision in the ampulla of Vater was executed, bile flow was observed; however, it was still difficult to insert a tube into the bile duct. Even though bile samples were collected occasionally from the hepatic portal area through a tube for amylase measurement, the amylase level was still as high as 7600 U/L, demonstrating that the previous EST had failed to prevent pancreatitis. During a period of one year after the second EST, pancreatitis recurred eight times in this patient, indicating the failure of the second EST. Biliary-pancreatic shunting was then performed based on evidence of biliary-pancreatic reflux. In January 2005, the patient received transection of the common bile duct and choledochointestinal anastomosis. During the operation, the bile duct pressure was measured to be 12 cmH₂O, and the bile amylase level was 690 U/L. In addition, pancreatoscopy revealed an 11-mm stenotic segment of the common duct after two ESTs, and the common duct was tortuous, which may lead to biliary-pancreatic reflux. During the two-year follow-up, pancreatitis did not recur. Moreover, a CT scan performed one year after the last surgery showed obvious shrinkage of the swollen pancreatic head, indicating an effective relief of the edema.

RESULTS

Based on our clinical observations, we added the double-duct type and the biliary-pancreatic reflux type of SOD to the Milwaukee classification, to achieve greater clarity in the SOD clinical symptoms and to guide the clinical treatment of SOD. The therapeutic methods and effects using this new classification are shown in Table 3.

Biliary type SOD

Among the 305 patients, 229 (75.1%) definitively demonstrated biliary-type SOD, and 6 of them were not eligible for EST due to a diverticulum near the ampulla of Vater; these patients were treated by open abdominal surgery. Of the 213 patients who underwent EST, 202 (94.8%) were successfully treated, but 10 (4.9%) of these patients had unsatisfactory therapeutic effects after EST, with biliary colic and bile duct distension. EST failed in 11 (5.2%) patients. Hence, a total of 21 patients had unsatisfactory therapeutic effects or EST failure. In addition to the 16 patients for whom open abdominal surgery was initially prescribed, only 29 patients actually underwent surgery (choledochointestinal anastomosis in 21 patients and sphincteroplasty in 8 patients) and had good therapeutic effects. The other 8 patients underwent conservative treatment, but showed no stable therapeutic effects.

Table 3 Clinical classification and features of sphincter of Oddi dysfunction

Features	Bile duct type (type I)	Pancreatic duct type (type II)	Double-duct type (type III)	Biliary-pancreatic reflux type (type IV)
Abdominal pain	Retrosternal pain	Epigastric pain	I + II	Same as type II
Radiating pain	The central point of the back	The left back or indefinite site	I + II	Same as type II
Timing of onset	After high-fat meals	After ordinary meals	I + II	At night or in the morning
Jaundice	±	-	±	-
Liver enzyme levels	±	-	+	±
Serum amylase levels	-	+	±	+
Bile duct distension	≥ 10 mm	< 10 mm	≥ 10 mm	≥ 10 mm
Pancreatic duct distension	-	++	+	+
Anterograde cholangiography				
Voiding time	> 10 min	Normal	> 10 min	Unexpected pancreatic duct visualization

Pancreatic type SOD

There were 16 (5.3%) cases of the pancreatic type of SOD, and 3 of them had multiple stones that were difficult to remove thoroughly because they were located deep in the pancreatic duct. Therefore, EPS was not indicated for these 3 patients. The other 13 cases were first treated by EPS, but the treatment failed in 3 cases (a success rate of 76.9%). Hence, a total of 6 cases were managed with pancreatico-jejunostomy subsequently. The therapeutic effect was satisfactory in these cases. The other 10 patients treated by EPS (5 patients with pancreatic duct stent placement) were followed up for more than two years. Satisfactory therapeutic effects were exhibited in 8 (73%) patients, and the other 2 patients were then treated by subsequent open abdominal surgery. These cases suggest that open abdominal surgery may be helpful for treating SOD regardless of whether pancreatic duct stones are involved.

Double-duct type SOD

There were 19 (6.2%) cases of double-duct type SOD, in which both the bile duct and the pancreatic duct of the sphincter of Oddi were affected. Among them, 11 patients were treated by EST, and 7 (63.6%) were cured. The remaining 8 cases were treated surgically. A total of 12 cases were treated by open abdominal surgery. Seven patients underwent choledocho-intestinal anastomosis plus pancreato-intestinal anastomosis, and one was treated with a resection of the pancreatic head with the duodenum preserved, followed by choledocho-intestinal anastomosis. Duodenopancreatectomy was performed on one patient. The other three received biliary sphincterotomies by EST and pancreato-intestinal anastomosis to treat distension of the pancreatic duct. Follow-up results revealed good curative effects in these cases.

Biliary-pancreatic reflux type SOD

Forty-one cases (13.4%) of SOD met the profile of the biliary-pancreatic reflux type, and EST was performed in 34, with a success rate of 58.8% (20/34). Surgical treatment was preferentially carried out for 2 cases, and then, a total 16 cases underwent laparotomic biliopancreatic diversion (BPD). During a follow-up period of 0.5-7 years, 14 patients showed satisfactory therapeutic effects, and in the other 2 cases, sclerotic changes were observed throughout

the pancreas. After surgery, abdominal pain occurred occasionally and was significantly mitigated in these 2 cases. Five cases without receiving any treatment are under observation, and their symptoms still often occurred.

DISCUSSION

Although the Milwaukee classification system has been widely accepted for the classification of patients with suspected SOD, it has some potential problems. For example, the description of typical biliary or pancreatic pain may be interpreted differently by different doctors, which may lead to inappropriate referrals for SOM. In addition, according to the Milwaukee criteria, a CBD diameter of at least 12 mm is required for the diagnosis of SOD. Most patients being investigated for SOD have had their gallbladder removed, and in the past, it was believed to be normal for a post-cholecystectomy CBD to be dilated by 2-3 mm^[10]. Moreover, there is a question of whether patients with both biliary and pancreatic pain should be classified into the biliary type or the pancreatic type. Freeman *et al*^[11] stated that for this group, all patients should undergo biliary sphincterotomy, and 40% should have pancreatic sphincterotomies. How to interpret these clinical data? The Milwaukee classification has some limitations. In our study, there were 60 cases that could not be accurately interpreted by the Milwaukee classification criteria. Our long-term observation of the clinical cases suggests that our new classification based on anatomy, symptoms, endoscopic tests and radiological imaging is superior to the Milwaukee criteria in guiding the treatment of SOD. According to the Milwaukee criteria, the two types of SOD (biliary and pancreatic) can be further classified into three subtypes each, making classification complex. The newly proposed classification of SOD and the clinical characteristics associated with each type are listed in Table 3.

The new classification system presented in this paper is simpler than the initial one, but continues to closely follow the Milwaukee classification criteria. For example, biliary-type SOD patients only have biliary pain, and pancreatic-type SOD patients only have pancreatic pain. The two types are no longer divided into subtypes. MRCP usually shows the distension of bile ducts for biliary SOD and dilation solely of the pancreatic duct for pancreatic-

type SOD. With respect to treatment, EST can often yield better results for patients suffering from biliary SOD, and EPS should be a good choice of treatment for patients with pancreatic SOD.

We have paid more attention to the clinical characteristics and significance of the other two types of SOD, i.e. the double-duct and biliary-pancreatic reflux types.

In double duct type of SOD, both the biliary and pancreatic ducts of the sphincter of Oddi are affected. Clinical cases of SOD meeting these criteria have previously been reported but have not been definitively classified^[12]. The characteristics of this SOD type include symptoms typical of both biliary- and pancreatic-type SOD that appear simultaneously or alternately, with mobile positions of abdominal pain and radiating pain. Meanwhile, laboratory tests indicate elevated levels of liver-related enzymes and amylase in the blood. These findings usually result in the diagnosis of chronic biliary pancreatitis, manifested as mild abnormal liver function due to edema of the pancreatic head. Imaging exams usually show distension of both the bile and pancreatic ducts and stones in the pancreatic duct. Notably, frequently recurrent pancreatitis and evident distension of the pancreatic duct could mislead surgeons to focus on pancreatitis, thus underestimating the severity of mild bile duct distension, causing the neglect of possible SOD diagnoses, and resulting in unsuccessful treatment. Therefore, it is necessary to outline the double-duct type of SOD so that patients with these symptoms can be effectively treated. Case 1 is a typical SOD of the double-duct type, with mild bile duct distension due to sphincter of Oddi stenosis, and not due to compression by the head of the pancreas. This was proved by the finding that the obstruction in the extremity of the bile duct was not relieved even when the head of the pancreas was resected in the first operation. In patients with the double-duct type SOD, the sphincter of common duct is short, but the inferior stenotic segment of the bile duct is relatively long. Hence, patients with this form of SOD can be treated by surgery rather than EPS.

The anatomical basis for biliary-pancreatic reflux type of SOD is probably as follows: Patients may have a congenital abnormality in the convergence of the biliary and pancreatic systems, i.e. an overlong duct (> 11 mm). Inflammation and stenosis mainly occur in the sphincter of the common duct, whereas the sphincters of the superior bile duct and the pancreatic duct remain relatively normal or only mildly affected. If fibrosis of the ampullary septum causes dysfunction in the anti-reflux valve, reflux between the bile and pancreatic ducts is likely to occur. Repeated reflux of small amounts of bile into the pancreatic duct will usually induce pancreatitis, including recurrent chronic biliary pancreatitis and even severe acute pancreatitis. Although the clinical manifestations of this SOD are quite similar to those of the pancreatic duct type, there are a few differences: (1) As the reciprocal reflux between the fluids in the bile and pancreatic ducts is structurally barrier-free, the pancreatic duct orifice has no evident stenosis or obstruction, and so distension of the pancreatic duct does not occur; (2) Because there was only mild biliary-pancreatic reflux in most cases, the pancreatic duct and

gland alveoli in the head of the pancreas near the biliary-pancreatic convergence were usually affected, inducing swelling of the pancreatic head, as indicated by imaging examination. Therefore, these cases are sometimes diagnosed as pancreatitis with a pancreatic head mass. Some authors have reported tumor-like features of chronic pancreatitis, and some of these cases may suffer from biliary-pancreatic reflux^[13,14]; (3) In this type of SOD, EST failed to incise the stenotic segment of the sphincter of Oddi due to the slender and tortuous common duct. EST was successful in only 56% of cases with this type of SOD at our center. However, the achievement ratio of incision on the normal ampulla of Vater was nearly 100%^[15,16]. Moreover, some patients can only be treated by open abdominal surgery due to failure to incise the stenotic common duct after several attempts by EST; and (4) The amylase levels in bile sampled from the biliary tract during ERCP in biliary-pancreatic-reflux-type SOD patients were about 2-10 times higher than normal.

Although distension does not always occur in the pancreatic duct and is usually slight in the biliary duct in the early stages of biliary-pancreatic reflux SOD, the symptoms of pancreatitis may be more serious than those in the simple pancreatic-type SOD. Because the common duct of the bile and pancreatic ducts is simply a potential duct due to the tension of the sphincter of Oddi, an image of an over long common duct was obtained from ERCP or MRCP in only a few cases. Case 2 mentioned above illustrates that the therapeutic effects of BPD were satisfactory for the biliary-pancreatic-reflux-type SOD patients who failed in EST. This demonstrates the significance of using the biliary-pancreatic reflux type classification for SOD.

In summary, the new classification of SOD proposed in this study demonstrates significant advantages for guiding the diagnosis and treatment of SOD patients in China, as compared with the conventional Milwaukee criteria. Nonetheless, further investigations on the applicability of this quaternary classification system to patients in other regions are needed.

COMMENTS

Background

Sphincter of Oddi dysfunction (SOD) is a pathological syndrome that is usually classified into the biliary type or the pancreatic type according to the Milwaukee criteria. However, this classification has some drawbacks in clinical practice, some of which result in flawed classification and failure to properly guide diagnosis and treatment.

Research frontiers

The conventional SOD classification system is insufficient in guiding the clinical treatment of all SOD patients. The authors conducted a retrospective analysis of 305 patients with SOD according to the clinical records in the past 10 years, and proposed a modified classification system based on the Milwaukee classification, which includes all SOD symptoms.

Innovations and breakthroughs

The authors proposed a new classification system for SOD according to anatomy, symptoms, endoscope tests and radiological imaging, i.e. SOD is divided into four types instead of two types: the biliary-type, the pancreatic-type, the double-duct type and the biliary-pancreatic reflux type. The new classification demonstrates significant advantages for guiding the diagnosis and treatment of SOD patients in China, as compared with the conventional Milwaukee criteria.

Applications

The new classification system has significant advantages for guiding the diagnosis and treatment of SOD patients, thus improving the clinical treatment of SOD.

Peer review

In order to solve some problems of the Milwaukee classification of SOD, the authors have proposed a new interesting classification. Compared with the Milwaukee classification, the new classification system presented in this paper is simpler than the Milwaukee classification, better explains clinical symptoms of the disease from the anatomical perspective, and should have some application values in guiding the diagnosis and treatment of SOD.

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S- Editor Sun H L- Editor Ma JY E- Editor Lin YP

Effect of preoperative biliary drainage on malignant obstructive jaundice: A meta-analysis

Yu-Dong Qiu, Jian-Ling Bai, Fang-Gui Xu, Yi-Tao Ding

Yu-Dong Qiu, Fang-Gui Xu, Yi-Tao Ding, Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital, Medical School, Nanjing University, Nanjing 210008, Jiangsu Province, China
Jian-Ling Bai, Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Author contributions: Qiu YD and Xu FG reviewed the articles and extracted data for further analysis; Bai JL performed the meta-analysis in accordance with the Cochrane Handbook for Systematic Reviews of Interventions; Qiu YD and Ding YT designed the study and wrote the manuscript.

Supported by Key Medical Center for Hepatobiliary Disease of Jiangsu Province, No. ZX200605

Correspondence to: Yi-Tao Ding, MD, Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital, Medical School, Nanjing University, No. 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China. yitaodding@hotmail.com

Telephone: +86-25-83304616 Fax: +86-25-83317016

Received: July 13, 2010 Revised: September 27, 2010

Accepted: October 4, 2010

Published online: January 21, 2011

Abstract

AIM: To evaluate the effect of preoperative biliary drainage (PBD) on obstructive jaundice resulting from malignant tumors.

METHODS: According to the requirements of Cochrane systematic review, studies in the English language were retrieved from MEDLINE and Embase databases from 1995 to 2009 with the key word "preoperative biliary drainage". Two reviewers independently screened the eligible studies, evaluated their academic level and extracted the data from the eligible studies confirmed by cross-checking. Data about patients with and without PBD after resection of malignant tumors were processed for meta-analysis using the Stata 9.2 software, including postoperative mortality, incidence of postoperative pancreatic and bile leakage, abdominal abscess, delayed gastric emptying and incision infection.

RESULTS: Fourteen retrospective cohort studies involving 1826 patients with malignant obstructive jaundice accorded with our inclusion criteria, and were included in meta-analysis. Their baseline characteristics were comparable in all the studies. No significant difference was found in combined risk ratio (RR) of postoperative mortality and incidence of pancreatic and bile leakage, abdominal abscess, delayed gastric emptying between patients with and without PBD. However, the combined RR for the incidence of postoperative incision infection was improved better in patients with PBD than in those without PBD ($P < 0.05$).

CONCLUSION: PBD cannot significantly reduce the postoperative mortality and complications of malignant obstructive jaundice, and therefore should not be used as a preoperative routine procedure for malignant obstructive jaundice.

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Key words: Malignant obstructive jaundice; Preoperative biliary drainage; Meta-analysis; Mortality; Incidence of complications

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Qiu YD, Bai JL, Xu FG, Ding YT. Effect of preoperative biliary drainage on malignant obstructive jaundice: A meta-analysis. *World J Gastroenterol* 2011; 17(3): 391-396 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/391.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.391>

INTRODUCTION

Surgery for patients with malignant obstructive jaundice carries an increased risk of postoperative complications

and a high mortality rate^[1,2] and preoperative hyperbilirubinemia is considered an important risk factor for postoperative complications and death. Hyperbilirubinemia due to obstructive jaundice damages hepatic function, clearance of circulating endotoxins, coagulation system, immune function, and gastrointestinal barrier^[3-6]. To avoid the poor outcome, preoperative biliary drainage (PBD) has been used to reduce the postoperative morbidity and mortality of these patients. However, PBD has also many drawbacks, such as biliary stent-induced bacterial contamination and risk of cholangitis due to clogging. In addition, biliary stenting generates a severe inflammatory response in the bile duct which may increase the risk of bile leakage at the biliodigestive anastomosis. Since 1970s, several randomized and retrospective studies have compared the effect of PBD and surgery without PBD on malignant obstructive jaundice^[7,8]. However, it is difficult to find evidence that routine PBD improves the outcome of patients with malignant obstructive jaundice in clinical practice.

Despite the scarcity of clinical evidence, most patients with malignant obstructive jaundice undergo either percutaneous transhepatic or internal PBD in many centers. This meta-analysis was to evaluate the effect of PBD on malignant obstructive jaundice.

MATERIALS AND METHODS

Search strategy and selection criteria

Studies on obstructive jaundice in the English language were retrieved from MEDLINE and Embase databases from 1995 to 2009 with the key word “preoperative biliary drainage”. The primary selection criteria for meta-analysis included patients with malignant obstructive jaundice, those with or without PBD, and those with their postoperative mortality and incidence of complications assessed. The exclusion criteria were patients who underwent different surgical procedures, and those with other severe diseases unrelated to obstructive jaundice. The included studies were reviewed by two independent reviewers, with disagreements settled by group discussion.

Data extraction

Data were independently extracted by two investigators in a standard form. The concordance rate between the two investigators was 100%. Following information was extracted from all included publications including study group, year, number of included patients, type of drainage, postoperative mortality, incidence of postoperative pancreatic and bile leakage, abdominal abscess, delayed gastric emptying and incision infection

Analysis of methodological quality

Methodological quality was analyzed as previously described^[8].

Statistical methods

Stata 9.2 software was used in meta-analysis of the data. Effect measures of interest were relative risks for cohort

Table 1 Characteristics of 14 studies included in this study

Study	Yr	Type of drainage	Patients (n)
Hochwald <i>et al</i> ^[9]	1999	Internal and external	71
Martignoni <i>et al</i> ^[10]	2001	Internal and external	30
Pisters <i>et al</i> ^[11]	2001	Internal and external	255
Srivastava <i>et al</i> ^[12]	2001	Internal and external	95
Hodul <i>et al</i> ^[13]	2003	Internal	212
Pešková <i>et al</i> ^[14]	2005	Internal	304
dos Santos <i>et al</i> ^[15]	2005	Internal	53
Tsai <i>et al</i> ^[16]	2006	Internal and external	303
Barnett <i>et al</i> ^[17]	2006	Internal	104
Bhati <i>et al</i> ^[18]	2007	Internal	50
Choi <i>et al</i> ^[19]	2008	Internal and external	49
Ferrero <i>et al</i> ^[20]	2009	Internal and external	60
Abdullah <i>et al</i> ^[21]	2009	Internal and external	82
Li <i>et al</i> ^[22]	2009	Internal and external	140
Total			1826

studies and corresponding 95% CI. Estimates of intervention effect on malignant obstructive jaundice were expressed as relative risks using a fixed effect model. χ^2 test or Fisher's exact test was used to calculate the probability values when appropriate. Pooled effect was estimated using a random-effect model. Publication bias was evaluated by funnel plots and Egger test.

RESULTS

Fourteen retrospective cohort studies that were relevant and eligible were retrieved according to the selection and exclusion criteria (Table 1). Of the 1826 patients with malignant obstructive jaundice included in the studies, 1028 were subjected to PBD and 798 were subjected to surgery but not to PBD. Furthermore, only internal PBD was used in 5 out of the 14 studies, and both internal and external PBD were described in the other 9 studies.

Overall mortality

No significant difference was observed in postoperative death rate reported in 9 studies between patients with or without PBD [risk ratio (RR) = 0.996, 95% CI: 0.669-1.484, Figure 1].

Incidence of postoperative pancreatic leakage

The incidence of postoperative pancreatic leakage was reported in 10 studies (Figure 2A). In our study, PBD intervention did not reduce the incidence of pancreatic leakage in patients without PBD (RR = 0.792, 95% CI: 0.478-1.311).

Incidence of postoperative bile leakage

The incidence of postoperative bile leakage was reported in 10 studies with no significant difference observed between experimental and control groups (RR = 0.935, 95% CI: 0.576-1.518, Figure 2B).

Incidence of postoperative incision infection

The incidence of postoperative incision infection was reported in 9 studies. A significant difference was observed

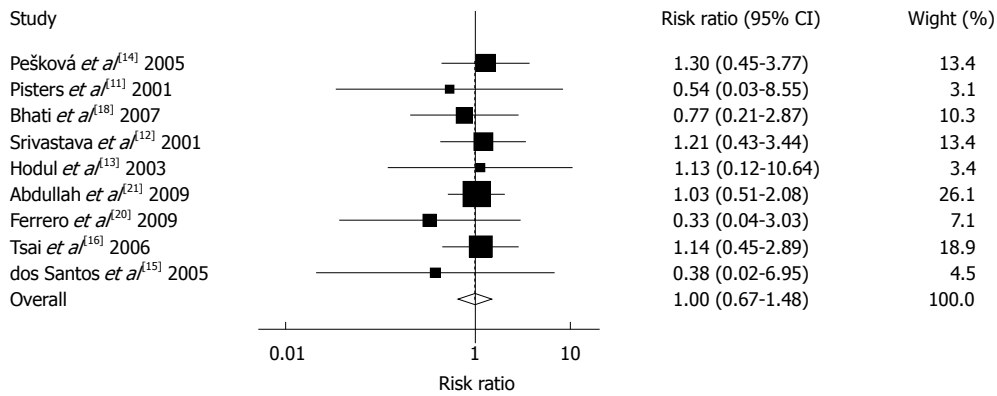
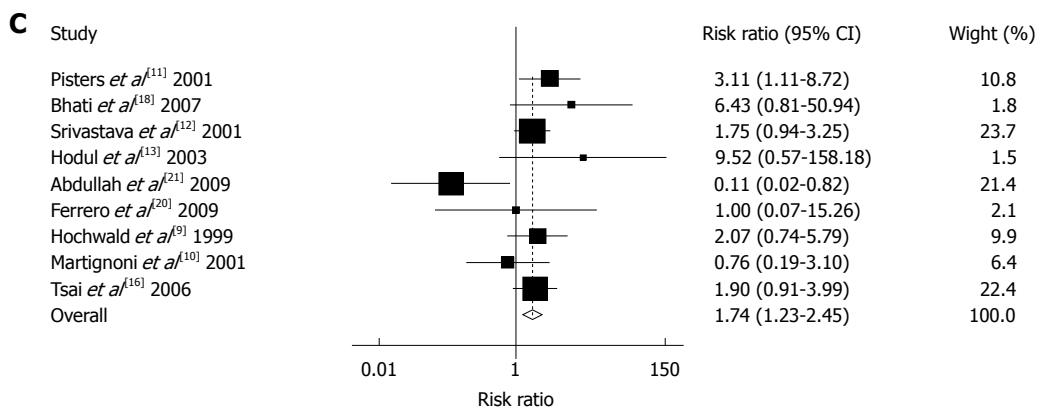
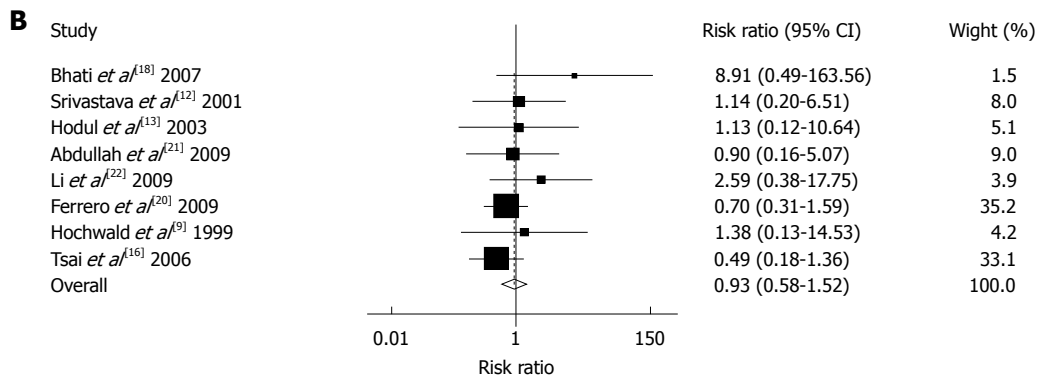
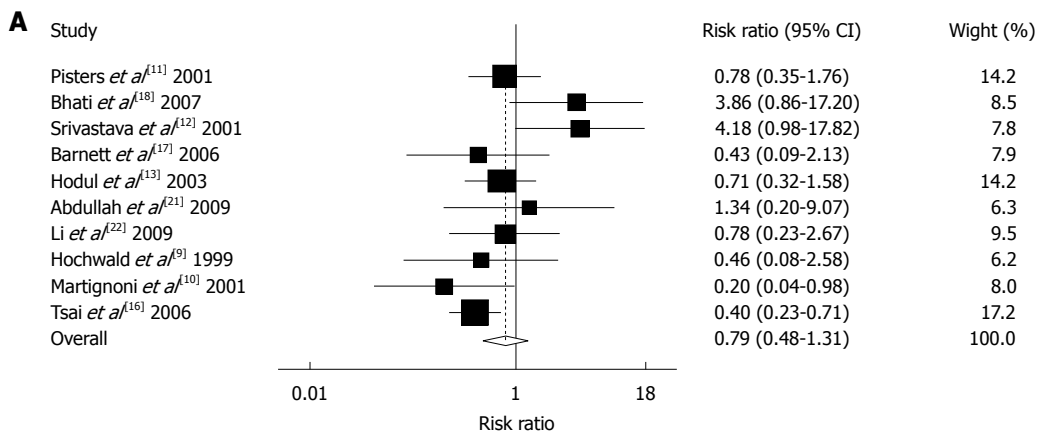


Figure 1 Overall mortality of patients with or without preoperative biliary drainage. Estimates of preoperative biliary drainage (PBD) effects of each study are presented on a log scale along with the 95% CI. The weight of each study is reflected by the size of square. The open diamond represents the global estimate of the PBD effect along with the 95% CI (random-effects model).



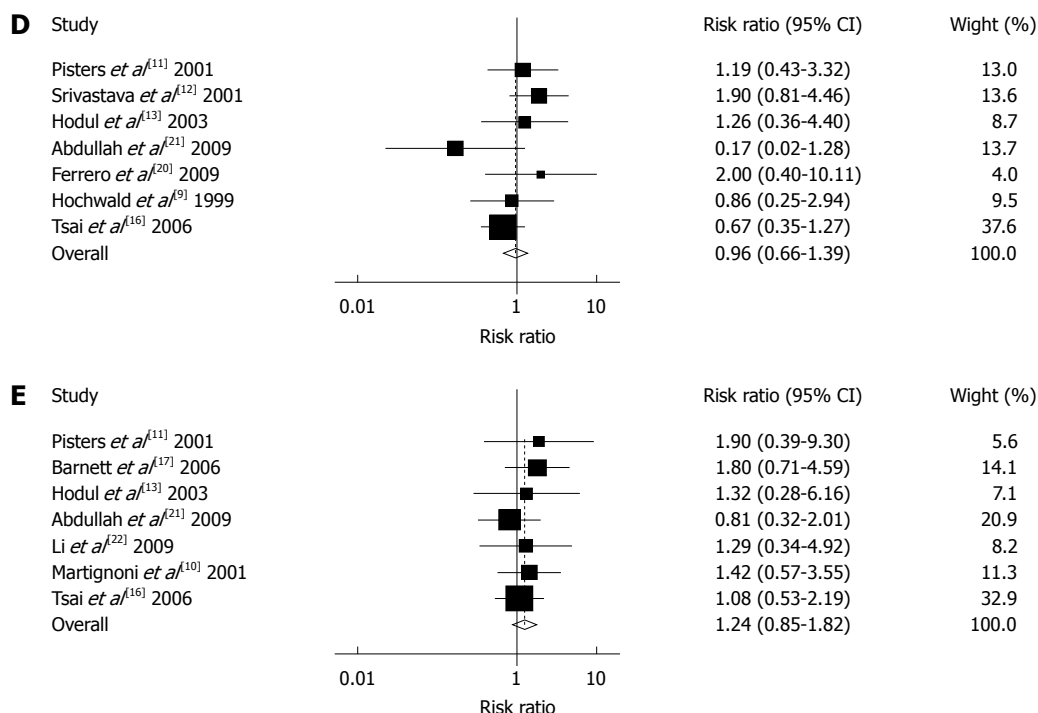


Figure 2 Incidence of postoperative pancreatic leakage (A), postoperative bile leakage (B), postoperative incision infection (C), postoperative abdominal abscess (D), and postoperative delayed gastric emptying (E). Estimates of preoperative biliary drainage (PBD) effect in each study are presented on a log scale along with the 95% CI. The weight of each study is reflected by the size of square. The open diamond represents the global estimate of the PBD effect along with the 95% CI (random-effects model).

between surgical patients with or without PBD (RR = 1.736, 95% CI: 1.229-2.450, $P < 0.05$, Figure 2C).

Incidence of postoperative abdominal abscess

The incidence of postoperative abdominal abscess in patients with or without PBD was reported in 9 studies. PBD could not decrease the postoperative abdominal abscess compared with surgery (RR = 0.957, 95% CI: 0.658-1.392, Figure 2D).

Incidence of postoperative delayed gastric emptying

The incidence of delayed postoperative gastric emptying was reported in 7 studies with no significant difference observed between patients with or without PBD (RR = 1.242, 95% CI: 0.849-1.819, Figure 2E).

DISCUSSION

Biliary obstruction has been identified to be an important risk factor for tumor which may result in alterations of glycogen metabolism, impaired hepatic and renal functions, decreased cell-mediated immunity, increased circulating endotoxins, and depressed synthesis of homeostasis factors^[23,24]. These factors can decrease the tolerance of patients to anesthesia and surgery, leading to increasing operative risks. For these reasons, in 1935, Whipple *et al*^[25] performed a staged surgery with a preliminary bypass to reduce jaundice and improve hepatic functions. In 1978, Nakayama *et al*^[26] found that the operative mortality is significantly reduced after PBD. Since then, more and more

investigators have accepted the concept that PBD can improve the hepatic functions of patients with malignant obstructive jaundice^[27-31].

With the great advances in surgical techniques and perioperative management, the postoperative complication rate has been dramatically declined in recent years. Whether PBD is still valuable in surgery for malignant obstructive jaundice is questioned by many experts. Several prospective randomized and retrospective studies compared the effect of PBD with surgery without PBD on malignant obstructive jaundice and showed that PBD cannot improve the postoperative outcome but can increase the overall complication rate^[32-35]. Although the controversy involves the indication of PBD for malignant obstructive jaundice, some centers still believe that PBD can improve the outcome for some time. To date, whether PBD should routinely be performed for malignant obstructive jaundice is still in debate. One of the reasons why the reported results are distinct is that the overwhelming majority of clinical trials were retrospective and some included heterogeneous groups of patients as well as a variety of different surgical procedures. Thus, unrecognized bias and differences in selection of patients may have affected the results. Another reason is that PBD failing to benefit patients with malignant obstructive jaundice may have a relatively short length of drainage, usually 2-3 wk. In fact, proliferation and fibrosis of bile duct epithelium may take 4-6 wk to recover, and avoid postoperative complications and impaired liver metabolism.

In the present study, the postoperative mortality, the

incidence of postoperative pancreatic and bile leakage, abdominal abscess, and delayed gastric emptying were not significantly different in patients with or without PBD, whereas the incidence of postoperative incision infection was significantly different in patients with or without PBD, which is consistent with other reports^[33,36,37]. Povoski *et al.*^[33] reviewed the effect of PBD and found that PBD has no beneficial effect on the postoperative outcome. In contrast, Trede *et al.*^[36] showed that the postoperative morbidity is significantly reduced in patients after internal PBD following pancreaticoduodenectomy. Lygidakis *et al.*^[37] also reported that the postoperative morbidity of obstructive jaundice is significantly decreased after internal PBD following pancreaticoduodenectomy. Due to the selective bias in choice of PBD, a well-selected subgroup of patients may benefit from PBD. Moreover, most patients with PBD, experiencing other serious diseases secondary to biliary obstruction, are in a relatively poorer condition than those undergoing surgery. Experimental and clinical evidence has shown that external PBD cannot improve the outcome of surgery, while internal PBD may have a beneficial effect because it can restore the nutritional and immune function^[7]. Since internal PBD can significantly reduce the number of postoperative laparotomies for bleeding, anastomotic leakage and abscess, many centers support the view that internal PBD reduces the morbidity rate of obstructive jaundice in patients undergoing surgery. However, Lai *et al.*^[38] did not support the routine use of internal PBD because of procedure-related complications, mainly cholangitis. In addition, biliary drainage for a proximal tumor with intrahepatic stenosis of the bile duct is also different from that for a distal obstruction. Therefore, various confounding factors affecting the prognosis should be taken into consideration in future clinical investigations.

In conclusion, there is no convincing evidence that supports the view that routine PBD improves postoperative outcome in patients with malignant obstructive jaundice. PBD has its own complications that partially cancel out its benefits. More randomized controlled trials are needed to identify patients who may benefit from PBD.

COMMENTS

Background

Preoperative hyperbilirubinemia in patients with malignant obstructive jaundice is considered an important risk factor for postoperative complications and death. Therefore, preoperative biliary drainage (PBD) has been used to reduce postoperative morbidity and mortality of such patients.

Research frontiers

Since 1970s, several randomized and retrospective studies have compared the effect of PBD with non-PBD on malignant obstructive jaundice. However, it is difficult to find convincing evidence that routine PBD can improve the outcome of patients with malignant obstructive jaundice in clinical practice.

Innovations and breakthroughs

To date, there is no convincing evidence that supports the view that routine PBD improves postoperative outcomes of patients with malignant obstructive jaundice. This is the first meta-analysis of the recent studies concerning the effect of PBD on malignant obstructive jaundice.

Applications

The present meta-analysis indicated that PBD could not significantly reduce the postoperative mortality and the complications of malignant obstructive jaundice. Therefore, PBD may not be regarded as a preoperative routine measure for malignant obstructive jaundice.

Peer review

A meta-analysis of the effect of PBD on malignant obstructive jaundice was performed by reviewing the publications between 1995 and 2009. The authors drew a conclusion that PBD may not be regarded as a preoperative routine measure for malignant obstructive jaundice. This is a very interesting topic for hepatologists and other digestive experts.

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S- Editor Sun H L- Editor Wang XL E- Editor Zheng XM

Adjuvant radiotherapy for gallbladder cancer: A dosimetric comparison of conformal radiotherapy and intensity-modulated radiotherapy

Xiao-Nan Sun, Qi Wang, Ben-Xing Gu, Yan-Hong Zhu, Jian-Bin Hu, Guo-Zhi Shi, Shu Zheng

Xiao-Nan Sun, Qi Wang, Ben-Xing Gu, Yan-Hong Zhu, Jian-Bin Hu, Guo-Zhi Shi, Department of Radiation Oncology of Sir Run Run Shaw Hospital, Sir Run Run Shaw Institute of Clinical Medicine of Zhejiang University, Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China
Shu Zheng, Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, Key Laboratory of Molecular Biology in Medical Sciences, Zhejiang Province, China), The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China
Author contributions: Sun XN and Wang Q contributed equally to this work; Zheng S, Sun XN and Wang Q designed the research; Sun XN, Wang Q and Hu JB analyzed the data; Hu JB, Gu BX, Zhu YH and Shi GZ collected the data; Sun XN and Wang Q wrote the paper.

Correspondence to: Shu Zheng, Professor, Cancer Institute, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China. zhengshu@zju.edu.cn

Telephone: +86-571-87214404 Fax: +86-571-87214404

Received: June 3, 2010 Revised: September 29, 2010

Accepted: October 7, 2010

Published online: January 21, 2011

1.8 or 2.0 Gy per fraction. CRT planning was compared with IMRT.

RESULTS: The most common reported acute toxicities requiring medication (Radiation Therapy Oncology Group, Radiation Therapy Oncology Group Grade 2) were nausea (10/20 patients) and diarrhea (3/20). There were no treatment-related deaths. Compared with CRT planning, IMRT significantly reduced the volume of right kidney receiving > 20 Gy and the volume of liver receiving > 30 Gy. IMRT has a negligible impact on the volume of left kidney receiving > 20 Gy. The 95% of prescribed dose for a planning tumor volume using either 3D CRT or IMRT planning were 84.0% ± 6.7%, 82.9% ± 6.1%, respectively ($P > 0.05$).

CONCLUSION: IMRT achieves similar excellent target coverage as compared with CRT planning, while reducing the mean liver dose and volume above threshold dose. IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose.

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Abstract

AIM: To assess the efficacy and toxicity of conformal radiotherapy (CRT) and compare with intensity-modulated radiotherapy (IMRT) in the treatment of gallbladder cancer.

METHODS: Between November 2003 and January 2010, 20 patients with gallbladder cancer were treated with CRT with or without chemotherapy after surgical resection. Preliminary survival data were collected and examined using both Kaplan-Meier and actuarial analysis. Demographic and treatment parameters were collected. All patients were planned to receive 46-56 Gy in

Key words: Gallbladder cancers; Adjuvant treatment; Surgery; Radiation therapy

Peer reviewer: Kyu Taek Lee, MD, PhD, Professor, Department of Medicine Samsung Medical Center, Sungkyunkwan, University School of Medicine, #50, Irwon-dong, Gangnam-gu, Seoul, 135-710, South Korea

Sun XN, Wang Q, Gu BX, Zhu YH, Hu JB, Shi GZ, Zheng S. Adjuvant radiotherapy for gallbladder cancer: A dosimetric comparison of conformal radiotherapy and intensity-modulated radiotherapy. *World J Gastroenterol* 2011; 17(3): 397-402 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/397.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.397>

INTRODUCTION

Gallbladder cancer is the fifth most common malignancy of the gastrointestinal tract^[1]. In general, the prognosis of patients with gallbladder cancers is poor, with an overall 5-year survival rate of less than 10%^[2].

Gallbladder cancer was associated with a uniformly poor prognosis due to its highly aggressive behavior, only 10%-30% of the patients had resectable tumors at presentation^[3]. Better understanding of the biological behavior of the disease, its pattern of dissemination, better diagnostic tools, and more aggressive therapy has resulted in some improvement in survival in the last decade^[4]. However, most long-term survivors are patients with incidentally diagnosed carcinomas confined to the mucosa of the gallbladder.

Surgical therapy is the standard treatment for patients presenting with resectable disease. Unfortunately, a large number of patients develop recurrent diseases after undergoing curative resection. The long-term outcome of the patients with recurrent gallbladder cancer is very poor. And local-regional failure is common and is a major cause of mortality^[5]. Because of this, adjuvant therapy has been used to improve loco-regional control and survival rate. Several studies have reported improvement in survival for patients treated with adjuvant chemoradiation^[6,7].

Actually, the definite role of adjuvant therapy after curative resection is still uncertain. Studies to improve the loco-regional control rates with adjuvant radiation with/without chemotherapy or chemotherapy alone, have been conducted. Although gallbladder cancer is considered to be radiation resistant, radiation has been administered in the form of external beam radiotherapy, intra-operative radiation therapy and brachytherapy^[8-11]. However, the relative rarity of this malignant disease made it difficult to conduct large phase III studies to guide management in both the adjuvant and comprehensive treatment. This study reports a single-institution series that used adjuvant radiation therapy with or without chemotherapy after resection in patients with locally advanced gallbladder carcinoma. The aim of the study was to evaluate conformal radiotherapy (CRT) and intensity-modulated radiotherapy (IMRT) planning parameters in the treatment of this malignancy.

MATERIALS AND METHODS

Between November 2003 and January 2010, a total of 20 patients with pathologically diagnosed primary adenocarcinoma of gallbladder were treated in the Department of Radiation Oncology in our hospital. All patients were diagnosed with adenocarcinoma of gallbladder. All patients had complete resection, with negative microscopic margins. Demographic data were collected regarding patient age, gender, histological classification, tumor staging (Table 1). In addition to radiotherapy, the majority of patients received concurrent fluoropyrimidine-based and oxaliplatin-based chemotherapy. Concurrent and adjuvant chemotherapy regimens are shown in Table 2. Six patients received postoperative radiotherapy alone. No clear pattern of che-

Table 1 Patient and tumor characteristics (*n* = 20)

Characteristics	<i>n</i> (%)
Median age (range, yr)	56 (33-73)
Gender	
Male	8 (40)
Female	12 (60)
ECOG performance status	
0	2 (10)
1	16 (80)
2	2 (10)
Tumor and node status	
PT1	0 (0)
PT2	13 (65)
PT3	6 (30)
PT4	1 (5)
NX	
PN0	13 (65)
PN	7 (35)
Tumor grade	
Well	8 (40)
Moderate	9 (45)
Poor	3 (15)

ECOG: Eastern Cooperative Oncology Group; NX: Node staging.

Table 2 Treatment details for the patients

Treatment	No. of patients
CT concurrent RT	9
5-fluorouracil	4
Oxaliplatin	5
Concurrent RT followed by CT	7
5-fluorouracil + oxaliplatin	4
Gemcitabine + oxaliplatin	3
RT followed by CT	3
5-fluorouracil + oxaliplatin	1
Gemcitabine + oxaliplatin	2
Postoperative CT before RT	2
5-fluorouracil bolus	
RT alone	6

Nine patients received concurrent radiochemotherapy, 7 of them received adjuvant chemotherapy. CT: Chemotherapy; RT: Radiotherapy.

mothy or standardized dosing regimen was evidenced from chart data available for review. All patients were simulated on a computed tomography-scanner (Siemens Definition AS 40) and were imaged using a slice of thickness 3.0 mm. All simulations were performed using a timed bolus of non-ionic intravenous contrast media to acquire images of early arterial/portal venous contrast phase, and a secondary venous contrast phase. Digital imaging and communications in medicine data were transferred to an inverse IMRT treatment planning station (Philips Pinnacle³ 7.6C). Gross target volumes and clinical target volumes (CTVs) according to ICRU 62 definitions^[12] were delineated on a slice-by-slice basis. External beam radiation therapy fields generally encompassed the tumor bed and regional lymph nodes (porta hepatis, celiac, pancreaticoduodenal) to a dose of 45 Gy in 1.8-2.0 Gy daily fractions. Reduced fields to tumor bed plus a 2-2.5 cm margin received an additional 5.0-10.0 Gy. A variety of multi-beam

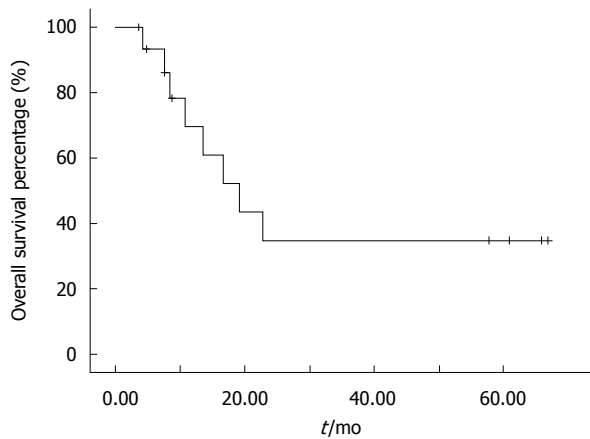


Figure 1 Overall survival for all the patients.

techniques were used to treat the tumor bed to a median total prescription dose of 52 Gy (range, 46-56 Gy). Organs-at-risk (OAR) delineated included the spinal cord, kidneys, and healthy liver. A planning tumor volume (PTV) was created by volumetric expansion of the CTV by 10-15 mm. Dose volume histograms (DVHs) were utilized to evaluate the plans. Prescribed doses to the initial PTV ranged from 46 to 56 Gy in daily doses of 1.8-2.0 Gy. Relative constraints included left kidneys constrained to a D100 of < 20 Gy and D66 < 18 Gy; right kidneys were specified to achieve D100 < 30 Gy, with D66 < 20 Gy. The total mean liver dose was specified to < 22 Gy, and liver V20 kept under 33%. Treatment was delivered using a 10 MV linear accelerator (Siemens Primus M) with 200 MU/min delivery capability using a multi-leaf collimator. Survival data were collected and examined using the Kaplan-Meier method.

By a comparison of CRT and IMRT, all fields of the patients were coplanar. DVHs were obtained for the PTV, kidneys, liver and spinal cord. Acute toxicity was scored using the Radiation Therapy Oncology Group (RTOG) morbidity scoring criteria^[13]. Dosimetric endpoints for the target and critical structures were compared using the two tailed paired *t* test.

RESULTS

Survival analysis

The median follow-up for patients alive at analysis was 14.0 mo (range, 3.0-66.9 mo). Nine patients were alive. The median preliminary survival from diagnosis in the 20 patients was 19.2 mo (range, 4.2-66.9 mo, 95% confidence interval: 10.1-28.3). Kaplan-Meier analysis revealed an estimated one-year survival rate of 40.48% (Figure 1).

Toxicity analysis

Twenty patients completed their planned course of treatment without breaks and no reduction of planned chemotherapy. For gastrointestinal (GI) toxicity analysis, radiotherapy or chemotherapy with CRT was well tolerated without > grade 3 acute GI toxicity occurring during radiotherapy, and 11/20 and 4/20 patients reported grade 1 upper and

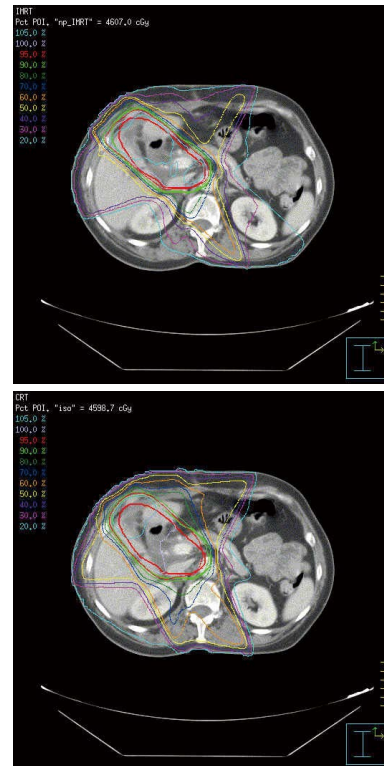


Figure 2 Isodose curves on an axial slice for a representative case. Intensity-modulated radiotherapy plan (upper picture) and conformal radiotherapy plan (lower picture).

lower acute RTOG GI toxicity scores. The most common reported acute toxicities requiring medication (RTOG Grade 2) were nausea (10/20 patients) and diarrhea (3/20). There were no treatment-related deaths.

Fourteen patients received chemotherapy (including concurrent chemotherapy), the major grade 3-4 adverse events were leukopenia (21%), neutropenia (29%) and anemia (14%). Compared with pre-treatment values, no abnormalities were detected in the laboratory test of kidney function for any of the 20 patients, either during treatment or at follow-up. Three patients had elevated liver enzymes about 6 mo after the completion of radiotherapy. No late toxicity was seen in this series.

Dosimetric comparison between 3D CRT and IMRT plans

To demonstrate the differences in dose distribution, Figure 2 shows isodose curves on an axial slice for one representative patient for IMRT and CRT. Figure 3 shows the DVH curves for the kidneys and liver at risk for one representative patient. Table 3 summarizes the mean doses to the PTV, kidneys and liver for CRT and IMRT plans. Compared with the CRT plan, IMRT significantly reduced the mean dose to the right kidney and liver, while the improvement in the dose to the left kidney was not significant. Both of CRT and IMRT limited the dose to spinal cord under 40 Gy. Table 4 summarizes the volume of critical structures receiving greater than the threshold dose^[14]. Compared with CRT planning, IMRT significantly reduced the volume of right kidney receiving > 20 Gy and the volume of liver receiving > 30 Gy. IMRT has a

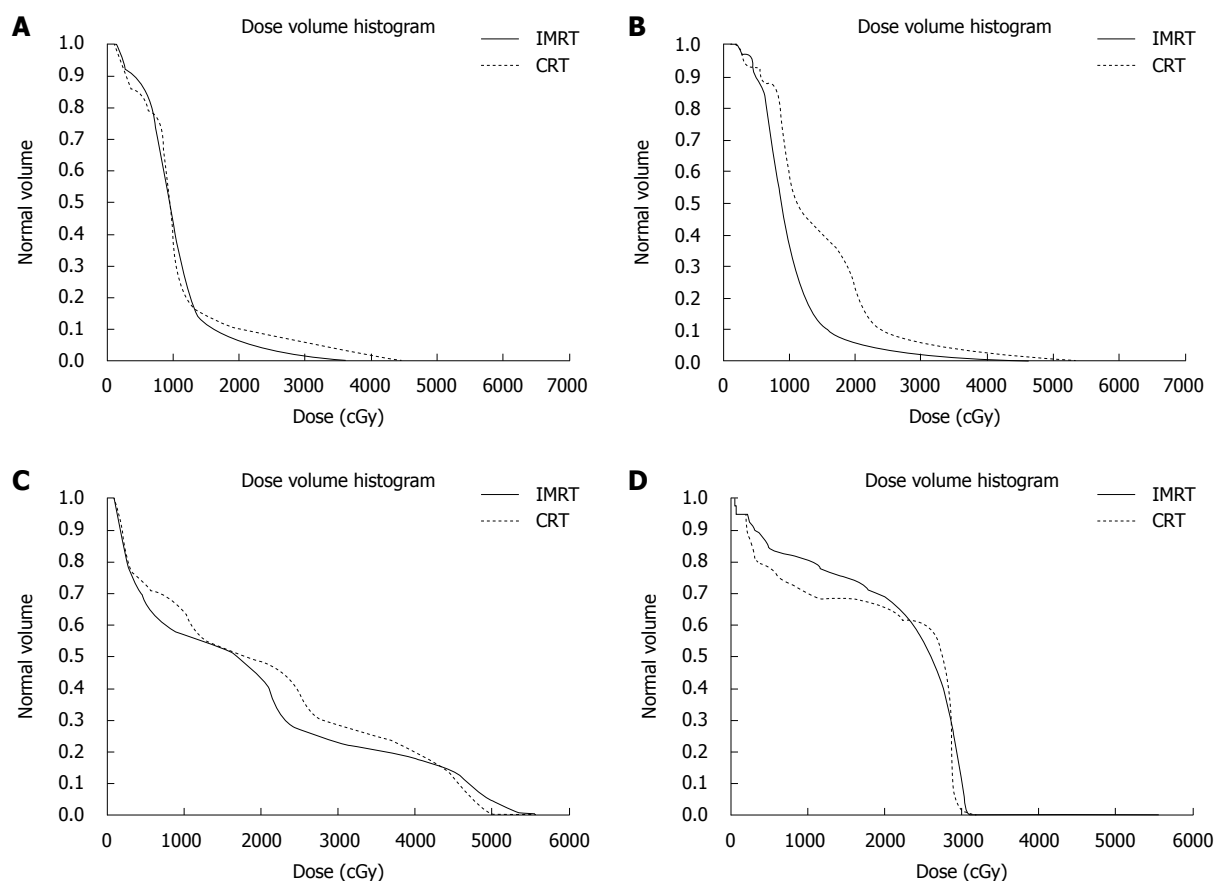


Figure 3 Dose volume histogram curves for the organs at risk in a representative case. Left kidney (A), right kidney (B), liver (C), and spinal cord (D). IMRT: Intensity-modulated radiation therapy; CRT: Conformal radiation therapy.

Table 3 Mean dose to the targeted structures/organs

Structure/organs	Mean dose (Gy)		<i>P</i> value ¹
	CRT	IMRT	
PTV	50.1 ± 2.8	51.5 ± 2.7	0.00015
Right kidney	10.6 ± 4.4	8.6 ± 2.4	0.032
Left kidney	8.1 ± 3.4	9.4 ± 4.6	NS
Liver	22.4 ± 3.9	21.0 ± 2.9	0.027

¹Two-tailed paired *t* test. Values are expressed as mean ± SD. CRT: Three dimensional conformal radiation therapy; IMRT: Intensity-modulated radiation therapy; PTV: Planning tumor volume; NS: Not significant (*P* > 0.05).

negligible impact on the volume of left kidney receiving > 20 Gy. The range of the 95% of prescribed dose for PTV using either 3DCRT or IMRT planning was 84.0% ± 6.7% and 82.9% ± 6.1%, respectively (*P* > 0.05).

DISCUSSION

Gallbladder cancer has a dismal prognosis and loco-regional recurrence has been described as the most frequent site of relapse, and death most commonly occurred due to complications and sequelae of loco-regional recurrence^[15]. Hepatic infiltration has been reported in 60%-70% and nodal involvement in 20%-40% in some series^[16,17]. The loco-regional pattern of recurrence after surgery provides

Table 4 Volume of structures/organs receiving greater than the threshold dose

Structure/organs	Dose (Gy)	Volume above threshold dose (%)		<i>P</i> value ¹
		CRT	IMRT	
PTV	95% of prescribed dose	84.0 ± 6.7	82.9 ± 6.1	NS
Organs at risk				
Right kidney	20.0	12.5 ± 11.7	5.4 ± 2.7	0.031
Left kidney	20.0	5.3 ± 6.0	3.7 ± 2.3	NS
Liver	30.0	34.5 ± 8.0	30.1 ± 6.3	0.0065

¹Two-tailed paired *t* test. Values are expressed as mean ± SD. CRT: Three dimensional conformal radiation therapy; IMRT: Intensity-modulated radiation therapy; PTV: Planning tumor volume; NS: Not significant (*P* > 0.05).

a rationale for the use of radiotherapy as a component of gallbladder cancer treatment. The radiosensitive nature of gallbladder cancer is evidenced by numerous clinical studies reporting tumor size reduction after radiotherapy for unresectable diseases^[18,19].

Our one-year survival rates and median preliminary survival time are similar to those reported in the limited radiotherapy literature and are generally better than those reported with surgery alone in patients with local advanced disease^[20,21].

IMRT and automated optimization have the abil-

ity to shape isodose curves, avoiding the dose to the OARs. The use of direct machine parameter optimization IMRT improves the PTV coverage compared with CRT for most patients, although dose escalation was only possible in a minority of patients. It is suspected that the dosimetric improvement with IMRT was less in these plans compared with CRT plans. However, these complex CRT plans are challenging to develop without automated optimization, and IMRT might have planning efficiency and time-saving advantages for these cases.

Our data demonstrated that IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose. IMRT achieves similar excellent target coverage as compared with CRT, while reducing the mean liver dose and volume above threshold dose. In summary, IMRT offers improved sparing of normal structures, however, it warrants further studies in the treatment of gallbladder carcinoma.

In conclusion, gallbladder carcinoma is an aggressive disease with a dismal prognosis. More effective adjuvant therapy is needed to improve overall survival. There was a clear association between adjuvant therapy use and improved survival in patients with loco-regional disease. The real benefit of adjuvant radiotherapy in gallbladder carcinoma remains unclear. A retrospective analysis^[21] was done about the surveillance, epidemiological, and end results survey by the American National Cancer Institute. The results showed that adjuvant radiotherapy is associated with improved survival in patients with locally advanced gallbladder cancer or gallbladder cancer with regional disease. Gallbladder cancer remains an aggressive disease that requires multimodality approach to individualize and optimize therapy. Prospective randomized trials of adjuvant therapy are needed in this disease. However, the low incidence of gallbladder cancer may make it difficult to successfully complete such trials, unless they are designed as inter-group studies within China or as international studies. In the future, methods of achieving earlier diagnoses may help improve the outcomes of the treatment. IMRT for dose escalation to improve tumor control and spare surrounding structure/organs from receiving radiation tolerance doses should be further studied.

COMMENTS

Background

Surgical therapy is the standard treatment for patients with resectable gallbladder cancer. Unfortunately, a large number of the patients develop recurrent disease despite curative resection. And local-regional failure is common and is a major cause of mortality. Because of this, adjuvant therapy has been used to improve loco-regional control and survival rate. Several studies have reported improvement for patients treated with adjuvant chemoradiation.

Research frontiers

Although gallbladder cancer is considered to be radiation resistant, radiation has been tried in the form of external beam radiotherapy, intra-operative radiation therapy and brachytherapy.

Innovations and breakthroughs

Intensity-modulated radiotherapy (IMRT) achieves similar excellent target coverage as compared with conformal radiotherapy (CRT) planning, while reducing

the mean liver dose and volume above threshold dose. IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose.

Applications

The mainstay of treatment has been surgery and the role of adjuvant therapy in the form of chemotherapy and/or radiation therapy remains to be defined. Some clinical studies suggest that adjuvant radiotherapy dosage was associated with a better local control of the tumor. IMRT may offer better sparing of the right kidney and liver compared with CRT planning, this makes it possible for dose escalation to improve tumor control and spare surrounding structure/organs.

Terminology

IMRT: A type of three-dimensional radiation therapy that uses computer-generated images to match radiation to the size and shape of a tumor, which is used to deliver a higher radiation dose to a tumor with less damage to the nearby healthy tissues.

Peer review

The role of adjuvant radiotherapy in gallbladder cancer is not definite. This paper deals with a methodological comparison of CRT and IMRT for gallbladder cancer. IMRT offered better sparing of right kidney and liver compared with CRT. This result can help clinicians understand the role and toxicity of radiotherapy in gallbladder cancer treatment.

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S- Editor Sun H L- Editor Ma JY E- Editor Lin YP

Hepatic veins as a site of clot formation following liver resection

Emmanuel Buc, Safi Dokmak, Magaly Zappa, Marie Helene Denninger, Dominique Charles Valla, Jacques Belghiti, Olivier Farges

Emmanuel Buc, Safi Dokmak, Jacques Belghiti, Olivier Farges, Department of Hepato-Biliary Surgery, Hôpital Beaujon, Assistance-Publique Hôpitaux de Paris, University Paris 7, 92118 Clichy, France

Magaly Zappa, Department of Radiology, Hôpital Beaujon, Assistance-Publique Hôpitaux de Paris, University Paris 7, 92118 Clichy, France

Marie Helene Denninger, Department of Biological Hematology, Hôpital Beaujon, Assistance-Publique Hôpitaux de Paris, University Paris 7, 92118 Clichy, France

Dominique Charles Valla, Department of Hepatology, Hôpital Beaujon, Assistance-Publique Hôpitaux de Paris, University Paris 7, 92118 Clichy, France

Author contributions: Buc E and Farges O produced the concept, designed the study, and drafted the manuscript; Buc E and Dokmak S were responsible for acquisition of data; Buc E, Zappa M and Farges O analysed and interpreted data; Valla DC, Belghiti J and Denninger MH revised the manuscript for important intellectual content; Farges O supervised the study.

Correspondence to: Olivier Farges, MD, PhD, Professor, Department of Hepato-Biliary Surgery, Hôpital Beaujon, Assistance-Publique Hôpitaux de Paris, University Paris 7, 100 boulevard du General Leclerc, 92118 Clichy,

France. olivier.farges@bjn.aphp.fr

Telephone: +33-1-40875222 Fax: +33-1-40870926

Received: July 29, 2010 Revised: September 6, 2010

Accepted: September 13, 2010

Published online: January 21, 2011

plan in a context of mutation of the prothrombin gene and inflammation induced biloma were the likely predisposing conditions. Clots disappeared following curative anticoagulation. We conclude that thrombosis of hepatic veins may occur after liver resection and is a potential source of pulmonary embolism.

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Key words: Hepatectomy; Hepatic veins; Thrombosis; Pulmonary embolism; Anticoagulants

Peer reviewer: Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Buc E, Dokmak S, Zappa M, Denninger MH, Valla DC, Belghiti J, Farges O. Hepatic veins as a site of clot formation following liver resection. *World J Gastroenterol* 2011; 17(3): 403-406 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/403.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.403>

Abstract

Pulmonary embolism occurs more frequently after hepatectomy than previously thought but is infrequently associated with peripheral deep vein thrombosis. In this paper, we report 2 cases of postoperative hepatic vein thrombosis after liver resection. Both patients had undergone major hepatectomy of a non-cirrhotic liver largely exposing the middle hepatic vein. Clots were incidentally found in the middle hepatic vein 4 and 17 d after surgery despite routine systemic thrombo-prophylaxis with low molecular weight heparin. Coagulation of the transition

INTRODUCTION

Patients undergoing liver surgery have long been considered to be at low risk of venous thromboembolism. Routine Doppler ultrasound following major hepatectomies identifies deep vein thrombosis in 2% of patients^[1], three to five times less than after general abdominal or colorectal procedures performed with adequate anticoagulation prophylaxis^[2-5]. However, pulmonary embolism has recently emerged as an increasingly frequent and potentially fatal complication following liver resections. Its incidence ranges between 1% and 3% in patients undergoing liver resections^[1,6] and has been reported to be as high as 10% in living-related donors undergoing a right hepatectomy^[7,8]. These figures are greater than the 0.3% incidence observed following general surgery and the 2%-3% incidence observed after high risk

procedures such as invasive neurosurgery, total hip arthroplasty, and radical cystectomy^[9]. Liver regeneration that follows major resections is indeed associated with an early and transient dysregulation of the haemostatic system resulting in a hypercoagulability state^[10].

This difference between a low incidence of deep vein thrombosis and a high incidence of pulmonary embolism is difficult to explain as more than 90% of pulmonary emboli are considered to arise from lower extremity and pelvic deep veins^[11]. Furthermore, less than 50% of patients developing a pulmonary embolism after liver resection have an associated deep vein thrombosis^[7].

We shed a new light on this discrepancy by reporting two patients who developed thrombi in their hepatic veins following hepatectomy. To our knowledge, this complication has not been previously reported which can be explained by the technical difficulty to visualise the hepatic veins on imaging studies in the early postoperative period.

CASE REPORT

Case 1

A 39-year-old woman underwent a right hepatectomy for a 13 cm large liver hemangioma responsible for incapacitating pain. Hepatic veins were patent and besides a body mass index of 32 kg/m² she had no known risk factors for thromboembolic disease^[12]. On the evening before surgery, tight-length graduated compression stockings were placed and she received a subcutaneous injection of 40 mg enoxaparin. Liver transection was performed using an ultrasonic dissector with two intermittent clamping of the hepatic pedicle of 11 and 15 min. Intrahepatic portal structures and hepatic veins were occluded with ligation, clips or bipolar coagulation as required. The right hepatic vein was closed extraparenchymally and the main trunk of the middle hepatic vein was retained with the left liver. Additional haemostasis of the transection surface was achieved with bipolar coagulation and the left liver was fixed to the diaphragm to prevent twisting of the hepatic veins under intraoperative ultrasound control^[13]. No transfusion was required, the patient was extubated 3 h after surgery and daily administration of 40 mg enoxaparin was reinitiated on the following morning.

The early postoperative course was uneventful with rapid normalization of liver function tests but on the fourth postoperative day, she developed shortness of breath and a temperature rise at 37.8°C at which time a computed tomography (CT) scan was performed. There was no obvious evidence of pulmonary embolism but three defects were found in the middle hepatic vein adjacent to the transection plan (Figure 1) that were confirmed to be 2-3 cm long clots by Doppler ultrasound. The inferior vena cava and termination of the middle hepatic vein had a normal flow pattern otherwise. Mild right pleural effusion, ascites and localised thrombosis of the right posterior tibial veins were also uncovered. Following administration of enoxaparin at 1.0 mg/kg twice daily, pulmonary symptoms disappeared within 48 h and control Doppler ultrasounds performed every other day

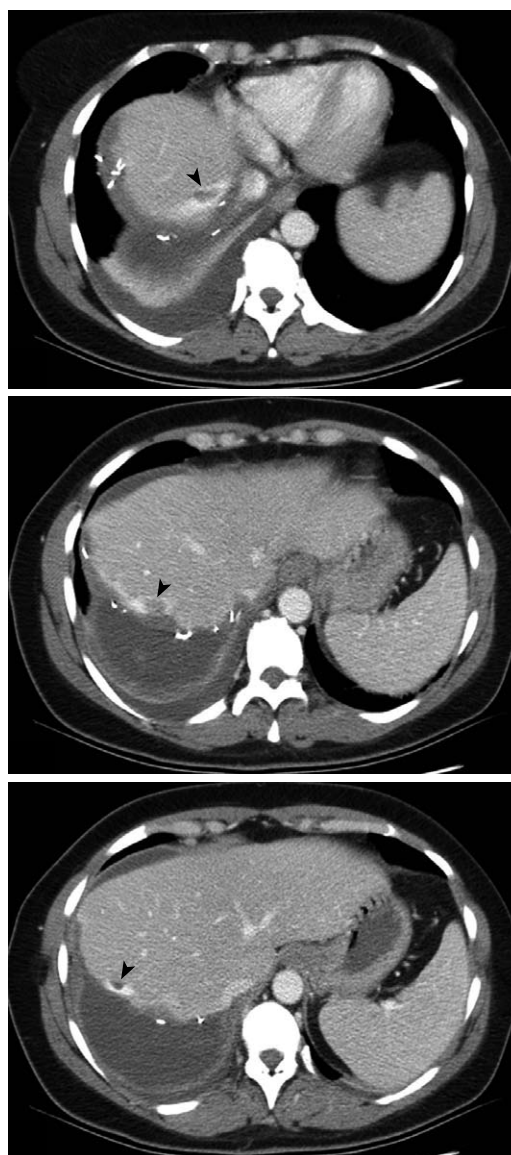


Figure 1 Postoperative computed tomography scan (day 4) in case 1. Defects are present in the middle hepatic vein close to the transection plan (arrowheads).

showed the progressive disappearance of two of the three clots and the reduction in size of the third at which time she was discharged (postoperative day 21). The postoperative course had otherwise been uneventful. A control CT scan performed 1 mo later showed the complete disappearance of the clots. Screening for inherited thrombophilia identified a heterozygote (20210AG) mutation of the prothrombin gene while other risk factors, including factor V Leiden mutation were absent.

Case 2

A 78-year-old man underwent simultaneous left hepatectomy extended to a part of segment 8 by laparotomy and laparoscopic sigmoidectomy for synchronous colorectal liver metastasis. He had no history of thromboembolic disease and surgery was preceded by 6 cycles of chemotherapy (FOLFOX regimen + cetuximab) that had induced a partial response according to RECIST criteria.

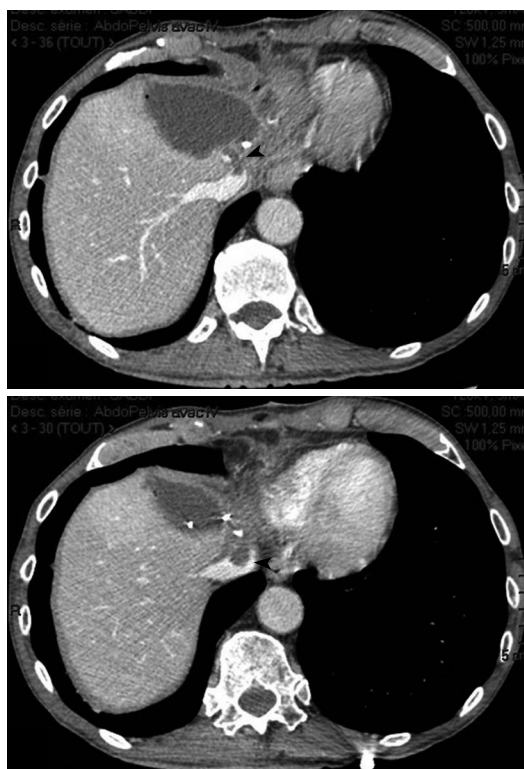


Figure 2 Postoperative computed tomography scan (day 17) in case 2. A biloma is present at the upper part of the transection plan and a defect is visible in the distal end of the middle hepatic vein extending in the inferior vena cava (arrowheads).

On the evening before surgery, tight-length graduated compression stockings were placed and he received a subcutaneous injection of 40 mg enoxaparin. Liver transection was performed as previously described, without vascular clamping. The middle hepatic vein was closed intraparenchymally while the left hepatic vein was closed extraparenchymally. No transfusion was required, the patient was extubated 3 h after surgery and daily administration of 40 mg enoxaparin was reinitiated on the following morning.

The early postoperative course was uneventful and the patient was discharged on postoperative day 11. He was re-admitted on postoperative day 17 for sepsis. CT scan showed bile collection close to the cut edge of the liver and a clot in the middle hepatic vein extending into the supra-hepatic vena cava (Figure 2). Percutaneous drainage of the collection was performed and anticoagulant therapy was administered, allowing complete regression of the thrombosis seven days later. Oral anticoagulation was then administered and the patient was discharged 10 d after admission (postoperative day 27). Screening for inherited thrombophilia was negative.

DISCUSSION

The present study reports two patients who developed early hepatic vein thrombosis following liver resection, a previously unrecognized complication. Both had several features in common: (1) they had undergone a major hepatectomy

within a non-cirrhotic liver; (2) the thrombosis was located within the hepatic vein adjacent to the transection plan; and (3) was discovered somewhat fortuitously.

We have previously shown that major resections of non-cirrhotic livers are associated with an early postoperative decrease in coagulation inhibitors protein C and antithrombin together with an increase in factor VIII and von Willebrand factor that induce a transient hypercoagulability state^[10]. Both patients underwent a major resection while already being at increased risk of thrombosis. One indeed retrospectively proved to be overweight and to have a heterozygote (20210AG) mutation of the prothrombin gene associated with a three- and two-fold increase in the risk of thrombosis, respectively^[14]. The other patient had an advanced malignancy that also increased the risk of thrombosis^[12,14] and is associated with increased procoagulant activity^[15].

The specific finding was, however, that thrombosis developed within the main trunk of a major hepatic vein adjacent to the transection plan. One patient (n°2) developed a biloma, an obvious local predisposing condition as local infection causes vein wall inflammation while general infection increases systemic procoagulant activity^[16]. This was not the case in the other (patient 1) and we believe that thrombosis may have been favoured by extensive coagulation of the raw surface of the liver. Bipolar coagulation, which is widely used to achieve hemostasis during parenchymal transection, may result in heat-induced endothelial injury when a major hepatic vein lies close to the transection plan which is the case during formal right and left hepatectomies. Portal triad clamping, which is frequently performed during major hepatectomies, may also favour through stasis these coagulation-induced thrombosis^[12].

The recognition of these thrombi in our patients was somewhat fortuitous which may explain why this complication might have previously been overlooked. CT imaging in the postoperative course of liver resections is generally indicated when a pulmonary embolism or an abdominal collection are suspected. Angio-CT scans are usually performed 15–20 s after the injection at which time hepatic veins are not visible. During more conventional CT scans, acquisition of images is similarly not always performed at the time hepatic veins are injected. Besides, the middle hepatic vein has a longitudinal direction and is therefore hardly visualized along a significant length by the transverse slices of CT scans.

Once present in major hepatic veins, these thrombi may easily migrate into the inferior vena cava as previously shown for tumour^[17] or hepatic fragments^[18]. Pulmonary embolism was not formally documented in our patients probably because anticoagulation therapy was initiated prior to clot migration.

In conclusion, thrombosis may occur in hepatic veins after liver resection as a result of intra- or postoperative local injury. This would explain why pulmonary emboli have been observed in the absence of peripheral deep vein thrombosis. This hazard should be taken into account when performing extensive coagulation of the raw surface of the liver when a major hepatic vein is exposed.

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S- Editor Shi ZF L- Editor O'Neill M E- Editor Lin YP

Comments on the article about correlation between computerized tomography and surgery in acute pancreatitis

Enver Zerem, Goran Imamović, Zoran Mavija, Bahrija Haračić

Enver Zerem, Bahrija Haračić, Department of Gastroenterology, University Clinical Center Tuzla, 75000 Tuzla, Bosnia and Herzegovina

Goran Imamović, Medical Division, Fresenius Medical Care BH, d.o.o. Sarajevo, 71000 Sarajevo, Bosnia and Herzegovina

Zoran Mavija, Department of Gastroenterology, Clinical Center Banjaluka, 78000 Banjaluka, Bosnia and Herzegovina

Author contributions: Zerem E, Imamović G, Mavija Z and Haračić B contributed equally to this letter to the editor.

Correspondence to: Enver Zerem, MD, PhD, Department of Gastroenterology, University Clinical Center Tuzla, Trnovac bb, 75000 Tuzla, Bosnia and Herzegovina. zerem@live.com

Telephone: +387-35-393300 Fax: +387-35-250474

Received: September 23, 2010 Revised: November 30, 2010

Accepted: December 7, 2010

Published online: January 21, 2011

Abstract

We read with great interest the article by Vege *et al* published in issue 34 of *World J Gastroenterol* 2010. The article evaluates the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections found at surgery. The results of their study indicate that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue and CECT has a limited role in differentiating various types of collections. However, there are some points that need to be addressed, including data about the stage of acute pancreatitis in which CECT was done and the time span between CECT examination and surgery.

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Key words: Acute pancreatitis; Pancreatic necrosis; Peripancreatic fluid collection; Contrast-enhanced computerized tomography; Surgery

Peer reviewers: Markus Raderer, Professor, Department of Inter-

nal Medicine I, Division of Oncology, Medical University Vienna, Wahringer Guertel 18 - 20, Vienna, A-1090, Austria; Julio Mayol, MD, PhD, Department of Digestive surgery, Hospital Clinico San Carlos, MARTIN-LAGOS S/n, Madrid, 28040, Spain

Zerem E, Imamović G, Mavija Z, Haračić B. Comments on the article about correlation between computerized tomography and surgery in acute pancreatitis. *World J Gastroenterol* 2011; 17(3): 407-408 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/407.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.407>

TO THE EDITOR

We read with great interest the article by Vege *et al*^[1] published in issue 34 of *World J Gastroenterol* 2010. The article evaluates the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections found at surgery. For that purpose the authors excluded false positive and negative collections found on CT and presented their results in a comparative analysis. The results of their study indicate that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue and CECT has a limited role in differentiating the different types of collections.

However, there are some points that need to be addressed. The authors neither specified in which stage of acute pancreatitis (pro-inflammatory or anti-inflammatory response) was CECT done nor they specified the time span between CECT examination and surgery. Since the clinical course of severe acute pancreatitis is very dynamic, and CECT and surgery were not performed concurrently, it may not be the matter of false negative and positive findings, but the collections could have rather be formed or disappeared in between CECT examination and surgery. Furthermore, the collections could have progressed from one stage to another, e.g. from necrotic to necrotic with pus or to liquefaction (as identified at surgery), which

could have also introduced significant bias into the analysis. We believe that this is a serious methodological limitation to this study which deserves attention, apart from having a significant number of unidentified collections with fluid but without necrosis on CECT.

By the way, the authors erroneously specified at the end of the 2nd paragraph in the Results section under the subheading *Peripancreatic collections* that 5 of 9 unidentified collections on CECT had associated necrosis and 4 had

only fluid without necrosis, whereas it is obvious from Figure 1 that 4 collections had associated necrosis and 5 had no associated necrosis.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Zheng XM



ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

A Mithat Bozdayi, MD, PhD, Hepatology Institute, Department of Gastroenterology, Ankara Medical Faculty, Ankara University, 06100 Cebeci Ankara, Turkey

Dario Conte, Professor, GI Unit - IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

Olav Dalgard, MD, PhD, Department of Medical, Rikshospitalet, 0027 Oslo, Norway

William Dickey, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Michelangelo Foti, Dr., PhD, MBA, Department of Cellular Physiology and Metabolism, Faculty of Medicine, University of Geneva, Centre Médical Universitaire, Geneva 1211, Switzerland

Jon C Gould, MD, FACS, Associate Professor of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States

Imran Hassan, MD, Assistant Professor, Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States

Klaus R Herrlinger, Dr., MD, Department of Gastroenterology, Hepatology and Endocrinology, Robert-Bosch-Hospital, Auerbachstrasse 110, D-70376 Stuttgart, Germany

Eberhard Hildt, Dr., Professor, Molecular Virology-NG1, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany

Takumi Kawaguchi, MD, PhD, Department of Digestive Disease Information and Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

Ali Mencin, MD, Assistant Professor of Pediatrics, Division of Pediatric Gastroenterology, Morgan Stanley Children's Hospital of New York, CHN-702, 3959 Broadway, New York, NY 10032, United States

Sandeep Mukherjee, MBBCh, MPH, FRCPC, Associate Professor of Internal Medicine, Section of Gastroenterology and Hepatology, Nebraska Medical Center, Omaha, NE 68198-3285, United States

Shmuel Odes, Professor, MD, Department of Gastroenterology and Hepatology, Soroka Medical Center, PO Box 151, Beer Sheva 84101, Israel

Stephan Johannes Ott, Dr., PhD, MD, Clinic for Internal Medicine I, University-Hospital Schleswig-Holstein (UK S-H), Campus Kiel, Arnold-Heller-Str. 3, Hs. 6, 24105 Kiel, Germany

Gustav Paumgartner, Professor, University of Munich, Klinikum Grosshadern, Marchioninistr. 15, Munich, D-81377, Germany

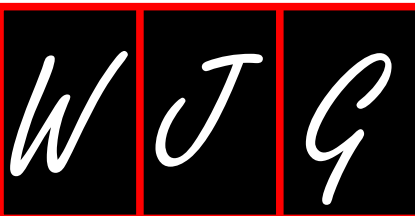
Douglas K Rex, Dr., MD, FACP, FACG, FASGE, Department of Medicine, Indiana University School of Medicine, 550 N. University Blvd., Indianapolis, IN 46202, United State

Ana Cristina Simões e Silva, MD, PhD, Professor, Faculdade de Medicina UFMG, Departamento de Pediatria, sala 267, Avenida Professor Alfredo Balena, 190, Bairro Santa Efigênia, Belo Horizonte, Minas Gerais 30130-100, Brazil

Eric WC Tse, Dr., MB, PhD, Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China

Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

Robert Christiaan Verdonk, MD, PhD, Department of Gastroenterology and Hepatology, University Medical Centre Groningen, Hanzplein 1, Groningen, 9700 RB, The Netherlands



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 04-05, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 07-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano
de Pediatria "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclu-

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

CSSN

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

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When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is <http://www.clinicaltrials.gov> sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

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Title page

Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, e.g. Telephone: +86-10-59080039 Fax: +86-10-85381893

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Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67 , $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

Instructions to authors

uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:....; B:....; C:....; D:....; E:....; F:....; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. ^a $P < 0.05$, ^b $P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, ^c $P < 0.05$ and ^d $P < 0.01$ are used. A third series of P values can be expressed as ^e $P < 0.05$ and ^f $P < 0.01$. Other notes in tables or under illustrations should be expressed as ¹F, ²F, ³F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, ▲, △, etc., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability^[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references^[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Please provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at <http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed> and <http://www.crossref.org/SimpleTextQuery/>, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date,

volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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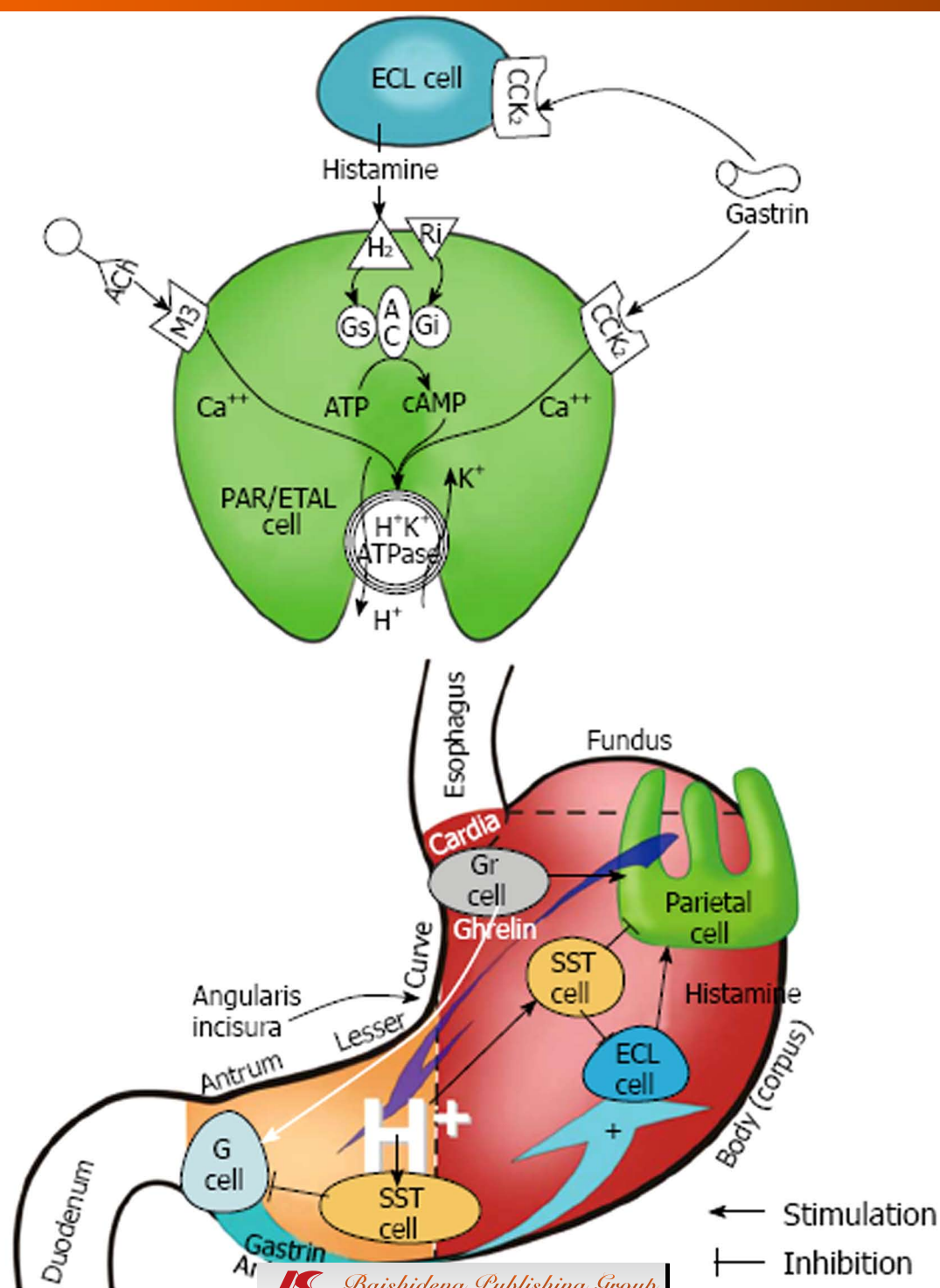
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World J Gastroenterol 2011; 17(4): 449-458
<http://www.wjgnet.com/1007-9327/full/v17/i4/449.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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SUBSCRIPTION
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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
January 28, 2011

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

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Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm. If you do not have web access please contact the editorial office.

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Nestin in gastrointestinal and other cancers: Effects on cells and tumor angiogenesis

Toshiyuki Ishiwata, Yoko Matsuda, Zenya Naito

Toshiyuki Ishiwata, Yoko Matsuda, Zenya Naito, Department of Pathology, Integrative Oncological Pathology, Nippon Medical School, Tokyo 113-8602, Japan

Author contributions: Ishiwata T conceived the topic, reviewed the literature, and prepared the initial manuscript; Matsuda Y and Naito Z contributed to the production of the final manuscript.

Supported by Grants (No. S0801035, to Naito Z) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and a Grant-in-Aid for Young Scientists (A, No. 22689038 to Matsuda Y)

Correspondence to: Toshiyuki Ishiwata, MD, PhD, Department of Pathology, Integrative Oncological Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. ishiwata@nms.ac.jp

Telephone: +81-3-38222131 Fax: +81-3-58146274

Received: September 10, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: January 28, 2011

Abstract

Nestin is a class VI intermediate filament protein that was originally described as a neuronal stem cell marker during central nervous system (CNS) development, and is currently widely used in that capacity. Nestin is also expressed in non-neuronal immature or progenitor cells in normal tissues. Under pathological conditions, nestin is expressed in repair processes in the CNS, muscle, liver, and infarcted myocardium. Furthermore, increased nestin expression has been reported in various tumor cells, including CNS tumors, gastrointestinal stromal tumors, pancreatic cancer, prostate cancer, breast cancer, malignant melanoma, dermatofibrosarcoma protuberances, and thyroid tumors. Nestin is reported to correlate with aggressive growth, metastasis, and poor prognosis in some tumors; however, the roles of nestin in cancer cells have not been well characterized. Furthermore, nestin is more specifically expressed in proliferating small-sized tumor vessels in glioblastoma and gastric, colorectal, and prostate cancers than are other tumor vessel markers. These findings indicate that nes-

tin may be a marker for newly synthesized tumor vessels and a therapeutic target for tumor angiogenesis. It has received a lot of attention recently as a cancer stem cell marker in various cancer cells including brain tumors, malignant rhabdoid tumors, and uterine, cervical, prostate, bladder, head and neck, ovarian, testicular, and pancreatic cancers. The purpose of this review is to clarify the roles of nestin in cancer cells and in tumor angiogenesis, and to examine the association between nestin and cancer stem cells. Nestin has the potential to serve as a molecular target for cancers with nestin-positive cancer cells and nestin-positive tumor vasculature.

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Key words: Cancer growth; Intermediate filament protein; Cancer invasion; Tumor migration; Nestin; Stem cell marker; Tumor angiogenesis

Peer reviewer: Cornelis FM Sier, PhD, Department of Surgery and Gastroenterology, Leiden University Medical Center, Albinusdreef 2, Bldg 1, D6-18, 2333 ZA Leiden, The Netherlands

Ishiwata T, Matsuda Y, Naito Z. Nestin in gastrointestinal and other cancers: Effects on cells and tumor angiogenesis. *World J Gastroenterol* 2011; 17(4): 409-418 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/409.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.409>

INTRODUCTION

Nestin is an intermediate filament (IF) protein that was originally described in 1990 as a neuronal stem cell/progenitor cell marker during central nervous system (CNS) development^[1]. Cytoskeletal proteins mainly consist of microfilaments, IFs, and microtubules. The diameter of microfilaments (actin) is 5-7 nm and that of microtubules is 20-25 nm, while the diameter of IFs falls between the two at 10 nm, giving them their name. IFs are

classified into six subtypes^[2,3], and the IF protein members are expressed in specific cell types, for example, keratin in epithelial cells, vimentin in mesenchymal cells, desmin in muscular cells, neurofilament in neuronal cells, and glial fibrillar acidic protein in glial cells.

Nestin is expressed in dividing cells during the early stages of development in the CNS, peripheral nervous system, and myogenic and other tissues. With differentiation, nestin is downregulated and replaced by tissue-specific IF proteins, and therefore, is widely used as a neuronal stem cell marker. Nestin is also expressed in immature or progenitor cells in non-neuronal cells in normal tissues^[4-7]. High levels of nestin expression have been detected in oligodendroglial lineage cells, ependymocytes, Sertoli cells, enteric glia, hair follicle cells, podocytes of renal glomeruli, pancreatic stellate cells, pericytes, islets, optic nerve, and odontoblasts^[8-14]. Recent work has shown that nestin is also expressed in follicle stem cells and their immediate, differentiated progeny, and the hair follicle bulge area has been noted as an easily accessible source of actively growing pluripotent adult stem cells^[15]. In adult organisms, nestin-expressing cells are restricted to defined locations, where they may function as a cellular reserve that is capable of proliferation, differentiation, and migration after reactivation^[16].

In pathological conditions, nestin is expressed in repair processes in the CNS, muscle, liver^[17-20], and infarcted myocardium^[21]. Furthermore, increased nestin expression has been reported in various tumor cells, including CNS tumors, pancreatic cancer, gastrointestinal stromal tumors (GISTs), prostate cancer, breast cancer, malignant melanoma, dermatofibrosarcoma protuberans, and thyroid tumors^[22-28] (Table 1). Expression of nestin in several tumors has been reported to be closely correlated with poor prognosis. Recently, nestin has also received attention as a cancer stem cell marker in various tumor cells including brain tumors, uterine and cervical cancers, prostate cancer, bladder cancer, head and neck cancers, ovarian cancer, testicular cancer, pancreatic cancer, and malignant rhabdoid tumors^[29-36]. In the tumor tissues, proliferating vascular endothelial cells also highly express nestin, and nestin is therefore closely correlated with tumor angiogenesis^[37-40] (Table 2). Detailed analyses of expression patterns of nestin in various tumor tissues and tumor angiogenesis, including gastrointestinal cancer, will be helpful for examining the roles of nestin in mechanisms of tumor growth and invasion and for finding novel therapeutic targets.

STRUCTURE AND REGULATION OF NESTIN

Nestin is a large protein (> 1600 amino acids) with a highly conserved α -helical core domain of 300-330 amino acids flanked by N- and C-terminal domains and classified into type VI IFs^[1,3,41]. Nestin contains a short N terminus and an unusually long C terminus, which interacts with other IFs including vimentin, desmin, or internexin, form-

Table 1 Expression and roles of nestin in cancers

	Expression patterns	Roles
Glioblastoma ^[66-69]	Tumor cells and tumor vessels Glioma << glioblastoma	<i>In vitro</i> and <i>in vivo</i> growth G1/S arrest Migration, invasion
Pancreatic cancer ^[87,90]	30% of PDAC	Nerve invasion, migration Initiation of PanIN
GIST ^[25,96]	Tumor cells and interstitial cells of Cajal	
Prostate cancer ^[27]	Androgen-insensitive cancer cells 75% of lethal androgen-independent prostate cancer	Migration, invasion <i>in vitro</i> Lung metastasis
Breast cancer ^[26,101-103]	Basal breast cancer subtype Triple-negative breast cancer Lymphovascular embolus of inflammatory breast cancer	Shorter survival Independent prognostic factor
Malignant melanoma ^[24,106-108,112,113]	Tumor cells and endothelial cells Ulceration of primary tumors Infiltrating front of tumors Primary tumor << metastatic tumor Stage IV >> III/IV with no evidence of disease in blood	Advanced stage Metastasis Shorter survival

PanIN: Pancreatic intraepithelial neoplasia; GIST: Gastrointestinal stromal tumor; PDAC: Pancreatic ductal adenocarcinoma.

Table 2 Expression and roles of nestin in tumor angiogenesis

	Expression patterns	Roles
Gastric adenocarcinoma ^[37]	Tumor blood vessels	Shorter survival
Colorectal cancer ^[38]	Small-sized and proliferating tumor vessels	Shorter survival
Prostate cancer ^[39,40]	Proliferating vascular cells	Shorter survival
	Endothelial cells in metastatic bone	Recurrence
		Skeletal metastasis
Glioblastoma ^[131]	Proliferating endothelial cells	
Malignant melanoma ^[108]	Endothelial cells	Shorter survival

ing heterodimers and mixed polymers^[42-44], but in contrast to other IFs, nestin cannot form homopolymers^[2]. Nestin is known to contribute to the disassembly of vimentin during mitosis^[45]. It has been suggested that the long C-terminal portion of nestin protrudes from the filament body and may function as a linker or cross-bridge between IFs and microtubules^[2]. The assembly and disassembly of cytoskeletal IFs modulate a variety of signaling cascades, and several lines of evidence suggest that nestin participates in this regulation^[46]. Nestin may thus play a role in

connecting the three components of the cytoskeleton and coordinate changes in cell dynamics.

Nestin has a high molecular weight (about 240 kDa), which differs among organs because of modifications to the protein^[47]. It has multiple phosphorylation sites and glycosylated side chains, and the phosphorylated and glycosylated forms of nestin may affect intracellular localization or act as a means of functional regulation in specific cell types or brain regions^[48]. Nestin is known to be phosphorylated at Thr³¹⁶ by cdc2 kinase^[49] and/or cyclin-dependent kinase 5^[50], and modulates mitosis-associated cytoplasmic reorganization during mitosis. However, the roles of glycosylation have not been closely examined^[51].

The minimal promoter of the mouse nestin gene resides in the region -11 to +183 of the 5' non-coding and upstream flanking regions, and two adjacent Sp1-binding sites are necessary for promoter activity. Sp1 and Sp3 proteins are reported to regulate the expression of the mouse nestin gene^[52]. The nestin gene has four exons and three introns, and neural cell-specific expression is reported to be regulated by the second intron, whereas nestin expression in tumor endothelium is enhanced by the first intron^[53]. Nestin expression in muscle precursor cells is regulated by temporally and spatially restricted enhancer elements in the first intron^[54]. Furthermore, the epigenetic regulation of nestin transcripts has been reported; histone acetylation is sufficient to mediate the activation of nestin transcription, but DNA demethylation is not^[55]. Tissue- and cell-specific and spatiotemporal regulation of nestin is important for cell behavior during development or in pathological conditions. These observations also suggest that nestin is more than just a structural protein that serves as a progenitor stem cell marker.

NESTIN IN CNS TUMORS

Nestin has been implicated in the rapid proliferation of progenitor cells during neurogenesis^[56]. However, when precursor cells differentiate into neural or glial cell types, nestin expression is downregulated or disappears^[54,57]. Nestin mRNA is expressed at a high level in the cerebrum of the developing rat embryo on embryonic day 15, and the level decreases toward postnatal day 12, disappearing from postnatal day 18 to the adult stage^[1]. Cells that express nestin have been found at the ventricular border in mammalian brains, and these cells give rise to neurons and glia in avian models^[58,59]. Nestin expression has not been detected in normal astrocytes but is transiently detected in reactive astrocytes accompanying, for example, trauma, tumor growth, or neurodegenerative diseases in brain tissue^[19,60-62].

Nestin expression has been reported in tumor cells originating from the CNS, including astrocytoma, ependymoma, oligodendroglioma, glioblastoma, and primitive neuroectodermal tumors^[63-66]. Nestin has been detected in human gliomas, including low and high grade, but its expression has been observed more frequently in high-grade than in low-grade gliomas, such as pilocytic astrocy-

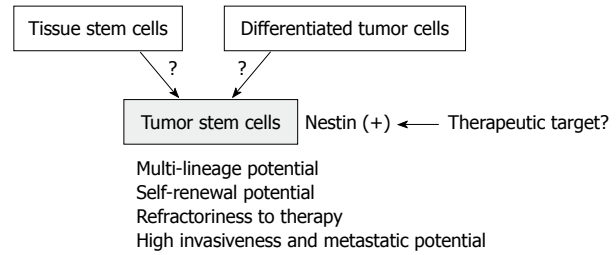


Figure 1 Tumor stem cells and nestin. Tumor stem cells have specific characteristics, including multi-lineage potential, self-renewal potential, refractoriness to therapy, and high invasiveness and metastatic potential. The origin of tumor stem cells has not been well clarified, but it is known that some tumor stem cells express nestin in their cytoplasm. Nestin in tumor stem cells thus is considered to be a novel therapeutic target.

tomas^[66,67]. The downregulation of nestin in glioblastomas induces cell cycle arrest at the G1/S phase^[68]. However, the roles of nestin in glial cell tumors have not been well clarified. Recent work has shown that nestin expression does not influence the *in vitro* proliferation of glioblastoma cell lines, while subclones characterized by high levels of nestin form larger tumors *in vivo* than those with low expression. Furthermore, blocking the expression of nestin in glioblastoma tumors via intratumoral injection of short hairpin RNA (shRNA) significantly slows tumor growth and volume^[69]. We have also found that expression of nestin correlates with cell growth, migration, and invasion in low- and high-grade gliomas. These findings demonstrate that nestin plays important roles in the development of glioblastomas and may potentially be a target for treatment of the disease.

Brain tumor stem cells (BTSCs), obtained by cell sorting of dissociated suspensions of tumor cells for the NSC marker CD133^[70,71], also express nestin but not differentiated neural lineage markers. These CD133⁺, nestin⁺ cells represent a minority fraction of the entire brain tumor cell population, exclusively generate clonal tumor spheres in suspension culture, and exhibit increased self-renewal capacity. These findings suggest that nestin serves as a BTSC marker. Furthermore, it has been reported that tumor stem cells play crucial roles in tumor proliferation, invasion, and metastasis; therefore, nestin may be closely associated with these tumor stem cell functions. The origin of tumor stem cells has been controversial, but nestin may be a novel therapeutic target to suppress them (Figure 1).

NESTIN IN PANCREATIC CANCER

During early embryonic development, neuronal and islet cells in the pancreas share many phenotypic properties, and developing islet cells express several neuronal-specific markers^[72-74]. In the adult pancreas, nestin-positive cells were initially described as a specific subpopulation of cells located in the endocrine islets, with a possible stem cell function^[75]. Nestin-expressing cells also reside in the pancreatic ducts, where they may function as possible progenitor cells^[76]. Nestin has been used as a selection marker for neuronal and pancreatic endocrine precursor cells^[77,78] dur-

ing differentiation assays using embryonic and adult stem cells. Additionally, the isolation of nestin-expressing cells from rat and human islets, and their *in vitro* differentiation into pancreatic endocrine and exocrine cells, has led to the suggestion that nestin-positive cells have a role as multipotent pancreatic stem cells^[76]. Moreover, nestin-positive cells do not necessarily serve as endocrine precursors during pancreas development in mice, rats and humans, or in a regenerating model of adult rat pancreas^[11,79-81].

Lineage-tracing experiments have indicated that exocrine cells are derived from nestin-expressing progenitor cells in the pancreas^[82-85]. In adult pancreas, localization of nestin has been reported in vascular endothelial cells and acinar cells at different levels but not in endocrine cells^[86-89]. In the regenerative process of a rat acute pancreatitis model, nestin expression was observed in proliferating capillary endothelial cells, stellate cells surrounding ductular structures, and submesothelial cells^[82].

Concerning nestin and pancreatic cancer, it has been demonstrated that activation of oncogenic K-ras in the nestin cell lineage is sufficient for initiation of premalignant pancreatic intraepithelial neoplasia in mice^[90]. We have reported that nestin immunoreactivity is present in the cancer cells in about 30% of pancreatic ductal adenocarcinoma (PDAC) cases, and nestin expression in pancreatic cancer cells correlates with nerve invasion and the presence of cancer cells at the tumor resection margins^[87]. We recently found that nestin expression also correlates with migration, invasion, and metastasis of pancreatic cancer cells.

Regarding pancreatic tumors other than PDAC, nestin expression has been reported in acinar cell carcinoma, pancreatoblastoma, solid-pseudopapillary neoplasm, and serous cystadenoma^[91]. However, nestin is rarely detected in intraductal papillary-mucinous neoplasms, mucinous cystic neoplasm, or undifferentiated carcinoma cases.

NESTIN IN GISTS

Mesenchymal tumors consisting of spindle-shaped cells develop in the gastrointestinal tract, and they were at first believed to originate from smooth muscle or neuronal cells. However, subsequent studies have shown that most of the tumors do not have the typical features of smooth muscle or neuronal cells; therefore, the most common mesenchymal tumors that differ from leiomyomas or schwannomas are designated as GISTs. Most GISTs express *c-kit* receptor tyrosine kinase (KIT) and CD34; both of which are expressed in hematopoietic stem cells^[92-95]. In some studies, nestin expression has been identified in most GIST cases examined but not in leiomyomas^[25,96]. However, a subsequent study from the same group has shown that nestin is also highly expressed in gastrointestinal schwannomas; thus, nestin may not be a definitive marker for GIST^[96]. Nestin expression has also been reported in granular cell tumors, considered to be benign neoplasms of Schwann cell origin in the gastrointestinal tract^[97].

In the normal gastrointestinal tract, intestinal cells of Cajal (ICCs), which are localized between the circular and longitudinal muscle layers, express KIT and CD34. ICCs are assumed to originate from mesenchymal progenitor cells that can also differentiate into smooth muscle cells^[98,99]. Expression of nestin in the ICCs and GIST supports the hypothesis that GIST is derived from ICCs.

NESTIN IN PROSTATE CANCER

Nestin is highly expressed in androgen-insensitive prostate cancer cell lines, but it has not been detected in androgen-dependent prostate cancer cells^[27]. Furthermore, nestin has been localized in 75% of lethal androgen-independent prostate cancer cases, but is undetectable in localized androgen-deprived tumors and in metastases without prior androgen deprivation. Work using shRNA against nestin has shown a marked decrease of migration and invasion of prostate cancer cells *in vitro*, and nestin knockdown in prostate cancer cells inhibits lung metastasis of the cells^[27]. Furthermore, it has been reported that nestin is a tumor stem cell marker of prostate cancer^[36,100]. The underlying mechanisms have not been well examined, but nestin may be a novel therapeutic target for preventing the metastatic and cancer stem cell potential of prostate cancer.

NESTIN IN BREAST CANCER

In normal human breast tissues, nestin is expressed in the cells within the basal/myoepithelial layers^[26,101,102] and may be used as a myoepithelial marker. In one of these cell types, nestin is co-expressed with Δ N-p63. This finding, coupled with the role of Δ N-p63 in preservation of self-renewal, suggests that nestin may be expressed in the regenerative compartment within the mammary gland. Furthermore, nestin and Δ N-p63 are coordinately expressed during pregnancy in the murine mammary gland^[26].

Among the breast cancer subtypes, nestin is highly expressed in basal breast cancer subtype (ER α ⁻/PR⁻/Her2) but not in the Her2 subtype (ER α ⁻/PR⁺/Her2⁺) or luminal epithelial phenotype (ER α ⁺/PR⁺)^[26]. The other group showed that triple-negative breast cancers, which do not express ER, PR, and Her2, have higher expression rates for nestin than other breast cancers^[103]. Furthermore, nestin expression has been associated with shorter survival and shown to be an independent prognostic factor of breast cancer^[103]. Another group has reported significantly high nestin expression in basal-like and triple-negative breast cancers in a cohort of 245 patients with invasive breast cancer treated with surgery followed by anthracycline-based chemotherapy^[104]. These findings indicate that nestin is a selective marker of the basal breast cancer subtype (triple negative), which displays aggressive growth and has a poor prognosis. Co-expression of nestin and melatonin receptor 1 (MT 1) in breast cancer cells has been reported in patients with higher stages (T II / III) and with a high risk of relapse. Co-expression of nestin and MT 1 may correlate with invasive breast cancer and ad-

vanced tumors^[102]. Lymphovascular emboli of inflammatory breast cancer, a particularly lethal form of breast cancer characterized by exaggerated lymphovascular invasion, express stem cell markers including nestin^[105]. These data indicate that nestin correlates with an aggressive growth phenotype and lymphatic invasion.

NESTIN IN MALIGNANT MELANOMA

Nestin is expressed in benign nevi and melanoma but not in basal cell carcinoma^[106]. Nestin expression is higher at the advanced stage in melanoma and in metastatic foci of melanoma cells^[24,107]. Nestin staining in stage I and II melanoma patients significantly predicts poor survival, with lower survival rates in cases with nestin positivity in tumoral and endothelial cells^[108]. Furthermore, the 5-year survival rate exceeded 80% in nestin-negative melanoma at all stages of tumor development^[109]. Nestin and SOX9 and SOX10 transcription factors are co-expressed in melanoma cells, and downregulation of SOX9 and SOX10 markedly decreases nestin levels^[110,111]. Furthermore, nestin has been significantly associated with the presence of ulceration in primary tumors of melanoma and with SOX9 in the more advanced state. These findings indicate that nestin and SOX9 may be negative prognostic markers in melanoma. Nestin protein has been shown to occur most abundantly at the infiltrating front of the tumors, suggesting that nestin plays important roles in melanoma cell migration and invasion^[106].

Nestin expression in the peripheral blood of melanoma patients has been examined using flow cytometry, and expression is higher in stage IV patients as compared with stage III/IV with no evidence of the disease^[112,113]. Nestin thus may be an additional marker of interest for circulating melanoma cells in the future.

Immunohistochemically, melanoma antigen-encoding-1 (MAGE-1), melanocyte-specific transcription factor, tyrosinase, and Melan-A have been reported as useful markers in the diagnosis of melanotic parts when HMB-45 is negative^[114]. However, nestin has been more specifically detected in HMB-45-negative melanoma cells in the dermal portions of melanotic and amelanotic nodular melanomas^[115]. Nestin thus also may be a useful diagnostic marker for HMB-45-negative melanoma.

NESTIN IN OTHER TUMORS

Few reports have addressed nestin expression in other types of tumor cells. Its expression has been reported in various kinds of thyroid tumors, and nestin mRNA has been detected in differentiated thyroid tumors but not in anaplastic carcinoma^[22]. Nestin mRNA is also expressed in normal thyroid tissues, therefore, the authors of the above study have suggested that nestin mRNA is not associated with the malignant characteristics of thyroid tumors. Dermatofibrosarcoma protuberans (DFSP) is a dermal and subcutaneous neoplasm of intermediate malignancy that is invasive and locally aggressive with frequent recurrence.

Histopathologically, the differential diagnosis between DFSP and dermatofibroma (DF) is important because DF is benign^[28]. In one study, nestin was found to be strongly expressed in DFSP, while all DFs examined were nestin negative. Based on these findings, nestin may serve as an additional marker for DFSP and for surgical margin evaluation of DFSP.

NESTIN IN TUMOR ANGIOGENESIS

Tumor angiogenesis is an important factor in the proliferation, metastasis, and drug sensitivity of human neoplasms. A possible explanation of this metastatic mechanism is that the increased number of tumor vessels increases the chances for tumor cells to enter the circulation. Newly formed tumor vessels or capillaries have leaky and weak basement membranes; thus, tumor cells can penetrate these more easily than they can mature vessels^[116]. Furthermore, increased tumor vessels supply abundant oxygen and nutrition to the tumor cells. Angiogenesis in malignant tumors, as measured by microvessel density (MVD), has been reported to correlate with clinicopathological factors or survival in breast, ovarian, esophageal, gastric, colorectal, and prostate cancers, malignant melanoma, and non-small-cell lung carcinoma^[117-124]. CD34, CD31, and factor-VIII-related antigen are commonly used as endothelial cell markers of tumor vessels, and MVD is determined based on staining of blood vessels with these markers^[125-127]. However, the markers identify not only newly formed small tumor blood vessels but also pre-existing large blood vessels^[128].

Nestin expression in endothelial cells accompanying the process of angiogenesis has been reported^[129]. In pathological conditions, nestin is strongly expressed in proliferating endothelial cells in acute pancreatitis^[82] and vascular malformations^[130]. Furthermore, nestin has been reported to be a marker of proliferating endothelial cells in brain tumor tissues^[131] (Table 2). In gastric adenocarcinoma, MVD determined by nestin correlates better with patient survival than MVD determined by CD34 when the size of the carcinoma exceeds 5 cm^[37]. We examined the effectiveness of nestin as an angiogenic marker in colorectal cancer^[38]. Diameters of nestin-positive and CD34-positive vessels were compared in subcutaneous colorectal cancer tumors formed in nude mice. Nestin was localized in the endothelial cells in small tumor blood vessels, whereas CD34 was localized in large blood vessels in nude mice. Furthermore, the diameter of nestin-positive vessels was smaller than that of CD34-positive vessels in human colorectal cancer. The ratio of proliferating cell nuclear antigen (PCNA)-positive to nestin-positive vascular endothelial cells was higher than that of PCNA-positive to CD34-positive cells. These findings indicate that nestin is expressed in small-sized and proliferating tumor vessels in colorectal cancer. Further, prognosis is worse in the highly nestin-positive MVD population of colorectal cancer patients. In pancreatic cancer tissues, CD31 is expressed in endothelial cells of most blood vessels, while nestin is

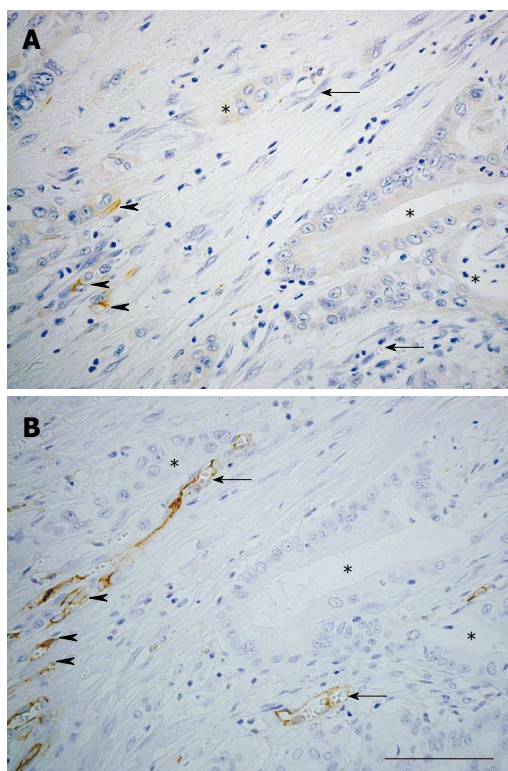


Figure 2 Expression of nestin and CD31 in pancreatic cancer tissues. Nestin is expressed in endothelial cells of small blood vessels (A, arrowheads) and cancer cells (asterisks), whereas it is not detected in large blood vessels (arrows). CD31 is expressed in endothelial cells of most blood vessels (B, arrowheads and arrows), but not in cancer cells (B, asterisks) in pancreatic tissues. Immunohistochemistry: bar, 100 μ m.

specifically expressed in those of small-sized blood vessels (Figure 2).

Recently, co-expression of nestin and Ki-67 has been shown to be a vascular proliferation marker in prostate cancer^[39], and vascular proliferation is of independent prognostic importance among prostate cancer. Furthermore, vascular proliferation is significantly increased in castration-resistant cases and metastatic lesions compared with localized cancers. Very recently, nestin expression has been reported in the endothelial cells of bone metastatic lesions of prostate cancer^[40]. These results indicate that nestin correlates with tumor angiogenesis in primary and metastatic lesions, and that nestin may be a novel molecular target for inhibition of tumor angiogenesis, as are vascular endothelial growth factor receptors (Figure 3).

CONCLUSION

Nestin is highly expressed in various kinds of cancer cells and proliferating tumor vasculature. The roles of nestin in cancer cells have not been clarified fully, although nestin correlates with growth, migration, invasion, and metastasis of some cancers. Nestin is also highly expressed in proliferating vascular endothelial cells in cancer tissues and metastatic lesions. These findings indicate that this protein may become a new molecular target for nestin-positive

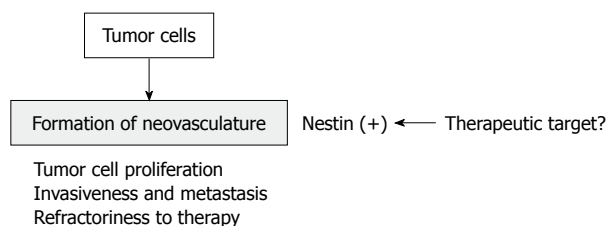


Figure 3 Tumor angiogenesis and nestin. Tumor cells induce neovascular formation, known as tumor angiogenesis. Formation of the neovasculature leads to tumor aggressiveness through tumor cell proliferation, invasiveness, metastasis, and refractoriness to therapy. Nestin expression has been reported in tumor vessels, and nestin may be a new molecular target for tumor angiogenesis.

cancer cells and tumor vessels. Furthermore, nestin is highly expressed in tumor stem cells in various tissues, and research concerning its expression and roles in various tumors may provide us important information about the origin of cancer stem cells and differentiation of cancer stem cells into cancer cells.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH

Peginterferon and ribavirin treatment for hepatitis C virus infection

Akihito Tsubota, Kiyotaka Fujise, Yoshihisa Namiki, Norio Tada

Akihito Tsubota, Kiyotaka Fujise, Yoshihisa Namiki, Norio Tada, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Author contributions: Tsubota A, Fujise K, Namiki Y and Tada N all generated the ideas and contributed to the writing of this manuscript.

Correspondence to: Akihito Tsubota, MD, PhD, Associate Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan. atsubo@jikei.ac.jp

Telephone: +81-4-71641111 Fax: +81-4-71668638

Received: June 28, 2010 Revised: August 2, 2010

Accepted: August 9, 2010

Published online: January 28, 2011

Peer reviewers: Roberto J Carvalho-Filho, MD, PhD, Hepatitis Section, Division of Gastroenterology, Federal University of Sao Paulo, Rua Botucatu, 740, 2.o andar, Vila Clementino, State of Sao Paulo, 04023-060, Brazil; Seung Woon Paik, MD, PhD, Professor, Division of Gastroenterology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Tsubota A, Fujise K, Namiki Y, Tada N. Peginterferon and ribavirin treatment for hepatitis C virus infection. *World J Gastroenterol* 2011; 17(4): 419-432 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/419.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.419>

Abstract

Pegylated interferon α (IFN α) in combination with ribavirin is currently recommended as a standard-of-care treatment for chronic hepatitis C virus (HCV) infection. This combination therapy has drastically improved the rate of sustained virological response, specifically in difficult-to-treat patients. Recently, individualized treatment, such as response-guided therapy, is being developed based on host-, HCV- and treatment-related factors. Furthermore, modified regimens with currently available medications, novel modified IFN α and ribavirin or combinations with specifically targeted antiviral therapy for HCV agents, are currently being investigated. The purpose of this review is to address some issues and epoch-making topics in the treatment of chronic HCV infection, and to discuss more optimal and highly individualized therapeutic strategies for HCV-infected patients.

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Key words: Pegylated interferon α ; Ribavirin; Chronic hepatitis C virus infection; Difficult-to-treat patient; Individualized treatment; Response-guided therapy; Specifically targeted antiviral therapy for hepatitis C virus

INTRODUCTION

Pegylated interferon α (peginterferon α , peg-IFN α) in combination with weight-based doses of ribavirin (RBV) is currently recommended as the first-line “standard-of-care” treatment for chronic hepatitis C virus (HCV) infection^[1]. The pegylated formulation prolongs the half-life of conventional IFN α by covalently binding it to the polyethylene glycol (PEG) molecule, leading to improvement in the overall rate of sustained virological response (SVR) from < 20% to > 60%; 40%-60% of “difficult-to-treat” genotypes 1 and 4 patients with 48-wk treatment, and 70%-90% of “easy-to-treat” genotypes 2 and 3 patients with 24-wk treatment^[1-5].

A recent trend in the treatment strategy of chronic HCV infection is the development of individualized treatment regimens based on strong predictors of SVR to IFN-based treatment, such as HCV genotype^[2-4,6-10] and the initial virologic response to treatment^[9,11-19]. Meanwhile, alternative options, such as modified regimens with currently available medications, novel modified IFN α and RBV or combinations with specifically targeted antiviral therapy for HCV (STAT-C) agents, are currently being investigated for the growing number of patients for whom current “standard-of-care” treatment has failed. For the

foreseeable future, however, peg-IFN α and RBV appear to remain the backbone of “standard-of-care” treatment.

This review addresses and summarizes some issues and epoch-making topics in the treatment of chronic HCV infection, and discusses more optimal and highly individualized therapeutic strategies for patients infected with HCV.

FACTORS ASSOCIATED WITH SVR

SVR is defined as an undetectable qualitative HCV RNA level (by using a qualitative polymerase chain reaction assay) at 24 wk after the completion of treatment. Identification of factors predictive of SVR, including host-, virus- and treatment-related elements, provides relevant insights about the mechanisms of action of IFN α and RBV. So far, numerous factors have been identified as significant predictors of SVR or non-SVR. Strong predictors of the response to non-pegylated IFN α monotherapy, such as HCV genotype, pretreatment viral load and fibrosis stage, are also significantly associated with the outcome of peg-IFN α plus RBV combination therapy. Most importantly, by recognizing these factors, therapy can be tailored to individual needs, helping to make decisions regarding whether treatment should be initiated, continued or stopped. Individualized treatment regimens determined according to outcome predictors should increase the SVR rate and reduce unnecessary patient and social burdens, such as medical costs, side effects/adverse events associated with treatment, and treatment-related absenteeism.

Host-related factors

A number of pretreatment factors are known to reduce the SVR rate: older age, presence of cirrhosis or advanced fibrosis, African American race, overweight, obesity, diabetes, low alanine aminotransferase (ALT), abnormal baseline fasting glucose, low level of cholesterol, low hemoglobin, low platelet count, insulin resistance and hepatic steatosis^[2-4,20-29]. The contribution of gender to treatment outcome is controversial as it varies among studies^[26,29-32].

The response to treatment of patients with genotype 1 has been reported recently to be strongly associated with a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene that resides on chromosome 19 and encodes IL28B or IFN-lambda-3^[32-34]. Patients with favorable genotypes at the SNPs (such as rs12979860^[33], rs12980275 and rs8099917^[32,34]) near the *IL28B* locus are more likely to achieve SVR than those with unfavorable genotypes. At present, the SNPs near the *IL28B* locus seem to be one of the strongest pretreatment predictors of SVR to peg-IFN α plus RBV or triple combination therapy including STAT-C agents, in addition to the HCV genotype. The population distribution of the favorable SNP genotype is significantly more prevalent in the Caucasian and Asian populations than in African Americans. Specifically, rs12979860 shows close correlations with the SVR rate and ethnicity: rs12979860 C-allele frequency is the highest in East Asians who show the highest rate of SVR among diverse ethnic groups^[33]. Therefore, racial dif-

ferences in treatment outcome may arise from divergence in host genomic genotype related to treatment response. Individualized therapeutic strategies should always consider ethnicity of individual patients as well as SNPs. However, it is highly unlikely that SNPs could be used alone to define different treatment strategies, since approximately 30%-40% of patients do not have the favorable genotype CC of rs12979860^[33].

HCV-related factors

HCV genotype, pretreatment viral load and initial virologic response are strong independent predictors of SVR to IFN monotherapy^[6-15,18,19,35]. These factors also have significant and independent impact on treatment outcome of conventional IFN α /peg-IFN α plus RBV combination therapy or triple combination therapy with telaprevir^[2-4,16,17,21,22,28,29,36-40]. Patients infected with “easy-to-treat” genotypes 2 and 3 respond much better than those with “difficult-to-treat” genotypes 1 and 4. Furthermore, those with low pretreatment viral load respond much better than those with high pretreatment viral load, although the cutoff value that discriminates between high and low viremia varies among studies. The earlier the serum HCV RNA becomes negative during the initial phase of treatment, the greater the likelihood of achieving SVR.

Although there are relatively few data regarding genotypes 4, 5, and 6, HCV genotypes can be ranked, in a decreasing order of susceptibility to IFN-based treatment, as follows: genotypes 2, 3, 4 and 1. Furthermore, genotype 1a rather than 1b and genotype 2a rather than 2b are likely to respond better to IFN-based therapy. Interestingly, resistant variants against telaprevir (an NS3/4a protease inhibitor) and viral breakthrough occur more frequently in genotypes 1a than in 1b for telaprevir alone or in combination with peg-IFN α (with or without RBV)^[40-42]. The different frequency results from nucleotide differences at amino acid position 155 of the nonstructural (NS3) protease region between genotypes 1a (aga, encodes R) and 1b (cga, also encodes R). The amino acid substitution of R with K at the position (R155K), which is most frequently related to telaprevir resistance, requires only one nucleotide substitution in genotype 1a and two substitutions in 1b. Similarly, the emergence of the resistant mutant R155K against boceprevir (an NS3 protease inhibitor) differs between genotype 1a and 1b^[43]. Thus, HCV sub-genotype as well as HCV genotype should be taken into consideration in triple combination therapy with peg-IFN α /RBV/STAT-C agent.

Two recent reports discuss the influence of wild and mutant types in the core or NS5 region of the HCV genome on treatment outcome of peg-IFN α plus RBV combination therapy in Japanese patients with genotype 1 and high pretreatment viral load^[31,44].

Treatment-related factors

The doses of both peg-IFN α and RBV are important and have significant impact on SVR^[2]. The likelihood of SVR increases as RBV dose (measured in mg/kg) increases^[2,4]: patients who receive peg-IFN α -2b plus a RBV dose of

10.6 mg/kg per day or more have a greater chance of achieving SVR than those receiving lower RBV doses^[2]. Furthermore, the moving average of SVR rates increases as RBV dose increases up to about 13 mg/kg, and is almost level between 13 and 15 mg/kg. Combination therapy with peg-IFN α -2a plus RBV for 48 wk is more effective in HCV genotype 1 patients if the ribavirin dose is 1000 or 1200 mg/d rather than 800 mg/d^[4]. In many cases, hemoglobin concentrations decrease drastically due to RBV-related hemolytic anemia (especially during the first 4 wk), and it may be necessary to reduce RBV dose and/or to initiate the use of erythropoietin. Any reduction in the RBV dose during the first 12-20 wk of therapy could have a larger negative influence on SVR rates than reductions in peg-IFN α dose^[21,45,46], although maintaining RBV exposure over the whole duration of therapy is crucial^[47,48]. Furthermore, a reduction in peg-IFN α dose during the first 12 wk could reduce the rate of early virologic response (EVR, defined as at least a 2-log decrease from the baseline levels or no detectable viremia) by 10%, but an additional reduction in RBV dose during this time was shown to decrease the EVR rate by another 37%^[45].

Adherence is important to achieve SVR^[49] and patients who take at least 80% of the prescribed total dose of the two drugs for at least 80% of the planned time are considered to be adherent (the “80/80/80” rule). For genotype 1 patients with unfavorable factors, more intensive therapy is recommended including a higher dosage of RBV and a longer duration of treatment or the use of STAT-C agents (such as telaprevir) as the initial therapy. Recent direct comparative trials, retrospective and meta-analysis studies demonstrated that treatment with peg-IFN α -2a is a significant and independent contributor to SVR in patients infected with genotype 1 or 3, compared to treatment with peg-IFN α -2b^[25,26,28,29], although the largest head-to-head trial (IDEAL study) failed to find a significant difference in SVR rates between the two peg-IFN α formulations^[27].

On-therapy response

HCV kinetics during the early phase of treatment are closely associated with SVR or non-SVR^[11-15,18,19]. Patients with rapid virological response (RVR), defined as undetectable HCV RNA levels at treatment week 4, have a better likelihood of achieving SVR, and this is a strong independent on-therapy predictor^[19,50-54]. The viral response is influenced by host-, virus- and treatment-related factors: young age, lean body, low baseline viral load and HCV genotype^[55]. Conversely, the probability of SVR is less than 5% in patients with a minimal fall in viral load of $< 1 \log_{10}$ from the baseline level at treatment week 4, even when peg-IFN α and RBV are combined with telaprevir^[40].

EVR is an important on-therapy indicator of the final treatment outcome: 65% of EVR patients have been reported to show SVR^[3]. Those with no detectable viremia at week 12 (complete EVR) had SVR compared with those who had only a 2-log decrease from the baseline level (75% *vs* 33%). In contrast, a lack of EVR was associ-

ated with no SVR in 97% of the patients^[3]. Such viral suppression during the initial phase is of crucial importance to resolve persistent viremia.

RESPONSE-GUIDED THERAPY

The extent of reduction in HCV RNA during the initial treatment phase is closely associated with the likelihood of achieving SVR^[11-19]. The more rapidly HCV RNA becomes negative during treatment, the higher the rate of SVR. This fact suggests that the rapidity of viral response could be used to modify the duration of treatment, and hence the design of response-guided therapy (RGT). RGT is a dynamic treatment algorithm that involves individualized treatment based on the virologic response. Based on the briskness of the viral response, the treatment duration of 48 wk could be shortened to 24 wk or extended to 72 wk in patients infected with genotype 1 or 4, whereas 24-wk duration could be reduced to 12-16 wk in “easier-to-treat” genotype 2 or 3 patients. The rationale for extending the duration of treatment is to increase the likelihood of achieving SVR and to reduce virological relapse after treatment^[56-58]. Conceivably, the shortened treatment would improve the overall tolerability and reduce exposure to unnecessary medication and economic burden. Likewise, a shorter therapy would avoid premature termination of treatment, while maintaining satisfactory SVR rates.

Genotype 1 or 4

The time points usually used to decide whether treatment should be stopped or continued are treatment weeks 4, 12 and 24, which constitute the basis for RGT^[11,19]. Among genotype 1 or 4 patients with RVR, the likelihood of SVR is approximately 80%-90% when treated for 48 wk^[19,27,51,59,60]. The existence of this patient subpopulation encouraged investigators to shorten the treatment duration to 24 wk without lowering the SVR rate. In patients with RVR treated for 24 wk, the SVR rate was 79%-89% for genotype 1 and 86%-87% for genotype 4^[50,52,55,60]. Specifically, these studies showed that abbreviated 24-wk regimens are best suited to genotype 1 or 4 patients with low baseline viremia who achieve RVR.

In contrast, patients who respond later have a lower likelihood of SVR and greater probability of virologic relapse when treated for 48 wk^[2,3,5,18,19,45,49]. Furthermore, the likelihood of achieving SVR is little or none in patients who do not show EVR or undetectable HCV RNA at week 24. Accordingly, a 12-wk stopping rule is widely accepted in patients who fail to achieve EVR^[1]. However, the negative predictive value for SVR in such patients could be reduced from over 95% to 85% by extending the treatment duration to 72 wk. Several studies investigated whether extending treatment to 72 wk increases the SVR rate in patients without RVR randomized to 48- or 72-wk regimens (Table 1)^[56-58,61]. In genotype 1 patients without RVR (including those with EVR who become HCV RNA-negative for the first time at treatment week 24, so called slow viral response), prolongation of treatment from 48 to 72 wk increases the likelihood of achieving

Table 1 Randomized, controlled trials for 48 wk *vs* 72 wk of peginterferon plus ribavirin in treatment-naïve patients infected with genotype 1 or 4

Authors	Country	Response criteria (time point) for randomization	Peg-IFN α /ribavirin	SVR rate in genotype 1 (48 wk <i>vs</i> 72 wk), <i>n</i> (%)	Difference (%)	<i>P</i> value	SVR rate in genotype 4 (48 wk <i>vs</i> 72 wk), <i>n</i> (%)
Sánchez-Tapias <i>et al</i> ^[57] , 2006	Spain	Non-RVR (at wk 4)	2a: (180 µg/wk)/ 800 mg/d	41/149 (28) <i>vs</i> 63/142 (44)	16	0.003	12/16 (75) <i>vs</i> 10/19 (53)
Berg <i>et al</i> ^[56] , 2006	Germany	Before treatment (Subgroup analysis for non-EVR)	2a: (180 µg/wk)/ 800 mg/d	17/100 (17) <i>vs</i> 31/106 (29)	12	0.040	-
Pearlman <i>et al</i> ^[58] , 2007	USA	Slow response (at wk 24)	2b: (1.5 µg/kg per week)/ 800-1400 mg/d	9/49 (18) <i>vs</i> 20/52 (38)	20	0.026	-
Ferenci <i>et al</i> ^[61] , 2010	Austria	EVR (at wk 12)	2a: (180 → 135 µg/wk)/ 1000-1200 mg/d	65/127 (51) <i>vs</i> 81/134 (60)	9	0.137	6/12 (50) <i>vs</i> 7/16 (44)

Peg-IFN α : Pegylated interferon α ; SVR: Sustained virological response; RVR: Rapid virological response; EVR: Early virological response.

SVR and reduces the probability of relapse. The rates of virologic response at the end of treatment and adverse events are similar between 48- and 72-wk regimens, although the rates of withdrawal from treatment and subsequent follow-up are higher in the latter than in the former.

However, some issues should be noted and carefully addressed to interpret the results of randomized controlled trials (RCTs), because they have differed in treatment regimens, criteria and time points for randomization, and study population. Firstly, to resolve these differences, investigators should identify patients who will benefit from 72-wk treatment, and the best time points and the response criteria to be used to prolong the duration of treatment. Secondly, it may be better to distinguish between “complete” (undetectable HCV RNA) and “partial” (> 2-log HCV RNA drop from baseline but detectable viremia) EVRs. Currently, we comply with the following recommendation for the extended 72-wk treatment in clinical practice: when patients do not achieve complete EVR but have slow viral response, they are advised to prolong the treatment to 72 wk. In the near future, it should be clarified whether extension of treatment to longer than 72 wk further would increase the SVR rate. Thirdly, it is not always clear whether patients with genotype 1 and 4 are treated in an identical manner in RGT, because the number of studied genotype 4 patients has been very small.

Genotype 2 or 3

Patients with genotype 2 or 3 are more susceptible to peg-IFN α plus RBV treatment than those with genotype 1 or 4, and the current recommendation advocates a 24-wk treatment course, because more than 80% of the former group will attain SVR^[4,22,37,38,45,62]. Several small studies showed that the treatment duration could be shortened from 24 to between 12 and 16 wk without adversely affecting outcome in patients with RVR at week 4^[36-38,62-64]. However, the results of large trials clearly indicated that shortening the treatment duration to 16, 14, or 12 wk significantly lessened the SVR rates, because of a higher rate of virological relapse^[39,62,65]. The results of these studies suggest that patients with RVR have high probability of achieving SVR regardless of treatment duration, but that the risk of relapse increases with abbreviated treatment. Still, the pros

and cons of the abbreviated treatment do not allow the making of a firm conclusion at present. Conversely, there is little information on the most suitable duration of treatment for patients with genotype 2 or 3 who do not achieve RVR. In this regard, there is a need to verify whether treatment week 4 is appropriate for prediction of outcome in genotype 2 or 3 patients, because the susceptibility to IFN-based therapy at the recommended duration of treatment apparently differs between genotypes 1/4 and 2/3.

Interestingly, there is ample evidence that peg-IFN α plus RBV treatment is more beneficial in patients infected with genotype 2 than those with genotype 3^[22,29,37,39,63,65]. This suggests that the two genotypes are not a homogeneous group, and that treatment regimens should perhaps be tailored or individualized for each genotype, with a special emphasis on the duration of treatment. Strictly speaking, the susceptibility to IFN-based treatment somewhat differs between sub-genotypes (e.g. genotype 1a *vs* 1b or genotype 2a *vs* 2b). For instance, treatment with peg-IFN α alone for 4 and 12 wk produced SVR rates of 91% and 100%, respectively, in genotype 2a patients with low viral load who became HCV RNA-negative at treatment week 1^[66]. Such subgrouping of patients with some strong predictors could further shorten the treatment duration with preservation of a high SVR rate.

Virologic response at critical time points, viral load at baseline, and HCV kinetics during the initial treatment phase provide useful information for tailoring or individualizing treatment to a given individual, leading to substantial reductions in both patient and society burdens without adversely affecting treatment outcomes.

DIFFERENCES BETWEEN PEG-IFN α -2a AND α -2b

Pegylation of therapeutic proteins modifies immunologic, pharmacokinetic and pharmacodynamic properties. Pegylation technology has been applied to improve these properties of conventional IFN α , to even out large fluctuating serum concentrations, and to resolve the inconvenient dosing regimens associated with conventional IFN α . The structure and size of the PEG moiety and covalent binding modes characterize the properties of the

Table 2 Randomized, controlled trials and cohort studies comparing efficacy of peginterferon α -2a *vs* α -2b in combination with ribavirin for treatment-naïve patients

Authors	Country	Study design	Peg-IFN α (μ g/wk)	Ribavirin (mg/d)	No. of patients	SVR rate (α -2a <i>vs</i> α -2b) (%)	P value
McHutchison <i>et al</i> ^[27] , 2009	USA	RCT, IDEAL study, industry-initiated, multicenter	2a: 180 2b: 1.0/kg or 1.5/kg	2a: 1000-1200 2b: 800-1400	2a: 1035 ¹ 2b: 1016 ¹ or 1019 ¹	40.9 <i>vs</i> 38.0 or 39.8	NS
Ascione <i>et al</i> ^[29] , 2010	Italy	RCT, investigator-initiated, single-center	2a: 180 2b: 1.5	2a: 1000-1200 2b: 1000-1200	2a: 160 ² 2b: 160 ²	68.8 <i>vs</i> 54.4	0.008
Rumi <i>et al</i> ^[28] , 2010	Italy	RCT, MIST study, investigator-initiated, single-center	2a: 180 2b: 1.5	2a: 1000-1200 2b: 800-1400	2a: 212 ² 2b: 219 ²	66 <i>vs</i> 54	0.020
Yenice <i>et al</i> ^[120] , 2006	Turkey	RCT, investigator-initiated, single-center	2a: 180 2b: 1.5	2a: 800-1200 2b: 800-1200	2a: 37 ¹ 2b: 37 ¹	48.6 <i>vs</i> 35.1	NS
Craxi <i>et al</i> ^[26] , 2008	Italy	Prospective, PROBE study, industry-initiated, multicenter	2a: 180 2b: 1.5	2a: 1000-1200 2b: 1000-1200	2a: 663 ¹ 2b: 354 ¹	36 <i>vs</i> 29	0.020
Witthoeft <i>et al</i> ^[75] , 2008	Germany	Retrospective, PRACTICE study, industry-initiated, multicenter, matched pair	2a: 180 2b: 1.5	2a: Not stated 2b: Not stated	2a: 848 ² 2b: 848 ²	59.3 <i>vs</i> 53.0	0.008
Backus <i>et al</i> ^[25] , 2007	USA	Retrospective, United States veterans, government-initiated, multicenter	2a: 180 2b: 1.5	2a: 1000-1200 2b: 800-1400	2a: 2091 ² 2b: 3853 ²	25 <i>vs</i> 18	< 0.001

¹Genotype 1 alone; ²Genotypes 1-3 or 1-4. Peg-IFN α : Pegylated interferon α ; SVR: Sustained virological response; RCT: Randomized controlled trial; NS: Not significant; IDEAL: Individualized Dosing Efficacy vs. Flat Dosing to Assess Optimal Pegylated Interferon Therapy; MIST: Milan Safety Tolerability; PROBE: Pegylated interferons and Real Optimization of Best Efficacy; PRACTICE: Pegylated Interferons and Ribavirin: Analysis of Chronic Hepatitis C Treatment In Centres of Excellence.

modified biomolecule. Peg-IFN α -2a has a large, branched 40-kDa monomethoxy PEG, comprising two linked 20-kDa chains, attached to the lysine residues of IFN α -2a *via* amide bonds that are not susceptible to hydrolysis, and consists of 4 major positional isomers^[67]. In contrast, peg-IFN α -2b is pegylated with a small, linear 12-kDa monomethoxy PEG moiety and involves 13 positional isomers, with the main isomer linked to His-34 of IFN α -2b *via* a urethane bond that is unstable and susceptible to hydrolysis^[68]. These differences between the two peg-IFN formulations have an impact on the pharmacokinetic/pharmacodynamic properties.

Pharmacokinetic properties

The absorption half-life for peg-IFN α is longer than for unmodified IFN α (50 h and 4.6 h for 2a and 2b, respectively)^[69-71]. The absolute bioavailability of peg-IFN α -2a is at least 60%. The time to maximum concentration (T_{max}) is 78-80 h after a single dose and 45 h after multiple doses of peg-IFN α -2a, while T_{max} times are 15-44 h and 22-29 h, respectively, in dosing of peg-IFN α -2b. The serum concentration after a single dose of peg-IFN α -2a is sustained up to 168 h (elimination half-life, 65 h), and up to 48-72 h (elimination half-life, 40 h) for peg-IFN α -2b. At steady phase, which is attained 5-8 wk after initiation of treatment, the peak-to-trough ratio of serum concentrations of peg-IFN α -2a and -2b is 1.5-2.0 and > 10, respectively, indicating that serum concentrations of peg-IFN α -2a are sustained during the 1-wk dosage interval. Since variations in the peak-to-trough ratios for peg-IFN α -2b are greater than for peg-IFN α -2a, viremia levels tend to fluctuate more with peg-IFN α -2b than peg-IFN α -2a (at least within the initial 4 wk of treatment). The volume of distribution is dependent on the body composition because of the wide distribution of peg-IFN α -2b throughout body fluids and tissues, whereas peg-IFN α -2a exhibits restricted

distribution with the highest concentrations in the liver. Thus, peg-IFN α -2b requires weight-based dosing, while peg-IFN α -2a is used at a fixed dose.

Pharmacodynamic properties

Comparative studies of the initial viral kinetics after treatment have shown either a greater HCV RNA decline in patients treated with peg-IFN α -2a than those treated with peg-IFN α -2b^[71] or vice versa^[72], or no difference^[73]. Pharmacodynamic profiling studies of the two formulations also showed conflicting results. Single dosing induced a similar degree or pattern of activity of 2'-5'-oligoadenylate synthetase and serum protein levels of neopterin and β 2-microglobulin, indicating no difference between both types of peg-IFNs (plus RBV)^[74]. In contrast, another study showed that peg-IFN α -2b up-regulated IFN response genes significantly more than peg-IFN α -2a during the first 72 h after single dosing of each peg-IFN α , both administered without RBV^[72]. The enzymatic activity and serum protein levels were assayed in the former study, while RNA transcription was measured in the latter study. Furthermore, the methods and duration applied to evaluate various indicators differed between the two studies. Peg-IFN α was administered in combination with RBV during the evaluation period in the former, but not in the latter. Collectively, it is difficult to draw definite conclusions by simply comparing the results of relatively small studies, which have varied in several respects including the aforementioned differences.

Head-to-head comparison

Which of the peg-IFNs is more effective in combination therapy with RBV for chronic hepatitis C? So far, several head-to-head studies have compared peg-IFN α -2a *vs* -2b in combination with RBV (Table 2).

Two investigator-initiated, independent, single-center,

randomized, controlled, head-to-head trials compared peg-IFN α -2a *vs* -2b in combination with RBV for 48 wk (genotype 1 or 4) or 24 wk (genotype 2 or 3). Peg-IFN α -2a plus RBV produced a significantly higher SVR rate than peg-IFN α -2b plus RBV in treatment-naïve patients^[28,29]. In a prospective observational cohort study (PROBE, sponsored by Roche), the rate of SVR was higher in genotype 1 patients treated with peg-IFN α -2a than with peg-IFN α -2b (36% *vs* 29%, $P = 0.02$)^[26]. In a retrospective observational cohort study (PRACTICE), matched pair analysis also showed a significant difference in the SVR rate between peg-IFN α -2a and -2b treatments (59.3% *vs* 53.0%, $P = 0.008$)^[75]. An observational retrospective cohort study at the Veteran Hospitals in the United States also reported that treatment with peg-IFN α -2a was associated with a higher likelihood of SVR than treatment with peg-IFN α -2b (25% *vs* 18%, $P < 0.001$)^[25]. These studies have highlighted the superiority of peg-IFN α -2a over peg-IFN α -2b in the critical end-point of efficacy.

In contrast, the largest multicenter, randomized, head-to-head trial (IDEAL study, sponsored by Schering-Plough) showed no statistical difference in SVR rates among treatment arms with low-dose (1.0 μ g/kg per week) or standard-dose (1.5 μ g/kg per week) peg-IFN α -2b or peg-IFN α -2a (180 μ g/wk), in combination with various RBV regimens (38.0%, 39.8% *vs* 40.9%, respectively)^[27]. However, there were some differences between the IDEAL and other studies that could be described as critical limitations: (1) RBV regimens differed in initial doses and dose reduction rules between the treatment arms or studies; (2) the IDEAL study compared treatment regimens but did not directly evaluate the difference between the two peg-IFNs; (3) the dosing rules seem inappropriate by current standards in some studies; and (4) the IDEAL study included higher proportions of overweight, obese, and black/Latino patients. Using the same dosing and dose reduction rules of RBV across all the treatment arms and studies might have provided a fairer comparison of the different performance of the two peg-IFNs without a confounding effect of various RBV regimens. In the two studies where RBV dosing was identical between the two arms (one RCT and one cohort-matched pair analysis)^[29,75], the difference in favor of peg-IFN α -2a was maintained. Interestingly, the safety and adverse-event profiles were similar among the treatment arms in the above studies, irrespective of the RBV regimen.

A recent Cochrane systematic review of randomized trials that compared both peg-IFNs identified 12 studies (5008 participants)^[76]. Meta-analysis using intention-to-treat analysis for SVR included 8 trials (4335 participants), and yielded an estimated effect in favor of peg-IFN α -2a [47% *vs* 41% with peg-IFN α -2b; risk ratio = 1.11; 95% confidence interval (CI): 1.04-1.19, $P = 0.004$]. Subgroup analyses with regard to HCV genotype yielded similar results for all subgroups. The meta-analysis of adverse effects leading to discontinuation of treatment included 11 trials and showed no significant differences between the two peg-IFNs. However, the study did not reach a definitive conclusion as to which of the two peg-IFN formulations in combination with RBV is superior across popula-

tions based on more appropriate RBV dosing.

Taken together, all these studies involve several relevant methodological flaws (such as mixed genotypes, inclusion of prior non-responders and co-infected patients, small samples, insufficient power, and different RBV doses and dose reduction rules) that preclude, at least for the time being, any firm conclusions about differences in efficacy between the two peg-IFN α formulations.

TREATMENT OF DIFFICULT-TO-TREAT PATIENTS

Advances in IFN-based treatment for chronic hepatitis C, such as the development of peg-IFN α and RBV and treatment modifications, have improved the SVR rates in patients with difficult-to-treat characteristics, such as genotype 1/4, high baseline viral load, previous non-response, overweight, and the presence of cirrhosis. However, the outcomes of treatments in such patients are still inadequate. Further treatment development for this difficult-to-treat population is necessary.

Re-treatment of non-responders

For the increasing number of non-responders to IFN-based treatment or patients with multiple difficult-to-treat features, retreatment with current standard combination^[21,77] or alternative options, such as maintenance therapy with peg-IFN^[78-81] or the use of higher doses^[82-84] and/or extended duration of treatment^[85], have been explored vigorously. Most retreatment options for non-responders have provided a limited chance of SVR and in fact have been associated with increased side effects. In contrast, treatment-naïve patients with several difficult-to-treat predictors of poor response are reported to gain from aggressive modification of the treatment regimens and show higher SVR rates.

The SVR rate with retreatment consisting of standard peg-IFN α -2b plus RBV regimen was 12% in non-responders to prior treatment with conventional IFN α /RBV or peg-IFN α /RBV who had detectable HCV RNA at retreatment week 12^[77]. The SVR rate with standard peg-IFN α -2a plus RBV regimen was 18% in non-responders (with advanced fibrosis/cirrhosis) to prior conventional IFN-based treatment who had undetectable HCV RNA at retreatment week 20^[21]. A randomized retreatment study for non-responders to prior peg-IFN α -2b plus RBV compared 48-wk *vs* 72-wk treatment duration using higher induction dose (360 μ g weekly for 12 wk) or standard dose peg-IFN α -2a (180 μ g weekly) plus RBV^[85]. Although the extended treatment duration rather than higher induction dose significantly increased SVR rates (16% for 72 wk *vs* 8% for 48 wk, $P < 0.001$), the SVR rate was unsatisfactorily low: 16% for the 72-wk/higher induction dose group, 14% for the 72-wk/standard dose group, 7% for the 48-wk/higher induction dose group, and 9% for the 48-wk/standard group. However, the study showed that SVR rates were higher in patients with undetectable HCV RNA at re-treatment week 12 (49%) compared to those with detectable HCV RNA at re-

treatment week 12 (4%). Despite differences in the study population and design among these retreatment trials, most of them have produced modest SVR rates ranging from 7% to 18%. A recent meta-analysis indicated that the pooled estimate of SVR rate was 16.3% with a 95% CI of 8.3%-29.6%, although there was a significant heterogeneity among the studies ($P < 0.0001$)^[86].

Difficult-to-treat naïve patients

In contrast, treatment-naïve patients, even with multiple unfavorable factors, may show a favorable treatment outcome with aggressive treatment regimens. A pilot study of peg-IFN α -2b administered twice weekly in combination with RBV showed a significantly higher SVR rate among treatment-naïve patients with genotype 1/4 and high baseline viral load (55% *vs* 17% with the standard regimen)^[83]. In another study, a very high dose of RBV based on an individualized schedule yielded a very high SVR rate with the combination of peg-IFN α -2a for treatment-naïve patients with genotype 1 and high baseline viral load, although this was a very small pilot study^[82]. A pilot, double-blind, RCT for treatment-naïve patients with multiple predictors of poor treatment response (genotype 1, high baseline viral loads of $> 800\,000$ IU/mL, and body weight > 85 kg) showed that high fixed doses of peg-IFN α -2a (270 μ g/wk) and RBV (1600 mg/d) increased SVR rates compared with lower, conventional doses of both agents (180 μ g/wk and 1200 mg/d, respectively)^[54]. Week 48 end-of-treatment virologic response and SVR rates were 55% *vs* 46% and 47% *vs* 28%, respectively, suggesting that a more aggressive treatment approach could improve the virologic response and suppress relapse, although increasing the dose of RBV alone did not reduce relapse or substantially improve the SVR rates. However, the initial 12-wk induction with high dose of peg-IFN α -2b (3.0 μ g/kg per day) failed to produce a positively favorable treatment outcome in treatment-naïve genotype 1 patients, compared to standard regimen^[84].

Taken together, non-responders to a prior 48-wk course of standard peg-IFN α plus RBV combination who have no virologic response at retreatment week 12 are the most difficult-to-treat population. Such non-responders may have a cluster of difficult-to-treat characteristics or yet undiscovered resistant factors. The overall modest efficacy in non-responders argues against an indiscriminate retreatment with peg-IFN α and RBV. Restricting retreatment to patients with favorable factors or less unfavorable conditions, using a 12-wk treatment stopping rule, would optimize the potential benefit with little likelihood of missing a curative response. For instance, relapsers with earlier virologic response to the prior treatment, and patients infected with genotype 2 or 3, would be possible candidates for successful retreatment. For treatment-naïve patients even with multiple predictors of poor treatment response, aggressive treatment regimens using currently available medications could significantly improve the likelihood of achieving SVR.

STAT-C AGENTS

A large number of STAT-C agents have been developed and are currently being tested in phase 1-3 trials^[87,88]. Adding STAT-C agents to peg-IFN α plus RBV should provide new treatment options for chronic hepatitis C. Recently, the Protease Inhibition for Viral Evaluation (PROVE, evaluating telaprevir) and Serine Protease Inhibitor Therapy (SPRINT, evaluating boceprevir) clinical trials have suggested that protease inhibitors combined with peg-IFN α plus RBV could produce SVR rates of 70%-75% in treatment-naïve genotype 1 patients^[40,89,90]. Telaprevir and boceprevir, orally bioavailable inhibitors of the HCV NS3 protease, are two of several investigational agents that specifically and directly target HCV with increased likelihood of SVR. In the PROVE studies, discontinuation of treatment because of adverse events was more frequent in telaprevir-based groups, with rash the most common reason for discontinuation. The frequencies of pruritus, rash and anemia were increased in telaprevir-based groups^[40,89,91]. In the SPRINT-1 study, the most common adverse events leading to discontinuation in boceprevir regimens were fatigue, nausea, depression, neutropenia and anemia. Incidence of rash-related adverse events was similar in boceprevir regimens and control^[90].

More recently, the results of the PROVE-3 study showed that retreatment with telaprevir in combination with peg-IFN α -2a plus RBV was more effective than retreatment with peg-IFN α -2a plus RBV alone in patients who failed to show SVR to the initial full course of peg-IFN α plus RBV^[91]. The SVR rates of the three telaprevir-treated groups: 51% in the T12PR24 group [telaprevir (1125-mg loading dose, then 750 mg every 8 h) for 12 wk and peg-IFN α -2a (180 μ g weekly) and weight-based RBV (1000 or 1200 mg/d) for 24 wk], 53% in the T24PR48 group (telaprevir for 24 wk and peg-IFN α -2a and RBV for 48 wk), and 24% in the T24P24 group (telaprevir and peg-IFN α -2a for 24 wk), were significantly higher than that of the PR48 (control) group (peg-IFN α -2a and RBV for 48 wk; 14%; $P < 0.001$, $P < 0.001$ and $P = 0.02$, respectively). Patients with a previous relapse in the T12PR24 and T24PR48 groups had SVR rates of 69% and 76%, respectively. Of note, those with a previous non-response had SVR rates of 39% and 38%, respectively, which are the highest reported to date and more than four times the SVR in the control group (9%). The higher discontinuation rates and the lower relapse rates in the T24PR48 group compared with the T12PR24 group suggest that an optimal retreatment regimen may consist of a shorter period of treatment with telaprevir combined with a longer period of treatment with peg-IFN α and RBV.

In the SPRINT-1 study, boceprevir in combination with peg-IFN α -2a (P) plus RBV (R) was more effective than P/R for 48 wk (control)^[90]. The SVR rates of four boceprevir-treated regimens: 56% or 75% after 4 wk of P/R lead-in followed by P/R/boceprevir for 24 or 44 wk, and 55% or 67% after P/R/boceprevir for 28 or 48 wk, were significantly higher than that of the control (38%; P

= 0.0048, < 0.0001, = 0.0082 and < 0.0001, respectively). 48-wk boceprevir regimens had very low relapse rates. However, P/low-dose R/boceprevir for 48 wk was associated with increased viral breakthrough (27%), relapse (23%) and lower efficacy (36%).

The addition of STAT-C agents, such as telaprevir, to current standard treatment adds promising antiviral activity and is one of the most powerful retreatment strategies, especially for the non-responder population.

LONG-TERM EFFECT OF IFN TREATMENT ON THE PROGRESSION OF LIVER DISEASES

Retrospective cohort studies and preliminary randomized controlled trials

There are few satisfactory medical treatments for patients who do not achieve SVR in response to IFN-based treatment. In such patients, the liver disease could progress to cirrhosis, hepatocellular carcinoma (HCC) and liver failure, culminating in liver disease-related death. Earlier retrospective cohort studies suggested that conventional IFN treatment reduces the risk of HCC even in patients who are treated with a single course as brief as 6 mo and who show transient biochemical response but fail to eradicate HCV^[92-95]. In these non-randomized analyses, the short- or long-term efficacy of conventional IFN or impact of the treatment outcome on the clinical end point were evaluated based on serum ALT levels, but not the degree of viral response because serum HCV RNA levels were not monitored. These studies included patients with various liver disease stages (degree of fibrosis) and perhaps those with SVR at a certain rate. The SVR induced by conventional IFN treatment apparently provides a long-term benefit by reducing liver-related death^[96]. As shown by other retrospective cohort studies, it is conceivable that achievement of SVR following IFN-based treatment would reduce the risk of adverse clinical outcomes (liver-related complications, HCC and liver-related mortality) even in patients with cirrhosis or advanced fibrosis, compared to non-SVR^[97-99]. Furthermore, a small, prospective RCT suggested that even a single, brief (24-wk) course of conventional IFN treatment for patients with compensated cirrhosis (grade A on the Child-Pugh scoring system) could slow liver disease progression and reduce the cumulative incidence of HCC and mortality in the very long-term clinical course^[100]. Another RCT of extended conventional IFN treatment to 30 mo showed suppression of HCV RNA levels and reduction in serum ALT levels and histologic findings (necroinflammation and fibrosis) in non-responders to 6-mo conventional IFN treatment but with a histologic response^[101]. In that preliminary study, the majority of patients did not have advanced fibrosis or cirrhosis, and the impact of maintenance treatment on morbidity and mortality was not assessed. These favorable results encouraged clinicians to prevent progressive liver disease, including development of HCC and progression to cirrhosis and liver failure, with IFN-based maintenance

treatment even in patients with advanced fibrosis or cirrhosis. However, most of the following prospective RCTs did not recommend long-term maintenance treatment for such patients.

Randomized controlled trials for advanced fibrosis and cirrhosis

A large, prospective RCT [the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial] also showed that maintenance treatment of peg-IFN α -2a at a dosage of 90 μ g weekly for 3.5 years correlated significantly with decreases in serum HCV RNA levels, serum ALT levels and histologic necroinflammatory scores, compared to no treatment ($P < 0.001$), in patients with advanced fibrosis who had not achieved SVR to a standard regimen of peg-IFN α -2a plus RBV^[80]. Nevertheless, the maintenance treatment failed to reduce the rate of disease progression, as indicated by death, HCC, hepatic decompensation, or increase in the fibrosis score, in those with or without cirrhosis: 34.1% in the treatment group *vs* 33.8% in the control group ($P = 0.90$). Among patients with bridging fibrosis at baseline, cirrhosis developed by year 3.5 at rates similar in the two groups (28.2% *vs* 31.9%, respectively). Conversely, the rate of at least one serious adverse event was higher in the treatment group (38.6%) than in the control group (31.8%, $P = 0.07$).

In a substudy of the HALT-C trial, profound viral suppression by $\geq 4 \log_{10}$ with standard-dose peg-IFN plus RBV during the 24-wk lead-in phase was significantly related to fewer clinically critical events ($P = 0.003$) over the ensuing 3.5 years, regardless of whether randomized to maintenance therapy or no treatment^[81]. Unexpectedly, persistent viral suppression by $\geq 4 \log_{10}$ with maintenance therapy did not lead to a further reduction in clinical events. Thus, there is no rationale for peg-IFN α maintenance therapy in patients without viral suppression to undetectable levels during the treatment. Strangely, profound viral suppression even for a relatively brief period during the lead-in phase may be associated with clinical benefits.

In a small RCT, patients with biopsy-proven compensated cirrhosis who had at least one risk factor of complications were randomized to either conventional IFN α -2a (3 MU three times weekly) or no treatment for 24 mo^[102]. In the Colchicine Versus Peginteron Long-term Therapy (COPILOT) trial, patients with advanced fibrosis or cirrhosis who were non-responders to either conventional IFN or peg-IFN α with or without RBV were randomized to receive either peg-IFN α -2b at a dose of 0.5 μ g/kg per week or colchicine for 4 years^[78]. In the study design of the Evaluation of Peginteron in Control of Hepatitis C Cirrhosis (EPIC3) trial, non-responders to a lead-in treatment phase of peg-IFN α -2b plus RBV were randomized to receive either peg-IFN α -2b at a dose of 0.5 μ g/kg per week or no treatment for up to 3 years^[79]. Despite differences in the study design, the results of these trials were similar to those observed in the HALT-C trial; maintenance therapy with conventional IFN or peg-IFN α has little or no impact on prevention of progressive liver disease or complication-free survival in patients with advanced fibrosis

or cirrhosis. In the COPILOT and EPIC3 trials, however, maintenance therapy reduced complications almost exclusively in patients with portal hypertension.

The results of these prospective RCTs contradict those of the majority of retrospective, non-randomized cohort studies and earlier preliminary prospective RCTs. There were some differences among studies: the end points of disease events, inclusion criteria for the study, patients' background involving wide-ranging fibrosis stage or advanced fibrosis/cirrhosis, race, and life-style (high calorie, cigarette smoking, or alcohol intake), and evaluation on the basis of the degree of viral suppression or decline in serum ALT. It is not clear why control patients in previous retrospective studies did not receive IFN-based treatment for long-term periods. These uncertainties may tip the balance in favor of the IFN-treated patient group. At the least, maintenance treatment with low-dose peg-IFN α or conventional IFN for 2-3.5 years does not appear to prevent disease progression in patients with advanced fibrosis/cirrhosis and persistent viremia, and thus provides no overall benefit to such patients. If maintenance therapy leads to profound viral suppression, it can potentially prevent progressive disease and liver-related complications.

NEW INTERFERON AND ALTERNATIVE FORMS OF RIBAVIRIN

The clinical trials of STAT-C agents, PROVE and SPRINT^[40,89,90], suggest that peg-IFN α -2a or -2b and standard-dose RBV are required as indispensable components even in new combination regimens with the first-generation protease inhibitors, because treatment arms without ribavirin in the PROVE2 trial and with low-dose RBV in the SPRINT-1 trial showed increased viral breakthrough, higher relapse, and lower SVR. However, the addition of telaprevir or boceprevir resulted in higher rates of treatment discontinuation because of adverse events (rash, pruritus, and anemia), compared with the control arm. Furthermore, pre-existing resistant variants and naturally occurring resistance-related mutations against STAT-C agents would disturb the efficacy of "add-on" triple combination therapy^[41,103]. To overcome these disadvantages resulting from the addition of STAT-C agents, alternative approaches to new treatment strategies are needed to increase the SVR rates and reduce adverse events by altering formulations of IFN and RBV.

Albinterferon

Recombinant human albumin-interferon α -2b (albinterferon, alb-IFN), a novel formulation of IFN α , is an 85.7-kDa protein consisting of IFN α -2b genetically fused to human albumin. In an *in vitro* study using liver cell-based and non-liver cell-based HCV replicon cell lines, alb-IFN preserved the antiviral properties of IFN α with significant suppression of HCV RNA at clinically relevant serum concentrations^[104]. In a study of monkeys, alb-IFN had a prolonged elimination half-life, and consequently provided greater exposure relative to IFN α ^[105]. In dose-

-ranging phase 1/2 studies involving IFN-experienced and naïve patients, alb-IFN administration of up to 1200 μ g at 14-d intervals demonstrated a favorable safety profile, the half-life was extended to approximate 144 h, and antiviral activity increased in a dose-dependent manner^[106,107]. Alb-IFN was detectable throughout the entire dosing interval, corresponding to viral dynamic changes observed at doses of 900-1200 μ g.

A phase 2b trial randomized naïve genotype 1 patients to four treatment arms: peg-IFN α -2a (180 μ g once weekly), alb-IFN [900 or 1200 μ g once every two weeks (q2wk), or 1200 μ g once every four weeks (q4wk)] plus weight-based RBV (1000 or 1200 mg/d) for 48 wk^[108]. The SVR rates in the 900- μ g q2wk- and 1200- μ g q2wk-alb-IFN groups (59% and 56%, respectively) were comparable to that in the peg-IFN α -2a group (58%), while SVR rate in the 1200- μ g q4wk-alb-IFN group was lower (51%, $P = 0.28$). The discontinuation rates due to adverse events were comparable among the 900- μ g q2wk- and 1200- μ g q4wk-alb-IFN and peg-IFN α -2a groups (9%, 12%, and 6%, respectively), and higher for 1200- μ g q2wk-alb-IFN (18%, $P = 0.04$). Another trial compared five alb-IFN-based regimens for non-responders to prior IFN-based treatment: 1200- μ g q4wk and 900-, 1200-, 1500-, and 1800- μ g q2wk plus RBV for 48 wk^[109]. Although the overall SVR rate was only 11% for previous genotype 1 non-responders to peg-IFN α plus RBV, the trial suggested the potential advantage of higher doses of alb-IFN (1800 μ g q2wk) and its promising antiviral activity. Taken together, alb-IFN is likely to have overall efficacy and safety profiles comparable to those of peg-IFN α -2a, with the convenience of a 2-wk to 4-wk interval dosing schedule. Interestingly, alb-IFN improves patients' psychological condition and reduces missed workdays, and could further reduce the immunogenicity of IFN, compared to peg-IFN α .

Taribavirin (Viramidine)

Hemolytic anemia induced by RBV can cause fatigue, affect quality of life, and consequently result in dose reductions, which could lower the chance of SVR^[2,3,45,49,110,111]. Erythropoietin preparations used to alleviate anemia and maintain RBV dose substantially increase medical expenses and may induce adverse effects^[46,112]. Some protease and polymerase inhibitors exacerbate anemia observed with peg-IFN α plus RBV combination treatment^[40,89]. Such cases strongly emphasize the need for an RBV analogue to alleviate hemolytic anemia.

Taribavirin (TBV, previously known as viramidine) is a prodrug of RBV being developed for combination treatment with peg-IFN α , in expectation of a lower incidence of anemia. The agent is a guanosine analogue that is primarily taken up by the liver and is rapidly converted to RBV by adenosine deaminase^[113-116]. TBV-derived RBV is concentrated in the liver at three-fold the rate of native RBV^[116]. Furthermore, TBV containing a positively charged 3-carboxamide group accumulates poorly in red blood cells (RBCs) and reduces RBV concentration in RBCs by half^[114-116].

A phase 2 dose-ranging comparison of TBV *vs* RBV combined with peg-IFN α -2a (180 μ g/wk) evaluated TBV doses of 800, 1200 and 1600 mg/d and RBV at a weight-based dose of 1000 or 1200 mg/d^[117]. The SVR rates were lower in the TBV groups than in the RBV group (23%, 37%, 29% *vs* 44%), although anemia was significantly less in the TBV groups. The phase 3, double-blind, ViraMidine's Safety and Efficacy versus Ribavirin 1 (ViSER1) study for naïve patients compared the safety and efficacy of flat-dose TBV (1200 mg/d) *vs* weight-based RBV (1000 or 1200 mg/d) in combination with peg-IFN α -2b (1.5 μ g/kg per week)^[118]. The SVR rates were 38% and 52%, respectively. The VR rate at every time point during the study was lower, and the relapse rate was higher in the TBV group. Thus, flat-dose TBV failed to show non-inferiority efficacy compared to weight-based RBV, suggesting that the dosage of TBV (1200 mg/d) is suboptimal or insufficient at least for a proportion of patients. However, the incidence of hemoglobin (Hb) events (Hb < 10 g/dL or a decrease of \geq 2.5 g/dL from baseline) was significantly lower with TBV (55%) than with RBV (84%, $P < 0.001$). More patients were encountered in the TBV arm with diarrhea (30%) compared to the RBV arm (20%). The incidence of moderate or severe diarrhea in the former group was double that in the latter (10% *vs* 5%, respectively).

The ViSER2 study showed similar results of efficacy and safety^[119]. It was performed with the same study design, except for the usage of peg-IFN α -2a (180 μ g once weekly, instead of peg-IFN α -2b). The SVR rate was 40% in the flat-dose TBV group and 55% in the weight-based RBV group. TBV was significantly superior to RBV in Hb event rates (54% *vs* 80%, $P < 0.001$). The rates of adverse events were similar between the groups except for diarrhea (TBV 30%; RBV 16%, $P < 0.0001$).

Similar to RBV, TBV appears to improve the SVR rate with higher TBV exposure based on body weight (mg/kg), and a dosage of > 18 mg/kg may be needed to produce SVR rates comparable to those of weight-based RBV^[118,119]. Therefore, further studies of TBV administered on a weight-based dosing schedule are required to determine the optimal dosage that would yield superior efficacy to, or at least comparable to, RBV, with preservation of the safety profile.

CONCLUSION

In summary, more optimal and highly individualized therapeutic strategies for HCV-infected individual patients are currently being investigated and developed, such as response-guided therapy, modified regimens with currently available medications, novel modified IFN α and RBV or combinations with STAT-C agents. In the foreseeable future, these ceaseless efforts will relieve a large number of HCV-infected patients all over the world.

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S- Editor Wang JL L- Editor Logan S E- Editor Lin YP

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Differentiation of Crohn's disease from intestinal tuberculosis in India in 2010

Anna Benjamin Pulimood, Deepak Narayan Amarapurkar, Ujjala Ghoshal, Mathew Phillip, Cannanore Ganesh Pai, Duvvur Nageshwar Reddy, Birender Nagi, Balakrishna Siddhartha Ramakrishna

Anna Benjamin Pulimood, Department of Gastrointestinal Sciences, Christian Medical College, Vellore, Tamil Nadu 632004, India

Deepak Narayan Amarapurkar, Department of Gastroenterology, Bombay Hospital and Medical Research Centre, Mumbai, Maharashtra 400025, India

Ujjala Ghoshal, Department of Microbiology, Sanjay Gandhi Postgraduate Institute, Lucknow, Uttar Pradesh 226014, India

Mathew Phillip, PVS Institute of Digestive Diseases, PVS Hospital, Kochi, Kerala 682017, India

Cannanore Ganesh Pai, Department of Gastroenterology and Hepatology, Kasturba Medical College, Manipal, Karnataka 576104, India

Duvvur Nageshwar Reddy, Asian Institute of Gastroenterology, Hyderabad, Andhra Pradesh 500082, India

Birender Nagi, Department of Gastroenterology, Postgraduate Institute, Chandigarh 160012, India

Balakrishna Siddhartha Ramakrishna, Department of Gastrointestinal Sciences, Christian Medical College, Vellore, Tamil Nadu 632004, India

Author contributions: Pulimood AB coordinated this review and contributed the section on pathology; Amarapurkar DN contributed the sections on clinical features and PCR for *Mycobacterium tuberculosis*; Ghoshal U contributed the sections on serology and cultures; Phillip M contributed the sections on Quantiferon TB Gold and surgery; Pai CG contributed the section on endoscopy; Reddy DN contributed the section on capsule endoscopy and small bowel enteroscopy; Nagi B contributed the section on radiology; Ramakrishna BS contributed the section on the role of therapy.

Correspondence to: Dr. Anna Benjamin Pulimood, Department of Gastrointestinal Sciences, Christian Medical College, Vellore, Tamil Nadu 632004, India. annapulimood@cmcvellore.ac.in

Telephone: +91-416-2282052 Fax: +91-416-2282486

Received: May 6, 2010 Revised: July 12, 2010

Accepted: July 19, 2010

Published online: January 28, 2011

ease (CD) is an important clinical challenge of considerable therapeutic significance. The problem is of greatest magnitude in countries where tuberculosis continues to be highly prevalent, and where the incidence of CD is increasing. The final clinical diagnosis is based on a combination of the clinical history with endoscopic studies, culture and polymerase chain reaction for *Mycobacterium tuberculosis*, biopsy pathology, radiological investigations and response to therapy. In a subset of patients, surgery is required and intraoperative findings with pathological study of the resected bowel provide a definitive diagnosis. Awareness of the parameters useful in distinguishing these two disorders in each of the different diagnostic modalities is crucial to accurate decision making. Newer techniques, such as capsule endoscopy, small bowel enteroscopy and immunological assays for *Mycobacterium tuberculosis*, have a role to play in the differentiation of intestinal tuberculosis and CD. This review presents currently available evidence regarding the usefulness and limitations of all these different modalities available for the evaluation of these two disorders.

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Key words: Tuberculosis; Crohn's disease; Clinical features; Endoscopy; Serology; Enteroscopy; Histology; Radiology; Surgery; Therapy

Peer reviewer: Jorgen Rask-Madsen, MD, FRCP, Professor of Gastroenterology, Department of Gastroenterology, Herlev Hospital, Skodsborg Strandvej 280A, 2942, Herlev, DK-2730, Denmark

Pulimood AB, Amarapurkar DN, Ghoshal U, Phillip M, Pai CG, Reddy DN, Nagi B, Ramakrishna BS. Differentiation of Crohn's disease from intestinal tuberculosis in India in 2010. *World J Gastroenterol* 2011; 17(4): 433-443 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/433.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.433>

Abstract

Differentiating intestinal tuberculosis from Crohn's dis-

INTRODUCTION

Tuberculosis (TB) of the gastrointestinal tract and Crohn's disease (CD) are chronic granulomatous disorders with similarities in their clinical presentation and pathology. The rising incidence of CD in countries like India where TB continues to be endemic has made the differentiation of these two disorders a diagnostic challenge^[1-4]. Misdiagnosis leads to delays in initiating effective therapy with increased morbidity and mortality, hence the importance of making an accurate diagnosis at the earliest possible stage. Despite the similarities of these two disorders a number of studies have identified specific differences in their clinical, endoscopic, radiological and pathologic findings. Other laboratory tests such as culture and molecular testing for *Mycobacterium tuberculosis* (MTB) have also been described to be useful in distinguishing them, as has been their response to therapy^[5,6]. In this review we present current evidence from the literature regarding conventional and new modalities that could be helpful in discriminating intestinal TB from CD.

CLINICAL FEATURES

Clinical features in both diseases include (1) constitutional symptoms such as fever, anorexia and weight loss; (2) symptoms due to mucosal ulceration such as diarrhea, hematochezia and malabsorption; (3) symptoms due to transmural involvement such as abdominal pain, and distention and vomiting due to luminal obstruction; a palpable lump; intestinal perforation and perianal and intestinal fistulization; (4) extra-intestinal manifestations such as arthritis and sclerosing cholangitis in the case of CD and involvement of other organs such as joints, lungs, peritoneum and lymph nodes in the case of TB; and (5) a family history of inflammatory bowel disease (IBD) in the case of CD or a history of family contacts in the case of TB^[7,8].

In patients with CD the duration of illness is generally more than 12 mo while it is shorter, lasting around 6 to 7 mo, in intestinal TB^[7]. Diarrhea and hematochezia are more commonly seen in CD while fever, ascites and co-existing TB at other sites are seen in gastrointestinal TB. Perianal disease, malabsorption and recurrence of disease after surgery are in favor of CD. A family history of IBD may be seen in 10% of patients with CD. Extra-intestinal involvement due to tuberculosis is seen in a third of patients with intestinal TB^[7,9]. A high index of clinical suspicion is required, however, to differentiate between the two conditions clinically.

SEROLOGY

Abnormalities in routine blood tests such as total and differential leukocyte count, raised ESR, C-reactive protein and low hemoglobin are seen in the active phase of both intestinal TB and CD^[5,7]. Platelet counts may be raised in the active phase of CD due to reversible hyposplenism and may increase the suspicion of CD over intestinal TB. One study on a small number of patients with intestinal

TB showed that only one of 14 (7%) patients had a positive result to enzyme-linked immunosorbent assay (ELISA) against anti-*Saccharomyces cerevisiae* antibody (ASCA) in serum in contrast to 49% with CD^[10], therefore, the authors recommended use of this test for differentiation between CD and intestinal TB. However, two studies from India involving a larger number of patients showed that ASCA was not useful in differentiating between CD and TB^[11,12]. Almost half of the patients with intestinal TB in both studies were ASCA-positive, which was comparable to the frequency in patients with CD. ASCA is a non-specific antibody resulting from macromolecular transport of food antigens (including antigens contained in baker's yeast), partly resulting from an increase in intestinal permeability. Since patients with intestinal TB have chronic inflammatory lesions of the small intestine, similar to patients with CD with increased small intestinal permeability, frequent positive results with the ASCA test in the former condition is quite expected^[11].

CULTURES

The most reliable method to differentiate between intestinal TB and CD is demonstration of acid-fast bacilli (AFB) either in smears or by culture. However, smear and culture to demonstrate MTB have low sensitivity. Furthermore, *Mycobacteria* take a very long time (4-6 wk) to grow in culture. Identification of AFB on intestinal biopsies has been reported with variable frequency (25%-36%)^[5,7,13]. MTB was cultured from mucosal biopsies only in one third of patients with colonic TB^[14]. The use of fluorescent stain for the diagnosis of intestinal TB increases sensitivity but lacks specificity and results are still poor due to the paucibacillary nature of the disease^[7]. The time to recovery of *Mycobacteria* from culture has been shortened to 2-3 wk by the use of automated culture systems such as BACTEC, *Mycobacteria* growth indicator tube (MGIT), MB/BacT mycobacterial detection system and the ESP culture system II^[15]. The sensitivity of the BACTEC system was found to be poor, however, for the diagnosis of intestinal TB^[7,8]. In the MGIT system, a fluorescent compound is embedded in silicone at the bottom of the tube. This compound is sensitive to dissolved oxygen in broth. As the actively growing bacteria consume the dissolved oxygen, the fluorescence is unmasked and can be observed in the tube under long wave UV light^[16]. MB/BacT is a colorimetric, non-radiometric method of detection of mycobacterial growth, which uses Middlebrook 7H9 media in an atmosphere of CO₂, H₂ and O₂ under vacuum. In ESP culture system II, each culture bottle is continuously monitored for any change in gas pressure due to metabolic activity of the microorganism^[16]. These systems, however, need to be evaluated for the diagnosis of intestinal TB.

POLYMERASE CHAIN REACTION FOR MTB

The TB polymerase chain reaction (PCR) assay is based on

augmenting oligonucleotides found in MTB chromosomes that are highly specific for the organism. TB PCR analysis of endoscopic biopsy specimens or surgical specimens can be done quickly and results can be obtained within 48 h^[17]. This test is very specific for TB but occasionally may be positive in patients with CD^[18,19]. Sensitivity of this test is modest and there is no correlation between PCR positivity and histological lesions such as caseation or granulomas^[17,18]. *In situ* TB PCR and analysis of fecal samples of patients with gastrointestinal TB have been shown to be useful in small studies^[14,20], but need validation in larger numbers of patients. The *in situ* TB PCR technique needs to be improved for better sensitivity. As currently used, TB PCR on biopsy samples has a high positive predictive value but a very low negative predictive value.

QUANTIFERON-TB GOLD

Quantiferon-TB Gold (QFT-G, Cellestis Limited, Carnegie, Victoria, Australia) is an *in vitro* ELISA which detects the release of interferon-gamma after stimulation by MTB antigen. The test, approved by the Food and Drug Administration (FDA) as an aid in diagnosing MTB infection, including both latent TB infection and TB disease, is performed by incubating fresh heparinized whole blood from sensitized persons with mixtures of synthetic peptides representing proteins present in *M. tuberculosis*: early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). The synthetic peptides used in QFT-G are absent from all BCG strains and most nontuberculosis mycobacteria except *M. kansasii*, *M. szulgai* and *M. marinum*.

Advantages and limitations

The advantages of QFT-G include lack of cross-reaction with BCG and most nontuberculous mycobacteria, avoidance of reader bias and the need for only a single patient visit. It may also be useful in monitoring the clinical response to anti-TB therapy.

The limitations include the need for incubation within 12 h of blood collection and its inability to differentiate infection with TB from latent TB infection. Though the sensitivity of QFT-G for detecting MTB infection in individuals with untreated culture-confirmed TB is approximately 80% in published studies, its sensitivity for latent TB infection seems to be less than that of the tuberculin skin test. The ability of the test to predict the risk of progression to subsequent TB disease in latent TB has not been determined. The test should be interpreted with caution as a patient with a negative test may still have latent TB infection or active TB disease. In a study of HIV sero-negative pulmonary TB patients from Chennai, QFT-G and the tuberculin skin test yielded diagnostic sensitivities of 90.6% (95% CI: 86.3%-94.9%) and 68.9% (95% CI: 60.6%-77.2%), and specificities of 55% (95% CI: 35.2%-54.8%) and 75.5% (95% CI: 66.8%-84.2%), respectively. The higher sensitivity noted in this study may be due to the exclusion of HIV patients^[21].

Role of QFT-G in intestinal tuberculosis

The role of QFT-G in intestinal tuberculosis is not clear. The test may have a possible role in follow up of patients on antituberculous therapy (ATT), in the diagnostic dilemma of CD *vs* TB and may be undertaken prior to starting biologicals in CD patients.

ENDOSCOPY

Gastrointestinal endoscopy - ileo-colonoscopy, device-assisted enteroscopy and gastro-duodenoscopy - plays a crucial role in the differentiation of intestinal TB from CD^[6,22]. Endoscopy permits direct visualization and biopsy of lesions for histological and other studies from virtually the entire alimentary tract^[23,24].

The ileo-cecal region is the most common site affected in either condition, and colonoscopy with retrograde intubation of the ileum is the initial procedure of choice. In patients with suspected or proven CD, ileo-colonoscopy provided similar sensitivity (67% *vs* 83%) but significantly higher specificity (100% *vs* 53%) compared to video capsule endoscopy^[25]. The incremental diagnostic yield of ileoscopy, low at 3.7%, may yet be important in a given patient^[26]. The diagnostic yield of histology increases with increasing number of biopsies from up to four segments in the colon^[1]. Endoscopic biopsies from segments upstream after dilating a stricture, and also from the normal looking ileum, increase the yield in patients with suspected TB^[27,28].

When the colon is spared, gastro-duodenoscopy and enteroscopy may be appropriate^[6,22,24]. Balloon-assisted and spiral enteroscopy are preferred modalities for evaluating the small bowel today because of biopsy and therapeutic capability^[23]. Biopsying small bowel lesions is important because the causes of ulcerating lesions cannot be differentiated based on endoscopic appearances alone^[23]. Biopsies from normal appearing colonic or gastro-duodenal mucosa may provide diagnostic clues in suspected CD^[1,29].

Characteristic endoscopic features have been described in intestinal TB and CD^[6,22,24]. Transversely placed ulcers, nodularity and hypertrophic lesions resembling masses are characteristic of TB. Aphthoid or longitudinal, deep, fissuring ulcers and a cobblestone appearance are said to be more typical of CD. Very few studies have directly compared these or evaluated their diagnostic value and inter-observer agreement.

In a small comparative study, ano-rectal lesions, longitudinal ulcers, aphthous ulcers, and a cobblestone appearance were significantly more common in CD, and involvement of fewer than four segments, a patulous ileocecal valve, transverse ulcers, and pseudopolyps were more frequent in intestinal TB. Assuming that a diagnosis of one or the other disease could be made based on which parameters were more common in a given patient, the endoscopic diagnosis would have been proved correct in 77 of 88 (87.5%) patients^[30]. These findings need to be validated before recommendation for routine use.

In a more recent prospective study, skip lesions in

the colon were significantly more frequent in patients with CD compared to patients with intestinal TB (66% *vs* 17%)^[31], as were aphthous ulceration (54% *vs* 13%), linear ulceration (30% *vs* 7%) and superficial ulceration (51% *vs* 17%). Cobblestoning of the colonic mucosa was seen only in CD (17% *vs* 0%). Nodularity of the colonic mucosa was significantly more common in patients with TB than in those with CD (49% *vs* 24.5%).

Capsule endoscopy

Capsule endoscopy has been established as a safe and non-invasive modality for the diagnosis of CD^[32,33]. A meta analysis comparing capsule endoscopy with other imaging modalities of the small bowel for inflammatory bowel disease established that capsule endoscopy has an incremental diagnostic yield of 25%-40% over other modalities^[34]. Capsule endoscopy has the unique ability to visualize small ulcers and early inflammatory lesions. Fidler *et al*^[35] defined a positive capsule result for CD as presence of four or more ulcers, erosions, or a region with clear exudate and mucosal hyperemia and edema. A scoring index developed to quantify mucosal changes detected by capsule endoscopy in any inflammatory processes of the small bowel is based on three variables: villous appearance, ulceration and stenosis. Each variable is assessed by size and extent of change. The index does not diagnose or measure a disease and does not have the discriminatory ability to differentiate between illnesses, but only measures mucosal changes. The International Conference on Capsule Endoscopy consensus statement declared that capsule endoscopy may play an important role in the diagnosis and monitoring of patients with known or suspected CD^[36]; playing a unique role in assessing mucosal healing after medical therapy, helping to assess early postoperative recurrence and guiding therapy. The consensus statement concluded that capsule endoscopy may identify sub-clinical markers in asymptomatic family members and contribute to the understanding of the natural history of IBD.

There are limited data regarding capsule endoscopy in intestinal TB. This can be attributed to non-affordability of capsule endoscopy in TB endemic countries and the inability of capsule endoscopy to take biopsies. A few case reports have described capsule endoscopic features of intestinal TB as multiple scattered short, oblique or transverse mucosal ulcers with a necrotic base in the jejunum and ileum^[37]. Cello *et al*^[38] also found that ulcers of the small bowel in intestinal TB were characteristically shallow with extensive irregular "geographic" borders, were usually not larger than 1-2 cm and were transverse rather than longitudinal. However, it is difficult to differentiate CD from TB based on capsule endoscopic features alone.

Small bowel enteroscopy

Double balloon and single balloon enteroscopy have the unique ability to visualize most of the small intestine by combined antegrade and retrograde approaches with biopsy capability and the possibility of therapeutic interven-

tions^[39,40]. However, they are more invasive than capsule endoscopy and need to be performed under sedation. A meta analysis compared capsule endoscopy and double balloon enteroscopy in patients with suspected inflammatory lesions and found no statistically significant difference in their diagnostic yield^[41]. A few case reports have described the double balloon enteroscopic findings in intestinal TB. Nakamura *et al*^[42] described multiple focal shallow irregular ulcers with necrotic bases of about 5 mm in the mid and distal ileum by double balloon enteroscopy through the retrograde route. In a series of 106 cases of single balloon enteroscopy, Ramchandani *et al*^[43] diagnosed 13 cases of CD and 3 cases of intestinal TB, which were confirmed by mucosal biopsies. There were focal longitudinal and aphthous ulcers in the jejunum and ileum in CD, whereas ulcers in TB were oblique or transverse with a necrotic base. Focal ulcerated strictures were noted in the distal jejunum and ileum in four cases of CD.

RADIOLOGY

Imaging plays an important role in diagnosing and differentiating intestinal TB from CD^[44-46]. Barium examination is the mainstay for the evaluation of the intestinal tract. Computed tomography (CT) is complementary to barium examination for the evaluation of extra-intestinal pathologies. CT enteroclysis is a hybrid technique that combines the advantages of both barium examination and CT.

In the vast majority of cases, differentiation of intestinal TB from CD is possible when radiological findings are correlated with clinical features, histopathological findings and response to treatment^[47-49].

Various points of distinction are summarized as follows.

Distribution of disease

The ileo-cecal region is the most common site involved in intestinal TB (Figure 1)^[49,50]. Isolated involvement of the ileo-cecal region is not seen in CD which typically involves the ileum with conspicuous sparing of the ileocecal valve. The cecum may rarely be involved in direct contiguity with the ileum or colonic disease.

Colorectal involvement with small bowel disease is more often seen in CD than in TB whereas the ascending colon may be involved in direct contiguity with the ileocecal region in TB. Isolated colorectal involvement in TB is rare as compared to CD.

Barium findings (small bowel)

Strictures are the most common finding in intestinal TB and are typically short, concentric and smooth in outline with significant prestenotic dilatation (Figure 1A). In CD, strictures are usually long (the result of fibrosis, irritability and inflammation), eccentric with sacculations at the anti-mesenteric border and without significant prestenotic dilatation (Figure 1B). Aphthous ulcers and an ulceronodular pattern are almost pathognomonic in the appropriate clinical settings (Figure 1C). Tubercular

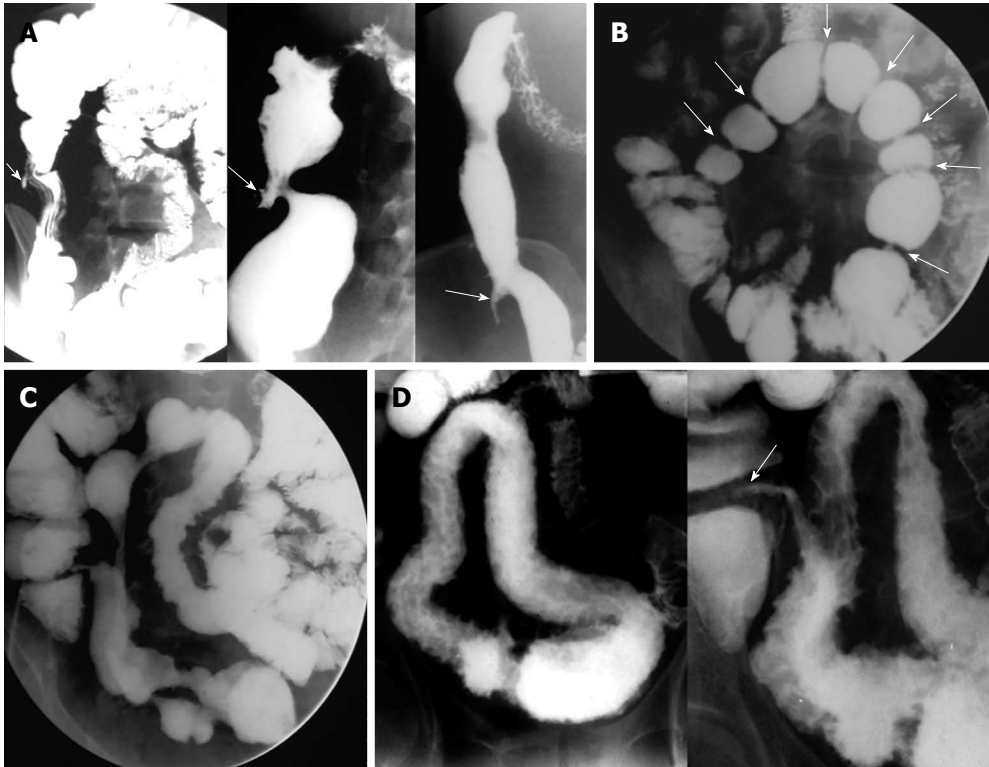


Figure 1 Findings on barium study of the small intestine. A: Barium study in three different patients with tuberculosis (TB) demonstrating shrunken, conical and retracted cecum (arrows); B: Multiple strictures (arrows) in ileal loops of a patient with TB. Note the short, concentric and smooth outline of the strictures; C: Multiple eccentric strictures seen in the ileum of a patient with Crohn's disease (CD). Note the normal ileo-cecal junction; D: Barium study showing an ulceronodular pattern involving a long segment of the ileum in a patient with CD. Note sparing of the ileo-cecal junction (arrow).

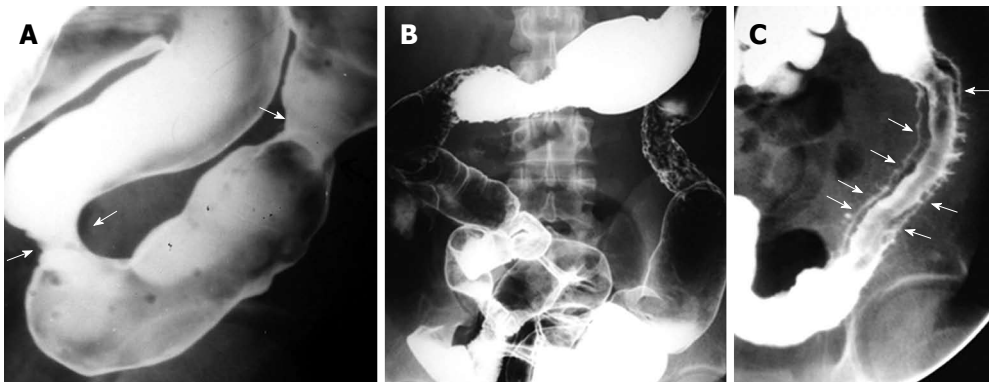


Figure 2 Findings on barium study of the colon in tuberculosis and Crohn's disease. A: Barium enema showing tubercular strictures (arrows) of the colon; B: Barium enema demonstrating skip lesions affecting descending and transverse colon as ulcers with areas of narrowing in a patient with Crohn's disease (CD); C: Deep ulcers with double tracking of sigmoid colon in a patient with CD (arrows).

ulcers are less common, longitudinally oriented and tend to be round to oval in configuration. Perforation and fistulae are more often encountered with CD, while enteroliths are more common in intestinal TB. Malignancy is a complication seen only with CD.

Barium findings (large bowel)

In TB, solitary or multiple strictures which are smooth and concentric are the most common finding (Figure 2A) while aphthous ulcers, segmental colitis and cobblestoning are the predominant findings in CD (Figure 2B and C).

Computed tomography

In CD, mural thickening with stratification is seen with active inflammation (Figure 3A) (Table 1). In addition, vascular engorgement of the mesentery (comb sign) and mesenteric fibrofatty proliferation are seen (Figure 3B and C). Mural thickening with contiguous ileo-cecal involvement is more often the presentation of intestinal TB (Figure 3D). Hypodense lymph nodes with peripheral enhancement in the mesentery and retro-peritoneum are characteristic of TB (Figure 3E), while in CD hypodense lymph nodes are seen.



Figure 3 Findings on computed tomography. A: Computed tomography (CT) enteroclysis with negative oral contrast showing mural thickening of ileum with skip areas and sparing of cecum in a patient with Crohn's disease (CD); B: Contrast-enhanced CT scan (CECT) showing asymmetrical mural thickening in ileal loop with deep ulcerations. Note fibro-fatty proliferation of mesentery; C: CECT in another patient with CD showing mesenteric vascular engorgement (Comb sign) with fibro-fatty proliferation of the mesentery; D: CT enteroclysis with negative oral contrast showing contiguous mural thickening of the terminal ileum and cecum in a patient with tuberculosis (TB); E: CECT in a patient with TB showing mural thickening of the terminal ileum and cecum (thick arrow) with multiple enlarged mesenteric lymph nodes showing central hypoattenuating and peripherally enhancing rims (thin arrow).

Table 1 Computed tomographic features of intestinal tuberculosis and Crohn's disease

Tuberculosis	Crohn's disease
Mural thickening without stratification	Mural thickening with stratification in active inflammation
Strictures concentric	Strictures eccentric
Fibrofatty proliferation of mesentery very rare	Fibrofatty proliferation of mesentery
Mesenteric inflammation but no vascular engorgement	Hypervascular mesentery (comb sign)
Hypodense lymph nodes with peripheral enhancement	Mild lymphadenopathy
High density ascites	Abscesses

PATHOLOGY

Surgical resections

Macroscopically, TB classically causes ulceration, short strictures, marked thickening of the bowel wall due to inflammation, fibrosis and adhesions, or a combination of these. The ulcers are transverse, often circumferential, with ill-defined, sloping or overhanging edges. The surrounding mucosa may show flattening of folds, ulcers, erosions and pseudopolyps. The cut section of the intestinal wall shows scarring and necrosis, often with loss of distinction of the different layers. The serosal surface may show 2-5 mm-sized nodules and adhesions. The regional lymph nodes are invariably enlarged and may show caseation^[51].

The histological hallmarks of TB are confluent, caseat-

ing granulomas containing acid fast bacilli and surrounded by a lymphoid cuff. These are found in all layers of the intestinal wall and in regional lymph nodes, but sometimes only in the latter^[51]. Early granulomas are usually found within lymphoid tissue^[52]. There may be extensive pyloric metaplasia. Occasional superficial fissuring ulcers that extend into the submucosa may be seen. Healing occurs by fibrosis, and epithelial regeneration begins at the edge of ulcers. Healing granulomas are surrounded by a rim of fibrous tissue in lymph nodes, but not in the intestinal wall^[51].

Macroscopically, CD also shows bowel wall thickening, skip lesions and strictures, but the latter are longer than in TB. Fat wrapping is common, as are adhesions, fistulae, sinuses and extra-intestinal abscesses^[53]. Mucosal aphthous ulcers are seen at an early stage, and coalesce to form larger stellate ulcers. Deep, longitudinal, fissuring ulcers are characteristic of CD, as well as smaller longitudinal ulcers separating edematous or uninvolved mucosa to create a cobblestone appearance^[54].

Microscopically, common features are aphthous ulcers over lymphoid follicles, fissuring ulcers that extend into the muscularis propria or deeper, distortion of the mucosal architecture, pyloric metaplasia, cryptitis and crypt abscess formation with moderate to severe chronic inflammation. These changes are often segmental or patchy and extend transmurally. Prominent lymphoid follicles in the submucosa and serosa are another characteristic feature of CD. Granulomas, characteristically small, are seen

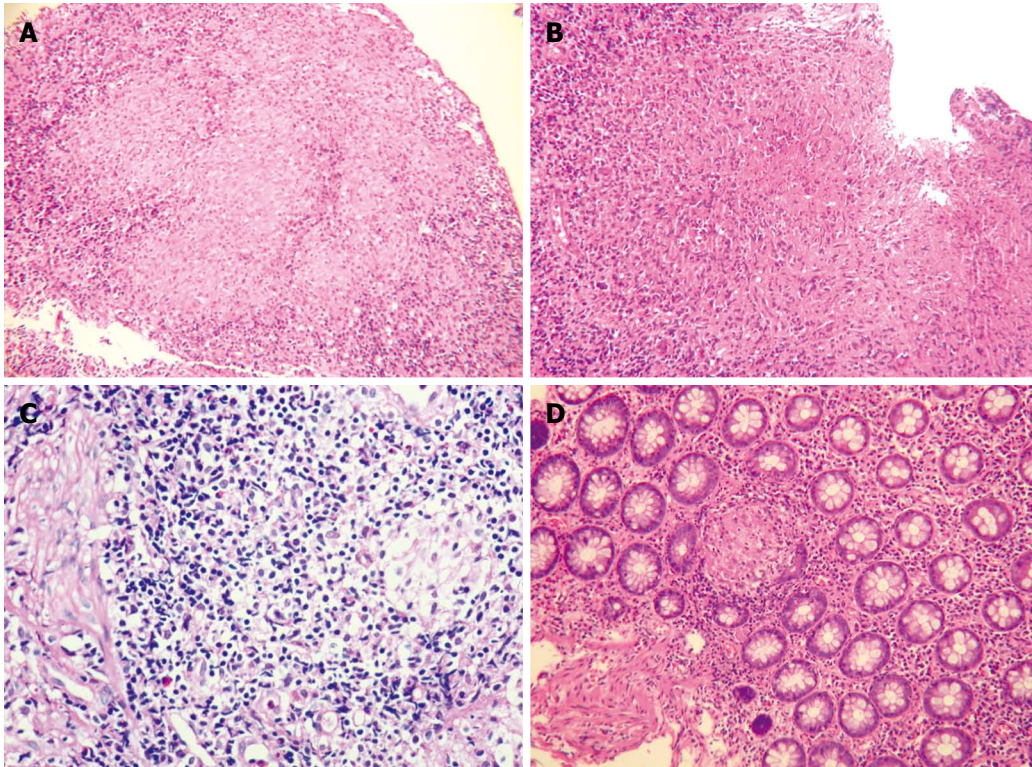


Figure 4 Histological features. A: Confluent granulomas in inflammatory granulation tissue from ulcerated colonic mucosa of a patient with tuberculosis (TB) [Hematoxylin and eosin (HE), 100 ×]; B: Large granuloma in the ulcerated mucosa of a patient with TB (HE, 100 ×); C: Microgranuloma composed of a small aggregate of macrophages in a lymphoid follicle from the mucosa of a patient with Crohn's disease (CD) (HE, 400 ×); D: Small pericryptal granuloma in the colonic mucosa of a patient with CD (HE, 100 ×).

in 50%-60% of resection specimens and may be found in the regional lymph nodes in about 25% of cases^[54].

Mucosal biopsies

With the increasing use of endoscopic procedures to visualize the intestinal lumen and obtain targeted biopsies from diseased areas, the histological differentiation of intestinal TB and CD is most commonly made on mucosal biopsies. One of the limitations of mucosal biopsies is that granulomas, the primary differentiating feature of TB from CD, are found in only 50%-80% of intestinal mucosal biopsies from patients with clinically confirmed TB^[51,55,56] and in 15%-65% of mucosal biopsies from patients with CD^[57]. Caseation and AFB, the diagnostic features of TB, are found in only 18%-33% of cases^[56,58] and in as low as 5% of cases^[58], respectively. Other features suggestive of TB include confluent granulomas (Figure 4A), a lymphoid cuff around granulomas, granulomas larger than 400 µm in diameter (Figure 4B), 5 or more granulomas in biopsies from one segment, granulomas located in the submucosa or in granulation tissue, often as palisaded epithelioid histiocytes, and disproportionate submucosal inflammation^[1,3]. Features that favor a diagnosis of CD on mucosal biopsies include infrequent (< 5), small (< 200 µm in size) granulomas that are poorly organized and discrete or isolated. Granulomas are located more commonly in the mucosa than in other sites in CD. Microgranulomas, or aggregates of histiocytes (Figure 4C), and

crypt-centered inflammation such as pericryptal granulomas (Figure 4D) and focally enhanced colitis are also features of CD^[1,3,59].

SURGERY

In India, due to a high prevalence of TB, the diagnosis of CD is often delayed. In one report nearly a quarter of patients with CD were being treated as tuberculosis^[60]. In a study published from India of 28 patients undergoing surgery for CD, elective surgery was performed in 23 and emergency intervention was required in 5. The commonest indication was subacute intestinal obstruction (53%), followed by enteroenteric and enterocutaneous fistulae (10.7%), chronic gastrointestinal blood loss (7%) and protein losing enteropathy (7%). The emergency indications included perforation and peritonitis (14%) and massive GI bleed (4%). Two patients required surgery for gastric outlet obstruction (4%). Eleven patients had combinations of pathology, such as stricture with fistula or perforations. Table 2 shows the clinical and operative findings of 68 patients who underwent surgery for CD and 41 patients for TB in a tertiary referral centre in South India. Indications for surgery in TB were diagnosis and relief of intestinal obstruction. In this study as in previous studies^[7], fever, altered bowel habits, clinical presentation as recurrent intestinal obstruction, diffuse small bowel involvement with multiple strictures and deep linear ulcers/cobblestone

Table 2 Clinical parameters and operative findings of 68 surgical patients with Crohn's and 41 patients with intestinal tuberculosis *n* (%)

Parameter	Crohn's disease (<i>n</i> = 68)	Intestinal Tuberculosis (<i>n</i> = 41)	<i>P</i> value ¹
Clinical parameters			
Male:female	38:30	28:13	NS
Mean age (yr)	31.2 (16-52)	36.8 (23-64)	NS
Fever	17 (25)	28 (68)	< 0.010
Pain	50 (73)	26 (63)	NS
Altered bowel habits	46 (67)	14 (34)	< 0.001
Fistula in ano	7 (12)	1 (2.4)	NS
Anemia	34 (50)	28 (68)	NS
Edema	22 (32)	14 (34)	NS
Growth retardation	14 (20)	4 (9.7)	NS
Treated as tuberculosis	18 (26)	8 (19)	NS
Pulmonary involvement	6 (8.2)	14 (34)	< 0.010
Abdominal distension	18 (26)	19 (46)	< 0.050
Abdominal lump	7 (12)	8 (19)	NS
Recurrent intestinal obstruction	40 (59)	14 (34)	< 0.020
Operative findings			
Peritoneal nodules	15 (22)	32 (78)	< 0.001
Ascites	19 (27)	28 (68)	< 0.001
Nodules over bowel/mesentery	14 (20)	14 (34)	NS
Site of involvement:			
Diffuse small bowel	22 (32)	6 (14.6)	< 0.050
Jejunum	14 (20)	6 (14.6)	NS
Ileum	44 (64)	32 (78)	NS
Colon	6 (8.8)	2 (4.8)	NS
Small bowel and colon	18 (26)	3 (7.3)	< 0.020
Multiple strictures	44 (64)	4 (9.7)	< 0.001
Skip lesions	17 (25)	4 (9.7)	NS
Internal fistula	14 (20)	1 (2.4)	< 0.01
Mesenteric fat creeping	44 (64)	28 (51)	NS
Shortened mesentery	18 (26)	14 (34)	NS
Aphthoid ulcers	32 (47)	15 (36)	NS
Deep linear ulcers	40 (59)	8 (19)	< 0.001
Cobblestone appearance	44 (64)	7 (17)	< 0.001
Stricture	40 (59)	18 (44)	NS

¹*P* value: by χ^2 . NS: Not significant.

appearance of bowel were significantly more common in CD than in TB. Conversely, pulmonary involvement, abdominal distension, ascites, peritoneal nodules and terminal ileal/ileocolic involvement without multiple strictures were significantly more common in TB.

ROLE OF THERAPY AND FOLLOW UP

Even when the best available diagnostic modalities are utilized, the differentiation of TB from CD remains a problem in 10%-15% of patients in India. Four decades ago, it was usual for these patients to have received anti-tuberculous treatment (ATT) for two years and the individuals who did not respond to such treatment were then diagnosed as having CD. However, advances in diagnosis and increasing experience have allowed Indian gastroenterologists to make a diagnosis of CD in the first instance. Today, nearly half of our patients with CD continue to have received ATT prior to the establishment of the diagnosis of CD. Clearly, a good response to ATT, confirmed

by endoscopic and histological clearance of disease, establishes and confirms the diagnosis of TB. Conversely, a poor response to ATT could indicate that the diagnosis is really CD, but could also represent non-responsive TB or drug-resistant TB. An understanding of the intestinal response to therapy of TB and of CD is therefore basic to the use of therapy in differentiating these two diseases.

Use of the directly observed treatment, short course (DOTS) strategy for 6 mo has become standard in the treatment of TB in India. In patients with newly diagnosed pulmonary TB, the "cure" rate after DOTS ranges from 75%-92%^[61,62]. Treatment success in extrapulmonary TB was 91% in one study, but this study did not further categorize extrapulmonary TB^[62]. In a study from Kerala of 47 patients treated with either DOTS or daily chemotherapy, complete mucosal healing was noted at colonoscopy in 35 at 2 mo and in all 47 at 6 mo^[63]. However, biopsies were not taken from these patients during follow up nor was there any long term follow up of the treated individuals. Similar observations have been made of mucosal healing soon after commencement of ATT in colonic TB, but the lack of long term follow up - to ensure that disease does not recur - is a drawback of these studies^[64]. In our anecdotal experience, it is common for a proportion of patients to have recurrent disease one to five years after cessation of ATT. At this point it is often difficult to decide whether the patient has recurrence of TB or whether the patient is actually suffering from CD. Drug resistance is increasingly common in strains of MTB and may contribute to recurrent or persistent disease in patients correctly diagnosed as having TB but not showing clinical, endoscopic or histological response to treatment with first line chemotherapy for TB. Multi-drug resistance (MDR) has been observed in 2.4% to 13.2% of strains of MTB isolated from newly diagnosed pulmonary TB patients and in 17.4% to 25.5% of previously treated patients^[65,66]. Extensive drug resistance (XDR) is found almost exclusively in previously treated patients and accounts for about 6% of MDR TB^[67]. Statistics regarding prevalence of MDR and XDR strains in intestinal TB are not available from India; however, in one series of 30 patients with colonic TB in Taiwan, 4 (13%) had MDR TB^[68].

Studies carried out in the last three decades clearly demonstrate that CD does not respond to conventional ATT including isoniazid, rifampicin and ethambutol^[69,70]. Prolonged treatment for *Mycobacterium avium* subsp. *paratuberculosis* with clarithromycin, rifabutin and clofazimine did lead to improvements in disease activity in patients with CD, but such improvement was not sustained^[71].

In summary, the response to an adequate course of ATT should differentiate patients with TB from CD in instances when this becomes necessary. Such response should be assessed by clinical and biochemical parameters, but also by evidence of mucosal healing at endoscopy and on segmental mucosal biopsies. A proportion of patients with intestinal TB (amounting in our experience to about 10%) will not show the desired response and may then be incorrectly diagnosed as having CD. Longer term follow up in these patients will eventually reveal the correct diagnosis.

CONCLUSION

In conclusion, differentiating intestinal TB from CD in countries like India, where both diseases are prevalent, is an important clinical problem. A combination of a good clinical history with colonoscopy, biopsies, cultures, and barium or simple CT studies can be utilized to make a diagnosis in the majority of cases. This article has highlighted important parameters that differentiate intestinal TB from CD in each of these diagnostic modalities. The role of newer techniques, such as capsule endoscopy, single and double balloon enteroscopy, CT enteroclysis, PCR and immunological assays for MTB, has also been highlighted. An adequate course of antituberculous therapy and longer term follow up may become necessary to differentiate patients with TB from CD in some instances. An understanding of the intestinal response to treatment of TB and of CD is, however, fundamental to the use of therapy in differentiating these two diseases.

ACKNOWLEDGMENTS

Ghoshal U would like to acknowledge the help of Ghoshal UC, Phillip M would like to acknowledge the help of Prakash K and Zacharias P, and Reddy DN would like to acknowledge the contribution of Rajesh Gupta towards the preparation of the manuscript.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

Is diabetes a causal agent for colorectal cancer? Pathophysiological and molecular mechanisms

Olga Giouleme, Michael D Diamantidis, Marios G Katsaros

Olga Giouleme, Michael D Diamantidis, Marios G Katsaros, Department of Gastroenterology, First Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, AHEPA General Hospital, 546 36, Thessaloniki, Greece

Author contributions: Giouleme O and Diamantidis MD contributed equally to this work; Giouleme O performed the research, analyzed the data and wrote the manuscript; Diamantidis MD performed the research, analyzed the data and wrote the manuscript; Katsaros MG assisted in writing parts of the paper.

Correspondence to: Olga Giouleme, MD, PhD, Assistant Professor, Department of Gastroenterology, First Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, AHEPA General Hospital, 1 S. Kiriakidi St, 546 36, Thessaloniki, Greece. olgagiouleme@yahoo.gr

Telephone: +30-2310-994687 Fax: +30-2310-994687

Received: March 15, 2010 Revised: April 24, 2010

Accepted: May 1, 2010

Published online: January 28, 2011

Abstract

The possible relationship between diabetes mellitus (DM) and colorectal cancer (CRC), concerning pathophysiological and molecular mechanisms is highlighted in this review. The most recent and complete articles and developments in this particular field were thoroughly reviewed. Common risk factors, such as obesity, sedentary lifestyle, and Western diet between DM and CRC, led to the theory that DM might be a causal agent for CRC development. Various studies have connected type 2 DM and CRC, either proximal or distal, in both sexes. Additionally, chronic insulin treatment has been linked with increased colorectal tumor risk among type 2 diabetic patients. Interestingly, elevated hemoglobin A1c has been proven to be an independent predictor of aggressive clinical behavior in CRC patients. These mechanisms include the insulin-like growth factor-hyperinsulinemia theory and the participation of oncogenic intracellular signaling pathways. Furthermore, it has been proposed that Cox-2 inhibitors might have a role in decreasing the incidence of CRC. Finally, the use of statins to reduce the risk for colon cancer in patients with diabetes has

remained controversial. Diabetic patients over 50 should receive counseling regarding their elevated risk for CRC, and screening colonoscopy should be recommended before initiating insulin therapy. However, there are no current guidelines, and this strategy is not yet applicable to some countries, as the corresponding risk would not allow screening colonoscopy to be adopted. There is strong evidence to indicate that DM is a causal agent for CRC development. This conclusion provides new impetus for re-evaluating CRC screening worldwide.

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Key words: Diabetes mellitus; Colorectal cancer; Molecular oncogenic pathways; Screening

Peer reviewers: Finlay A Macrae, MD, Professor, Department of Colorectal Medicine and Genetics, Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia; Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Giouleme O, Diamantidis MD, Katsaros MG. Is diabetes a causal agent for colorectal cancer? Pathophysiological and molecular mechanisms. *World J Gastroenterol* 2011; 17(4): 444-448 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/444.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.444>

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related death in the Western world with 655 000 deaths per year^[1]. In addition, the incidence of diabetes mellitus (DM) is increasing rapidly. In 2000, at least 171 million patients suffered from diabetes worldwide, and it is estimated that by the year 2030, the number will almost double^[2]. Serious long-term complications of DM include cardiovascular, retinal, and nerve disease, along with chronic renal failure and high tendency for infections. Common etiologic factors including obesity, sedentary

lifestyle, and Western diet between these two widespread diseases, led to the hypothesis that there might be a connection between diabetes and CRC, rendering diabetes a causal agent for CRC.

Diabetes has been linked with ovarian^[3], pancreatic^[4,5], liver^[5], renal^[6], breast cancer^[4], melanomas^[6], and cancers of the urinary tract^[6], stomach^[7], cervix^[7], and endometrium^[7]. Patients with type 1 and type 2 diabetes might be at an increased risk for cancer, but there is more evidence available for patients with type 2 diabetes^[6]. As far as CRC is concerned, type 2 DM has also been linked with the aforementioned cancer.

RELATIONSHIP BETWEEN DIABETES MELLITUS AND COLORECTAL CANCER

Various studies have connected diabetes with men or women suffering from CRC, with proximal or distal CRC, or with both sexes and colorectal subsites. Part of these discrepancies might be attributed to different sample sizes, follow-up duration, inclusion/exclusion criteria of the studied population, classification systems, disease heterogeneity, or interplay between biological and environmental factors. A large retrospective study conducted in 2006 reported that there is a significantly elevated risk for proximal CRC in type 2 diabetic men, but no significant increase in risk for diabetic women^[8]. Cigarette smoking has been reported to positively modify diabetes-associated CRC risk^[8]. Moreover, CRC has been connected with type 2 diabetic men^[8-10], diabetic women^[11], or both sexes^[12]. Interestingly, type 2 diabetes predisposes patients to an increased risk for proximal^[8] or distal CRC^[9], or both proximal and distal, colonic and rectal cancers^[12]. Thus, the relationship between type 2 diabetes and CRC has been proposed and reported in several studies.

Chronic insulin therapy has been associated with an increased colorectal tumor risk among type 2 diabetic patients^[13,14]. Specifically, a three-fold risk increase for patients with insulin-dependent type 2 DM in comparison to the general population has been observed^[15]. On the other hand, there is limited information on the short and long term outcome for diabetic patients diagnosed with CRC. An association between the control of type 2 DM, as determined by the levels of hemoglobin A1c (HbA1c), has been examined. Elevated HbA1c has been proven to be an independent predictor of aggressive clinical behavior in patients with CRC. Siddiqui *et al.*^[16] reported that patients with poorly controlled type 2 DM have more right sided and advanced colorectal cancers, a younger age of presentation, greater use of exogenous insulin, and a poorer 5-year survival. Interestingly, neoadjuvant chemotherapy in rectal cancer is less effective in diabetic patients than in non-diabetics^[17]. Nevertheless, according to a Norwegian study, diabetes does not seem to affect the short-term survival or the overall cancer-specific survival in patients with CRC. Indeed, the shorter overall survival in diabetic patients suffering from colorectal cancer in comparison to non-diabetics is attributed to cardiac diseases and higher age^[18].

PATHOPHYSIOLOGICAL AND MOLECULAR MECHANISMS

Several pathophysiological mechanisms have been proposed to explain the possible relation between DM and CRC. The insulin-like growth factor (IGF-1)-hyperinsulinemia theory implies that elevated insulin and free IGF-1 levels support the proliferation of colon cells, thereby leading to a survival benefit, resulting in CRC^[19]. In patients with type 2 DM, elevated insulin levels are present in an effort to overcome peripheral insulin resistance by increased insulin production. Moreover, hyperinsulinemia is further augmented by exogenous application of insulin. The aforementioned molecular model suggests that hyperinsulinemia, IGF-1, and the relative binding proteins play important roles in metabolism, cell growth, proliferation, and the regulation of apoptotic process in colon cells^[6]. Thus, normal colon cells acquire neoplastic characteristics, displaying cancerous transformation.

The aforementioned theory supports the view that not only does insulin stimulate the growth of colon cells *in vitro*^[20], but its proliferative effect is also mediated by insulin cognate and IGF-1 receptors^[21]. It has been demonstrated that the epithelium of colon carcinoma cell lines shows an increased insulin receptor density in comparison to normal colon epithelium. Furthermore, insulin leads to increased bioavailability of IGF-1^[19]. Insulin and IGF-1 signaling pathways either enhance proliferation, or inhibit apoptosis of colon epithelial cells, leading to carcinogenesis.

Oncogenic intracellular pathways mainly involving kinase neoplastic proteins [mitogen activated protein kinases, extracellular signal regulated kinase, phosphatidylinositol-3-kinase, protein kinase B and mammalian target of rapamycin (mTOR)] are activated^[19] (Figure 1). However, there have been conflicting results concerning the link between IGF-1 and CRC, as there are studies both supporting and disputing this link^[6]. In addition, the actions of insulin and IGF-1 are mediated by the activation of Ras, which may lead to increased sensitivity of colon cells to growth factors and accelerated progression from adenoma to carcinoma^[19]. The time from adenoma formation to development of CRC is believed to be between 10 and 15 years^[6].

Interestingly, insulin stimulates cell proliferation and c-Myc expression in various colon cancer cell lines, in the intestinal non-cancer cell line IEC-6, and in fetal rat intestinal cell cultures (Figure 1). The effects of insulin involve activation of the mTOR signaling pathway in combination with nuclear translocation of β -catenin, an effector of Wnt signaling (Figure 1). Hence, both Wnt and mTOR pathways participate in insulin-stimulated oncogene expression in intestinal cells^[22,23]. Furthermore, it has been reported that insulin stimulates the phosphorylation and activation of p-21-activated protein kinase-1 (PAK-1) in an *in vivo* hyperinsulinemic mouse model, indicating that PAK-1 serves as an important link between insulin and Wnt signaling pathways, promoting intestinal carcinogenesis^[24]. Finally,

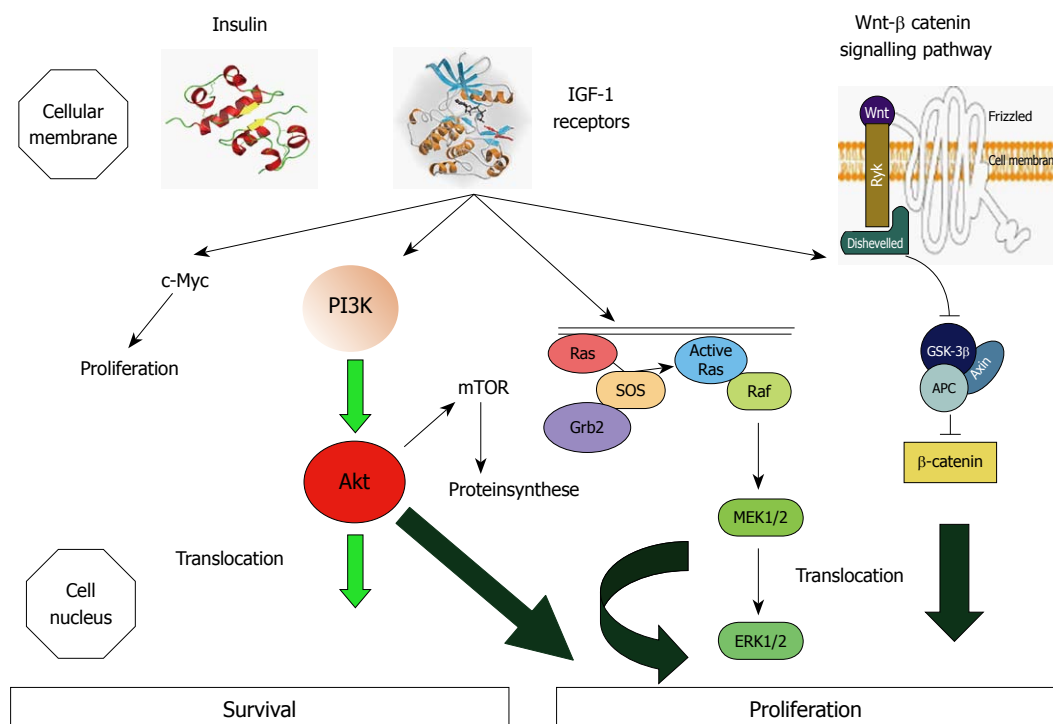


Figure 1 Schematic review of the molecular mechanisms linking diabetes mellitus and colorectal cancer. Insulin and insulin-like growth factor (IGF-1) signaling pathways are shown, along with their effect on apoptosis, proliferation, and protein synthesis. The activated mitogen activated protein kinases (MAPK) pathway is associated with proliferation and the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway mainly leads to survival (inhibition of apoptosis). Moreover, protein synthesis is induced via the Akt pathway. The complexity of the system and the cross-talk between the individual pathways are presented. MEK: Mitogen extracellular kinases; ERK: Extracellular signal-regulated kinases; mTOR: Mammalian target of rapamycin; Ras: "Rapid accelerating sarcoma" serine/threonine-kinase; Raf: "Rapid accelerating fibrosarcoma" serine/threonine-kinase; Ryk: Related to tyrosine kinases; SOS: Silencing of survival signals; Grb2: Growth factor receptor binding protein 2; APC: Activated protein C; GSK-3 β : Glycogen synthetase kinase-3 β ; Axin: Activated extracellular index.

the peroxisome proliferators-activated receptor- γ is associated with CRC, including insulin and inflammation related mechanisms, given its association with insulin, diabetes, obesity, and inflammation^[25] (Figure 1).

Another possible molecular mechanism linking diabetes and CRC involves the hormone glucagon-like peptide-1 (GLP-1), secreted by the intestinal endocrine L cells, along with the participation of Wnt signaling pathway and the oncogenes c-Myc and cyclin D1. Specifically, in type 2 diabetic patients, GLP-1 secretion is reduced, due to insulin resistance. Reduction of GLP-1 secretion causes compensatory activation of the Wnt pathway in combination with increased expression of proto-oncogenes, such as c-Myc, resulting in intestinal cell proliferation and, plausibly, CRC development^[26]. Nevertheless, cross talk exists between individual oncogenic pathways, which regulate either apoptosis or survival of colon cells. Thus, the theory defined remains controversial, with several aspects requiring thorough investigation, further complicating the relationship between DM and CRC.

Slower bowel transit time is another appealing concept connecting diabetes and CRC. Patients with diabetes are more prone to suffer constipation (bowel movements less than once a day) and slower colonic transit times, possibly because of diabetic neuropathy affecting the intestine. Certain fecal contents are cancer promoting, such as bile acids, ammonium acetate, and fecapentaene-12^[6]. Nevertheless, there is no epidemiological

evidence that constipation is associated with CRC.

Risk factors such as sedentary lifestyle, obesity, Western diet, and the metabolic syndrome are common in both type 2 DM and CRC^[27]. Obesity has a role in increased CRC risk of the diabetic population, because of a variety of factors including insulin resistance, increased inflammatory markers [interleukin (IL)-1, IL-6, tumor necrosis factor- α], and high levels of androgens and estrogens^[6,27].

PROPHYLAXIS FOR COLORECTAL CANCER

Given the increased CRC risk in patients with type 2 DM, it would be reasonable to postulate that these patients might benefit from any potential prophylaxis against CRC. The use of potential chemoprotective agents, such as aspirin, non-aspirin nonsteroidal anti-inflammatory drugs (NSAIDs), and cyclooxygenase 2 (Cox-2) inhibitors, have been considered and investigated. The basis for applying these agents is that they inhibit the production of prostaglandins, which are inflammatory mediators participating in the formation and proliferation of cancers^[28].

Data from two large randomized trials (the British Doctors Aspirin Trial and the UK-TIA Aspirin Trial) show that the use of high-dose aspirin (1200-300 mg/dL) for a minimum period of five years reduces the incidence of CRC^[6]. However, applying both high-dose aspirin and

NSAIDs over the long term is associated with gastrointestinal complications. Therefore, benefits and risks have to be considered on an individual basis. It would also be helpful to establish if smaller doses of aspirin could be effective for chemoprevention.

The association between Cox-2 expression, adenomatous polyps, and CRC has generated much interest. Specific Cox-2 inhibition might have a role, because colorectal adenomas and cancers show a higher than normal Cox-2 expression, which has been associated with worse survival^[6,28]. Thus, Cox-2 inhibitors have been shown to decrease the incidence of CRC. The effects of Cox-2 inhibitors are regulated by inhibition of prostaglandin synthesis. Cox-2 and prostaglandins mediate resistance to apoptosis, modulate tumor angiogenesis, and increase metastatic potential in the intestine. These effects are controlled through prostaglandin E2 and the epidermal growth factor receptor. Although there is some evidence that Cox-2 inhibitors might reduce the incidence of CRC, the cardiovascular risk (acute myocardial infarction) outweighs the potential benefit^[6,28].

An interesting theory has been proposed recently, implicating homocysteine as the missing link between type 2 DM and CRC^[29]. Recent findings indicate that a high homocysteine level is a risk factor for developing DM. In addition, hyperhomocysteinemia is associated with aberrant methylation of DNA, which might lead to inactivation of tumor suppressor genes and CRC growth^[29].

Moreover, the use of statins has been associated with a small reduction in the risk for colon cancer in diabetic patients. However, the causal link is not clear^[30]. There are also studies, which do not connect statins with a reduced risk of CRC in large cohorts of patients, indicating that long-term regular use of statins does not protect against CRC^[31-34]. Nevertheless, the application of statins has been linked with a relative reduction in the CRC risk^[35], along with a reduced risk for stage IV CRC^[31]. Interestingly, simvastatin therapy results in inhibition of the release of IL-8 and IL-6 from colorectal cell lines. Hence, CRC risk is reduced because of decreased synthesis and release of the aforementioned proinflammatory cytokines by the tumor cells^[36]. Thus, the conflicting results necessitate further investigation to elucidate this controversial topic.

PREVENTING COLORECTAL CANCER

A large number of epidemiological studies have demonstrated that the risk for CRC is increased in patients with type 2 DM. A recent meta-analysis showed that patients with type 2 DM have a 30% increased risk for CRC versus the general population and that this risk is doubled in diabetic patients treated with insulin. The increased risk correlates with the duration of insulin therapy and might be due to increased levels of insulin and free IGF-1. The increased risk in patients with type 2 DM treated with insulin is comparable to a positive family history.

A better way to prevent CRC in patients with type 2 DM is screening. Over the past decade, tests generally offered in screening guidelines include colonoscopy every 10

years, fecal occult blood testing (FOBT) annually, double contrast barium enema every five years, or flexible sigmoidoscopy every 5-10 years. Current guidelines in most Western countries recommend colonoscopy every 10 years, beginning at the age of 50 years for people with average risk, or alternatively screening with FOBT every year combined with flexible sigmoidoscopy every five years.

Most patients with type 2 DM will be over 50 at diagnosis and are eligible for screening according to screening guidelines. Diabetic patients over 50 should receive counseling regarding their elevated risk for CRC and screening colonoscopy should be recommended before initiating insulin therapy. In younger diabetic patients, the decision should be made individually and be based on the presence of other risk factors, such as smoking or previous insulin therapy. Bearing in mind that the action of insulin and IGF-1 is partly mediated by the activation of Ras, which might lead to accelerated progression from adenoma to carcinoma, screening intervals concerning the population with a positive family history should not exceed five years, especially in patients under insulin treatment. However, there are no current guidelines for this group of patients, and these recommendations only reflect the opinion of some experts. Hence, this strategy may not be applicable to some countries, as the corresponding risk would not allow screening colonoscopy to be adopted. Unfortunately, patients are often reluctant to undergo screening for CRC, and a high percentage of colorectal cancers could have been prevented; the disease is often diagnosed at advanced stages. Thus, the duty of the medical community is to inform the patients concisely, accurately, and clearly, convincing them to be checked regularly.

In conclusion, more clinical trials are required to elucidate not only the relationship between type 2 DM and CRC, but also CRC outcome in diabetic patients. Furthermore, the precise molecular mechanisms should be determined, which will lead to targeted therapy for CRC in diabetic patients.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Lin YP

Ghrelin and gastrin in advanced gastric cancer before and after gastrectomy

Anna Zub-Pokrowiecka, Kazimierz Rembiasz, Peter C Konturek, Andrzej Budzyński, Stanisław J Konturek, Marek Winiarski, Władysław Bielański

Anna Zub-Pokrowiecka, Kazimierz Rembiasz, Peter C Konturek, Andrzej Budzyński, Stanisław J Konturek, Marek Winiarski, Władysław Bielański, Department of Physiology, Medical College, Jagiellonian University, 31-531, Cracow, Poland
Anna Zub-Pokrowiecka, Kazimierz Rembiasz, Peter C Konturek, Andrzej Budzyński, Stanisław J Konturek, Marek Winiarski, Władysław Bielański, 2nd Department of General Surgery, Medical College, Jagiellonian University, 31-531, Cracow, Poland
Anna Zub-Pokrowiecka, Kazimierz Rembiasz, Peter C Konturek, Andrzej Budzyński, Stanisław J Konturek, Marek Winiarski, Władysław Bielański, Department of Medicine, Thuringia Clinic Georgius Agricola Saalfeld Teaching Hospital University Jena, 07318, Saalfeld, Germany

Author contributions: Zub-Pokrowiecka A, Rembiasz K, Winiarski M and Budzyński A interviewed and examined patients; Konturek PC and Bielański W performed reverse transcription-polymerase chain reaction and radioimmunoassays; Konturek SJ designed the study and drafted the manuscript; all authors read and approved the final manuscript.

Supported by The Ministry of Science and Higher Education in Poland Grant K/PBP/000012 for 2006-2008 and by Jagiellonian University Medical College Research Project for 2006-2009

Correspondence to: Stanisław J Konturek, Professor MD, Department of Physiology, Medical College, Jagiellonian University, Grzegorzewska 16, 31-531, Cracow, Poland. mpkontur@cyf-kr.edu.pl

Telephone: +48-12-605604 Fax: +48-12-211578

Received: June 4, 2010 Revised: August 6, 2010

Accepted: September 13, 2010

Published online: January 28, 2011

Abstract

AIM: To investigate plasma ghrelin, gastrin and growth hormone secretagogue receptor (GHS-R) expression in advanced gastric cancer (GC) before and after resection.

METHODS: Seventy subjects in whom endoscopy of the upper gastrointestinal tract was performed in the Department of General Surgery at Cracow University during the past decade: (1) 25 patients with GC associated with *Helicobacter pylori* (*H. pylori*) infection; (2)

10 patients with GC 4-5 years after (total or subtotal) gastrectomy; (3) 25 healthy *H. pylori*-negative controls, matched by age and BMI to the above two groups; and (4) 10 GC patients 4-5 years after total gastrectomy. Ghrelin and gastrin plasma concentrations were measured by specific radioimmunoassay under fasting conditions and postprandially at 60 and 90 min after ingestion of a mixed meal. GHS-R expression was examined in biopsy samples from intact healthy mucosa and GC tissue using semi-quantitative reverse transcription-polymerase chain reaction.

RESULTS: In healthy controls, fasting plasma ghrelin levels were significantly elevated and declined markedly at 60 and 90 min after a mixed meal. The concomitant enhanced ghrelin, GHS-R and gastrin expression in GC tissue over that recorded in intact mucosa, and the marked rise in plasma gastrin in these subjects under fasting conditions indicate the role of these hormonal factors in GC formation. Fasting plasma levels and postprandial response of ghrelin and gastrin appear to be inversely correlated in healthy subjects. Feeding in the controls resulted in a significant fall in plasma ghrelin with a subsequent rise in plasma gastrin, but in *H. pylori*-positive GC patients submitted to total or distal gastrectomy, feeding failed to affect significantly the fall in plasma ghrelin that was recorded in these patients before surgery. Fasting ghrelin concentrations were significantly lower in patients 4-5 years after total gastrectomy compared to those in healthy controls and to these in GC patients before surgery.

CONCLUSION: Elevated plasma gastrin and suppression of fasting ghrelin in patients with GC suggest the existence of a close relationship between these two hormones in gastric carcinogenesis.

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Key words: Ghrelin; Gastrin; Gastric cancer; Gastric resection

Peer reviewer: Guida Portela-Gomes, Professor of University of Lisbon, Department of Medicine, Rua Domingos Sequeira-128, Estoril, 2765-525, Portugal

Zub-Pokrowiecka A, Rembiasz K, Konturek PC, Budzyński A, Konturek SJ, Winiarski M, Bielański W. Ghrelin and gastrin in advanced gastric cancer before and after gastrectomy. *World J Gastroenterol* 2011; 17(4): 449-458 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/449.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.449>

INTRODUCTION

Ghrelin, a natural ligand for growth hormone secretagogue receptor (GHS-R), was originally identified in rat gastric mucosa and shown to be expressed by the endocrine ghrelin (Gr)-cells of this mucosa^[1,2]. These Gr-cells, represent about 20% of the total population of endocrine cells in human gastric mucosa, and are located in the lower parts of the oxyntic glands in the fundus and body of the stomach. Gastric acid secretion is stimulated physiologically by gastrin and histamine as well as by ghrelin itself^[2-6] (Figure 1).

Ghrelin is a stimulant of GH secretion, which acts *via* release of GHS-R in the stomach, but characteristically, it is also the only circulatory gastrointestinal hormone that is known to be secreted by the empty (fasting) stomach, to enhance food intake and maintain energy homeostasis following its central or peripheral administration^[6]. Ghrelin is currently considered as the most powerful endogenous orexigenic (appetite-stimulating) hormone, which results in a positive energy balance^[4,6,7]. Its action on gastric mucosa might contribute to carcinogenesis, but the role of ghrelin/GHS-R in gastric cancer (GC) pathogenesis^[8] awaits explanation, and possible use of ghrelin in severe gastric-originated cachexia remains unknown.

The reduction in the ghrelin levels after gastrectomy by > 60% supports the notion that the gastric mucosa is the main site of ghrelin production^[3,4,9]. Gastric cancer modifies the expression of ghrelin in gastric mucosa and this could be attributed, at least in part, to severe atrophic gastritis^[10,11], which usually precedes and might lead to gastric carcinogenesis. Mottershead *et al*^[12] have observed a lack of expression of mRNA for ghrelin in GC cells by immunohistochemical and reverse transcription-polymerase chain reaction methods. According to our recent experience, the expression of mRNA for ghrelin in pronounced atrophic gastritis is relatively low, so major changes in plasma ghrelin cannot originate from the *Helicobacter pylori* (*H. pylori*)-related atrophic gastritis tissue^[12].

The aim of the present study was to analyze the plasma concentrations of the ghrelin-GHS-R complex in patients with advanced GC before and after total or distal gastrectomy. In addition, we analyzed plasma ghrelin concentrations and mucosal gene expression of ghrelin and its receptor, GHS-R, in intact mucosa and in *H. pylori*-infected GC tissue. Moreover, the analysis of fasting and postprandial plasma ghrelin levels *vs* plasma gastrin con-

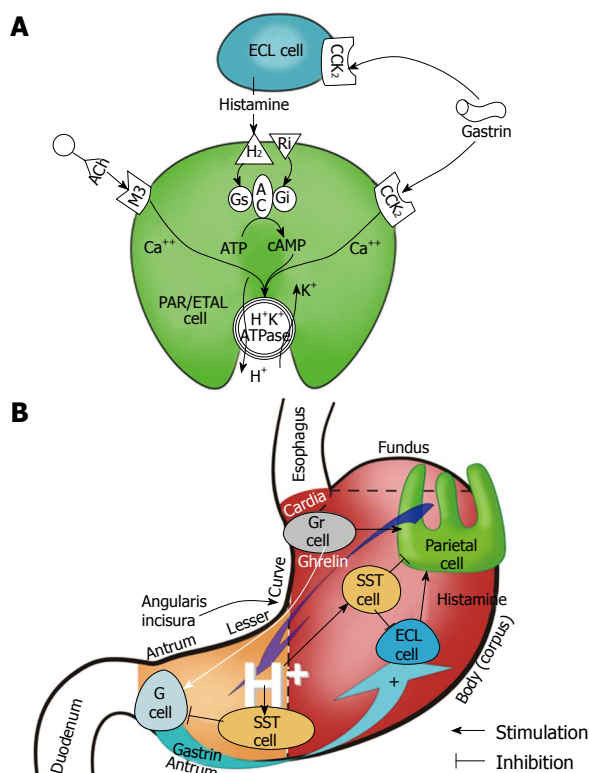


Figure 1 Gastric secretory mechanism at the parietal cell (oxyntic) level (A) and at the level of the whole stomach (B).

centrations in patients at 4-5 years after total and distal gastrectomy was assessed to elucidate the possible compensatory extragastric production of these hormones.

MATERIALS AND METHODS

The study included 70 patients, aged 18 and over who, from January 2006 to May 2008, underwent upper gastrointestinal tract endoscopy in a specialized unit of the Department of Surgery, Jagiellonian University Medical College, Cracow.

Exclusion criteria included any of the following: age < 18 years; no consent to participate in the study; a history of diabetes, thyroid disease and neuroendocrine tumors, use of glucocorticoids, progesterone or testosterone, renal and/or liver failure; prior chemotherapy (not applicable to a group of patients 4-5 years after gastrectomy); drugs and/or alcohol addiction; and body mass index (BMI) > 30 kg/m². Patients included in the research program based on the above criteria were assigned to one of the following groups: (1) 25 patients with GC associated with *H. pylori* infection; (2) 10 patients with GC 4-5 years after (total or subtotal) gastrectomy; (3) 25 healthy *H. pylori*-negative controls, matched by age and BMI to the above two groups; and (4) 10 GC patients 4-5 years after total gastrectomy. The basis of this categorization was the initial diagnosis upon admission to our gastrointestinal unit (Table 1). The study was approved and supervised by the Institutional Research Ethical Committee and informed consent was obtained from each participant in this study.

Table 1 Characteristics of study groups (median and range)

	Patients with GC and <i>H. pylori</i> infection (before operation)	Patients 4-5 yr after total gastrectomy	Controls without pathology in the gastric mucosa and <i>H. pylori</i> infection
<i>n</i>	25	10	25
Age (yr)	64.6 (43-81)	60.8 (49-69)	51 (22-67)
Male/female	14/11	5/5	12/13
BMI (kg/m ²)	23.1 (18.7-29.7)	22.4 (20.3-24.5)	23.4 (19.5-30)

GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; BMI: Body mass index.

All subjects underwent gastroscopy with mucosal biopsy. The study was performed after at least 12 h of fasting. Topical lidocaine (Lignocaine aerosol 10%, Glaxo-Wellcome, UK) was used for local throat anesthesia if there was no history of allergy to it. All gastroscopies were performed by the same highly experienced endoscopists. During each endoscopy procedure, visual assessment was made of the mucosa of the esophagus and gastric cardia, fundus, corpus, pylorus and antrum, and the first portion of the duodenum and fundus of the stomach in inverted gastroscopy. In post-gastrectomy patients, during initial endoscopy, esophago-jejunal anastomosis was evaluated and tissue samples were taken for histological examination.

In patients with GC and in the control group, the rapid urease test was performed at the time of endoscopy. The biopsy samples from the antral mucosa (or in cases of cancer involvement of the distal stomach from any part of the gastric mucosa) were placed on the yellow colored gel that contained urea. The change in color of the indicator contained in the gel from yellow to pink demonstrated the presence of urease in the specimen. In accordance with the attached instructions (Urease Test "GUTplus", Lencomm Trade International, UK), the results were read at 30 min and 3 h after collection to decide whether or not the mucosa was infected by *H. pylori*.

In controls during endoscopy, samples from the gastric fundus mucosa, body and antrum were obtained for histopathological examination. In patients with GC, biopsy specimens were also taken from the tumor itself. The morphological form of the cancer was assessed according to the Bormann classification system. Patients with GC underwent surgery; in 10 cases, it was total gastrectomy, and in eight, it was subtotal (distal) gastrectomy.

Venous blood was collected from all patients with an empty stomach (i.e. at least 12 h without eating solid or liquid meals), at 08:00 h, and postprandially at 60 and 90 min after a mixed carbohydrate/protein/fat meal plus 250 mL milk of about 600 Kcal (given at 10:00 h). In the patients with GC, the above-mentioned protocol was repeated twice, that is, before treatment and 10 d after surgery, when the patients started to eat.

Venous blood samples were collected into tubes that contained a 10% aqueous solution of disodium EDTA to prevent clotting (0.05 mL 10% EDTA in 5 mL of blood). The centrifugation was performed within 10 min from of

blood collection. The blood was centrifuged for 10 min at 3000 r/min (MPW-340 centrifuge; Precision Mechanics, Warsaw, Poland). Plasma obtained after separation was divided into three portions and frozen (at about -80°C) until quantification analysis of ghrelin, GHS-R and gastrin by radioimmunoassay.

Laboratory tests were performed in the specialized Hormonal Research Laboratory of Isotopic Diagnostics at the Department of Physiology, Jagiellonian University Medical College.

The concentration of ghrelin in the test plasma samples was determined using S-2227RIKU4864 radioimmunoassay kits (Peninsula Laboratories, San Carlos, CA, USA). All measurements were performed in duplicate. Standard curves were prepared by appropriate dilution of 12.8 µL lyophilized peptide. Initial incubation of 100 µL plasma samples and subsequent dilutions of ghrelin standards (1, 2, 4, 8, 16, 32, 64, and 128 pg) was carried out for 24 h at 4°C, after addition of 100 µL highly specific rabbit antibodies for human ghrelin. Later, 100 µL of tracer (¹²⁵I-ghrelin, 10000-15000 cpm) was added to each sample and incubation was continued for another 24 h at 4°C. Immunoprecipitation by addition of second antibody (goat anti-rabbit IgG) and centrifugation after 90 min incubation at 25°C was performed for the final separation of the free and bound fractions. The concentration of ghrelin in plasma samples was calculated based on the calibration curve that was obtained by "Spline" method, based on measurements of radioactivity (gamma counter) for consecutive concentrations of the standards. Assay sensitivity was 3.0 pg/mL, and the specificity of the antibodies for the labeled human ghrelin was 100%. In accordance with the attached attestation (Peninsula Laboratories, USA) antibodies do not fall (0%) in cross-reaction with motilin, growth hormone releasing factor, orexin, secretin, VIP and galanin.

Assessments of gastrin levels were performed using a commercial kit (GAS-PR RIA; CIS Bio-International, France) following the manufacturer's recommendations as described before^[5,6,13]. The plasma samples (100 µL) were incubated in duplicate at 25°C for 3 h with 100 µL of tracer (¹²⁵I-gastrin) and 300 µL of anti G-17 antibody. The antibody equally recognized and had affinity to the gastrins G-17 and G-34. Points of the standard curve at concentrations of 11.2, 28.4, 68.4, 255.8, 651.2 pmol/L were prepared from lyophilized synthetic G-17 and incubated as above. Separation of the free from bound fraction was obtained by immunoprecipitation. Final radioactivity in the samples was assayed, and standard curve points were measured in a gamma counter (1574 Clinigamma, Wallac-LKB, Sweden), using the computer program Spline in order to calculate the concentration of gastrin.

Histopathological examination of gastric endoscopic biopsies was performed at the Department of Pathomorphology, Jagiellonian University Medical College. Specimens were fixed in 10% buffered formalin and used for paraffin preparations stained with hematoxylin and eosin, alcian blue and periodic acid-Schiff stain at pH 2.5, and the standard Giemsa method. Fixed specimens

Table 2 Plasma ghrelin concentrations (median and range) recorded under fasting conditions and postprandially (at 60 and 90 min after a meal) in gastric cancer patients before surgery, 4-5 yr after gastrectomy, and healthy controls (95% CI) (pg/mL)

	Median fasting plasma ghrelin level	Median plasma ghrelin level 60 min postprandial	Median plasma ghrelin level 90 min postprandial
Patients GC (before surgery)	193.3 ¹ (150-220)	161 (125-220)	160 (153-248)
Patients 4-5 yr after gastrectomy	157 ² (107.1-229.4)	153.11 (108-217.3)	142 (98.4-205)
Controls	293 (179-480.4)	234 (148.3-368.4)	243.2 (151-392)

¹Indicates significant difference between fasting levels in controls and those in gastric cancer (GC) patients before surgery ($P = 0.0578$); ²Indicates significant difference in fasting ghrelin levels between controls and patients operated upon 4-5 years earlier ($P = 0.0113$).

Table 3 Plasma gastrin concentrations (median and range) in gastric cancer patients before surgery, 4-5 yr after surgery, and in healthy controls, under fasting and postprandial (60 and 90 min after a meal) conditions (95% CI) (pmol/L)

	Median fasting plasma gastrin level	Median plasma gastrin level 60 min postprandial	Median plasma gastrin level 90 min postprandial
Patients with GC (before surgery)	29 (20-43)	38 (24.5-54.2)	37 (26.4-53)
4-5 yr after gastrectomy	25.4 (14-45.5)	25.1 (14.5-43.2)	23 (13.5-39)
Controls	18.3 ^{1,2} (8.4-39.5)	39 (19.1-79)	40 (20-79)

¹Indicates significant difference between control and postprandial value recorded at 60 min after a meal ($P = 0.0027$); ²Indicates significant difference between control and postprandial value recorded at 90 min after a meal ($P = 0.0036$). GC: Gastric cancer.

were immersed in a series of alcohols and xylenes with increasing concentrations, and then embedded in a paraffin block. The paraffin blocks were cut into sections of 3 μ m, deparaffinized in a series of xylenes and alcohols with decreasing concentration, and stained by pathological methods. The Sydney classification system was used for the assessment of inflammation and atrophy, and Lauren's classification system for the evaluation of the type of gastric cancer.

Comparison of average hormone concentrations was performed using the general linear model, and more specifically, analysis of covariance. If a factor was statistically significant (at $\alpha = 0.05$), the post-hoc Tukey test (according to unequidistributed groups variant) was used to compare all possible pairs of means resulting from categorization according to factor.

RESULTS

In fasting and 60 and 90 min postprandial plasma samples from healthy controls, ghrelin levels decreased significantly at 60 and 90 min after food intake (Table 2, Figure 2). In contrast, plasma gastrin, which showed the lowest values under fasting conditions, almost doubled at 60 and 90 min after ingestion of a mixed meal (Table 3, Figure 2), so an inverse relationship was seen between fasting and postprandial ghrelin and gastrin concentrations in healthy subjects. Neither group of GC patients, just before surgery or 4-5 years after gastrectomy, showed this clear inverse relationship between fasting and postprandial plasma ghrelin and gastrin (Table 2).

Plasma ghrelin originates mainly from the Gr/endocrine/neurocrine cells that are present mainly in the gastric mucosa, and predominantly in the lining of the

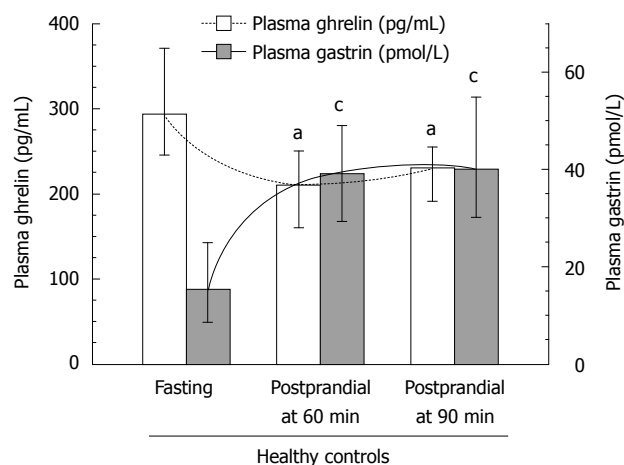


Figure 2 Plasma ghrelin and gastrin concentrations in healthy subjects under fasting and postprandial conditions at 60 and 90 min after a meal. Median values (with range). "a" and "c" indicates significant ($P < 0.05$) change compared to fasting ghrelin or gastrin plasma concentrations.

proximal portion of the stomach. Therefore, we first analyzed the fasting and postprandial plasma ghrelin levels in patients with GC located in the proximal (fundus and corpus) or distal (antrum) stomach. Mean plasma levels of ghrelin (fasting, 60 and 90 min postprandially) in cancer patients before surgery in each of subsequent measurements (fasting, 60 and 90 min after a meal) were significantly lower in those with tumor located in the proximal stomach (fundus or corpus) compared with that in the distal stomach (antrum). As shown in Figure 3 and Table 2, in GC of the proximal stomach, the plasma ghrelin levels were as follows: mean fasting concentration was 193 pg/mL (95% CI: 150-220 pg/mL), and postprandial ghrelin level at 60 min was 161 pg/mL (95% CI: 125-

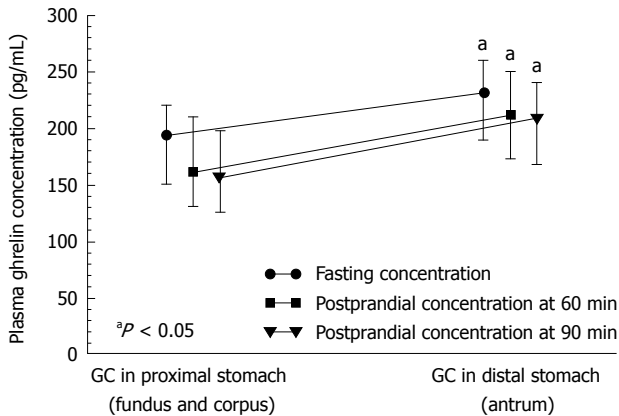


Figure 3 Median values (and range) of fasting and postprandial plasma ghrelin concentrations recorded in patients with gastric cancer localized in the proximal versus distal stomach. "a" indicates significant (^a $P < 0.05$) increase above the ghrelin levels in gastric cancer (GC) localized in proximal stomach.

220 pg/mL), and at 90 min, it was 160 pg/mL (95% CI: 153-248 pg/mL). There was no significant difference between fasting and postprandial ghrelin levels recorded in patients with proximal GC, whereas plasma ghrelin levels in patients with distal GC showed a small but significant increase. However, no difference was found between fasting and postprandial ghrelin levels in patients with GC localized in the proximal or distal portion of the stomach ($P = 0.3394$). As shown in Figure 3, in patients with distal GC, the corresponding plasma ghrelin values in subsequent samples were as follows: fasting, 230 pg/mL (95% CI: 182-252 pg/mL); 60 min postprandially, 201 pg/mL (95% CI: 165-235 pg/mL); and 90 min postprandially, 201 pg/mL (95% CI: 162-225 pg/mL).

The mRNA expressions for all three hormones, ghrelin, GHS-R and gastrin, in intact antrum and the gastric corpus and fundic mucosa are shown in Figure 4. Expression of gastrin and GHS-R mRNA was high in the GC tissue, whereas that of ghrelin was low. Expression of GHS-R and gastrin reached a high level in the intact antral mucosa and GC tissue, whereas ghrelin expression was low in GC but high in intact corpus and fundus mucosa (Figure 4).

Statistically significant differences in plasma levels of ghrelin under fasting conditions and after meal (at 60 and 90 min postprandially) were found in patients following total gastrectomy [in patients with proximal GC (fundus and body)] compared with those after distal gastrectomy (in patients with distal GC), both under fasting conditions and postprandially (Figure 5). Plasma ghrelin levels were significantly lower in the patients with total gastrectomy compared to those recorded after distal gastrectomy.

The plasma ghrelin levels in patients after total gastrectomy were as follows: fasting, 134 pg/mL (95% CI: 104-174 pg/mL); 60 min postprandial, 134 pg/mL (95% CI: 100.2-178 pg/mL); and 90 min postprandial, 136 pg/mL (95% CI: 100.6-161 pg/mL). In patients after subtotal (distal) gastrectomy, fasting ghrelin was a mean 241 pg/mL (95% CI: 205-300 pg/mL); at 60 min postprandially, it was

215 pg/mL (95% CI: 180-260 pg/mL) (subtotal gastrectomy); and at 90 min postprandial, it was 218 pg/mL (95% CI: 175-270 pg/mL). Plasma ghrelin concentrations under fasting conditions and postprandially at 60 and 90 min in patients following total gastrectomy were significantly lower ($P = 0.0183$) than those after subtotal gastrectomy (Figure 5).

Plasma gastrin recorded preoperatively under fasting conditions and postprandially (Figure 6) was significantly higher ($P < 0.05$) than that assessed after distal gastrectomy in patients operated upon for distal GC compared to those who underwent total gastrectomy. Unlike healthy controls, the GC patients who underwent surgery showed no significant difference between fasting and postprandial gastrin levels (at 60 and 90 min) after total or distal gastrectomy ($P = 0.4893$). It should be mentioned that both fasting and postprandial plasma gastrin levels tended to reach relatively lower values in patients with GC located in the proximal (fundic) portion of the stomach when compared to those recorded in patients with GC located beyond the fundus (Figure 7), but the difference between these levels was not statistically significant.

Plasma gastrin level showed a strong and statistically significant difference between fasting and postprandial conditions in the control subjects (Table 3, Figure 6). The lowest plasma gastrin occurred under fasting conditions, and the highest concentrations were observed at 60 and 90 min after food intake. Basal gastrin level in healthy controls was a mean 18.3 (95% CI: 8.4-39.5) pmol/L and it was almost doubled after 60 and 90 min upon food intake, reaching respectively, 39 pmol/L (95% CI: 19.1-79 pmol/L, $P = 0.0027$) and 40 pmol/L (95% CI: 20-79 pmol/L, $P = 0.0036$) (Table 3). In GC patients, fasting median plasma gastrin was 29 pmol/L (95% CI: 20-43 pmol/L), which was significantly higher than that observed in healthy controls. However, after food intake, mean plasma gastrin showed a relatively small, though significant increase at 60 min (38 pmol/L; 95% CI: 24.5-54.2 pmol/L) and at 90 min after food intake (37 pmol/L; 95% CI: 26.4-53 pmol/L). These elevations in plasma gastrin after feeding in GC patients were statistically significant only before surgery (Figure 6). At 4-5 years after gastrectomy (combined total and distal gastrectomy), there was no significant change in plasma gastrin after food intake, but median fasting gastrin levels (25.4 pmol/L; 95% CI: 14-45.5 pmol/L) were still significantly higher than those in healthy controls (Table 3).

After distal gastrectomy, fasting plasma gastrin fell significantly from about 25 pmol/L (95% CI: 21-32 pmol/L) before surgery to 15.7 pmol/L (95% CI: 11-21.1 pmol/L) after gastrectomy, and the differences between these concentrations were statistically significant (Figure 8). The decrease of median gastrin concentration after total gastrectomy was relatively stronger than that observed after subtotal gastrectomy (Figure 8).

The decrease in fasting and postprandial plasma gastrin was observed in both gastrectomy groups though postprandial gastrin level was relatively more reduced after

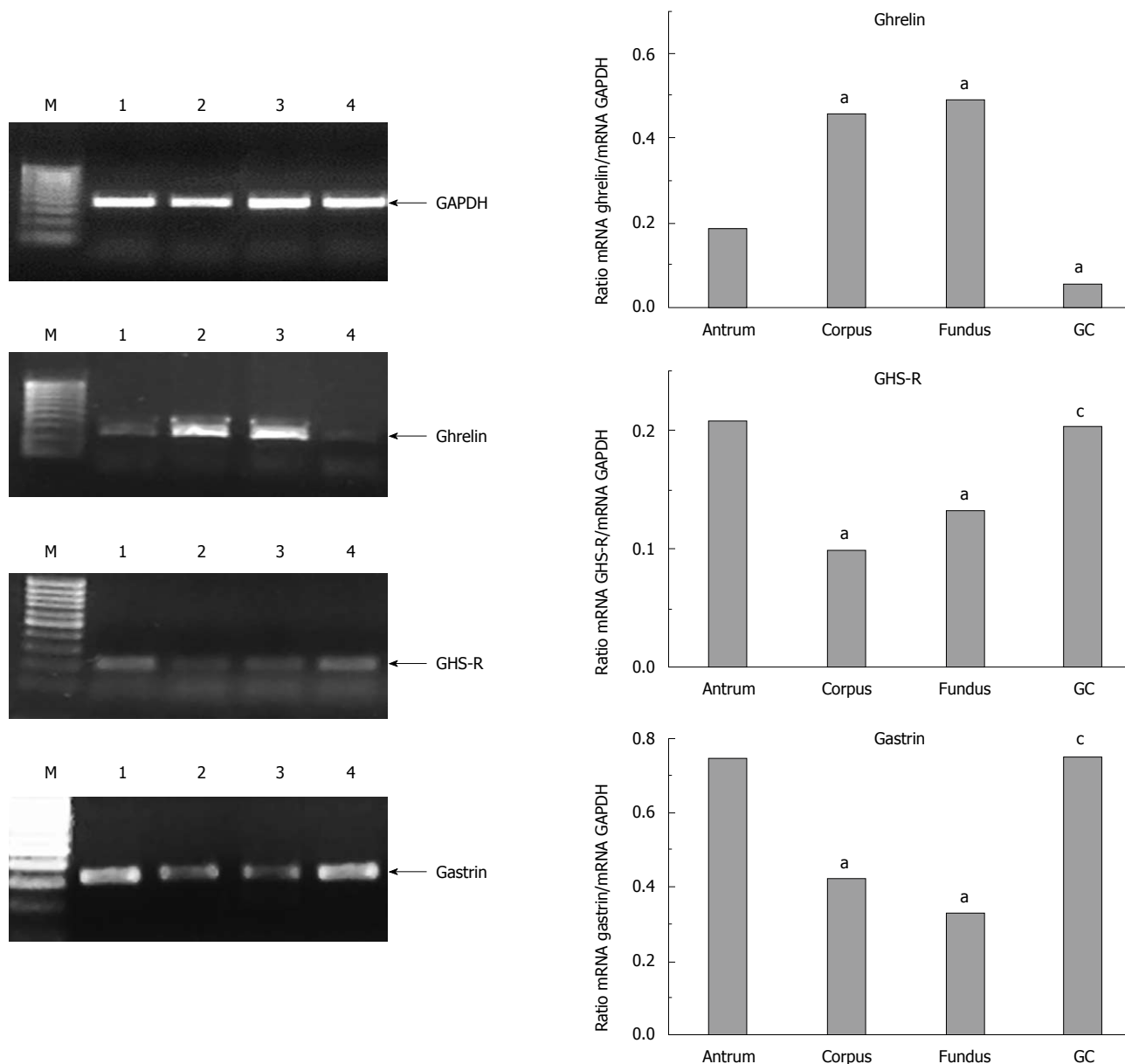


Figure 4 mRNA expression in mucosal and cancer tissue for ghrelin, growth hormone secretagogue receptor and gastrin. "a" indicates significant change in ghrelin, growth hormone secretagogue receptor (GHS-R) and gastrin expression as compared to that in the antral mucosa. "c" indicates significant change in the hormone expression in gastric cancer (GC) tissue compared to that in intact gastric mucosa. M: Marker; 1: Antrum; 2: Corpus; 3: Fundus; 4: GC.

total than distal gastrectomy after which small but significant increase in plasma gastrin was observed postprandially in the last 90 min postprandial sample (Figure 9).

DISCUSSION

The aim of this study was to determine the relationship between plasma ghrelin and gastrin levels under fasting and postprandial conditions in healthy subjects and patients with GC who underwent total and distal gastrectomy. In addition, we analyzed gastric mRNA expression for ghrelin, GHS-R and gastrin in intact gastric mucosa and cancer tissue. It was found that in healthy controls, an inverse relationship existed; such that, when under fasting conditions plasma ghrelin increased, plasma gastrin decreased, and postprandially, when plasma ghrelin was

markedly attenuated, plasma gastrin was significantly increased compared to preprandial levels. The reason for this inverse relationship in healthy subjects is not known, but it is likely that, in the fasting stomach, the high level of ghrelin release, that activates gastric motility and stimulates appetite, causes excessive release of gastrin immediately after the entrance of a food bolus into the stomach. The stimulatory synergistic action of ghrelin on gastrin and gastric acid secretion has been observed previously, but mostly in experimental animals^[14], and this effect has been attributed to hormonal stimulation of vagal nerves and histamine release from the gastric enterochromaffin-like cells. Our results suggest that increased ghrelin release in the fasting (empty) stomach promotes gastrin secretion immediately after food intake. Our recent but unpublished data with parenteral administration of synthetic ghrelin

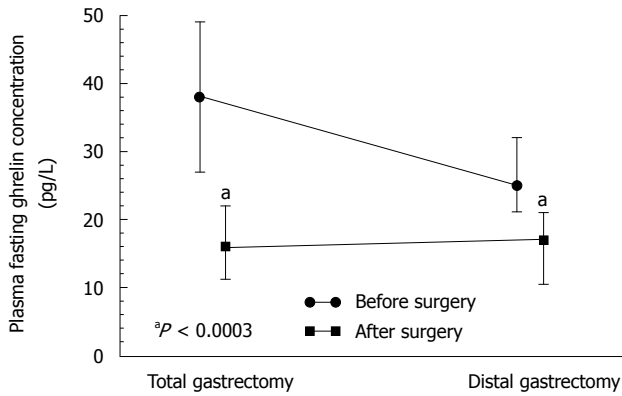


Figure 5 Plasma ghrelin concentrations in total and distal gastrectomy patients. "a" indicates significantly ($P < 0.05$) lower values recorded after total gastrectomy when compared to those recorded after distal gastrectomy.

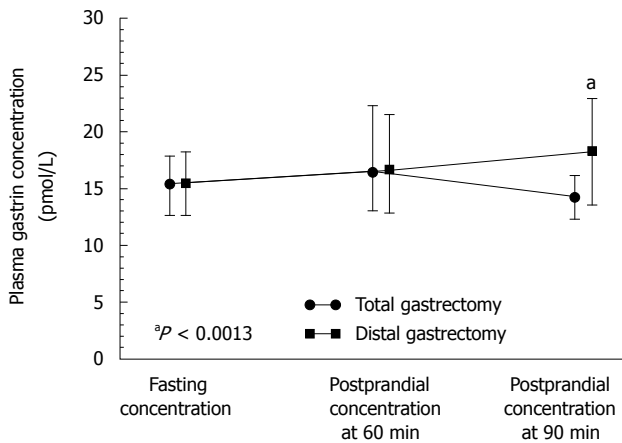


Figure 6 Plasma levels of gastrin under fasting conditions and postprandially (at 60 and 90 min after a meal) in patients after total or distal gastrectomy. "a" indicates significant difference ($P < 0.05$) compared to fasting level.

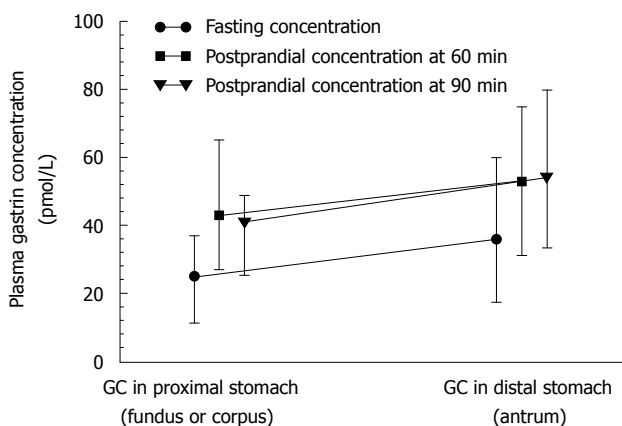


Figure 7 Plasma gastrin levels (median and range) under fasting and postprandial conditions (at 60 and 90 min after a meal) in patients with gastric cancer located in fundus and beyond the fundus (distal stomach).

analog^[12] showed that it potently enhances plasma gastrin level, thus providing direct evidence that ghrelin is capable of stimulating gastrin release in humans, but it remains to be elucidated why plasma ghrelin falls immediately after

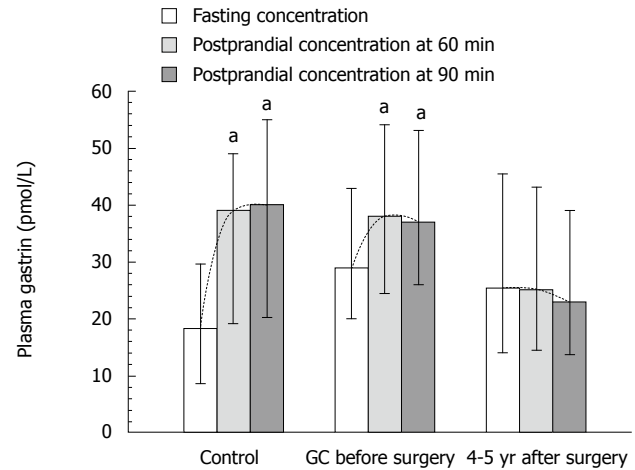


Figure 8 Plasma gastrin levels under fasting and postprandial conditions (at 60 and 90 min after a meal) in healthy controls, gastric cancer patients and patients after 4-5 yr of gastrectomy patients due to gastric cancer. (Medians and range). "a" indicates significant change ($P < 0.05$) compared to fasting level. GC: Gastric cancer

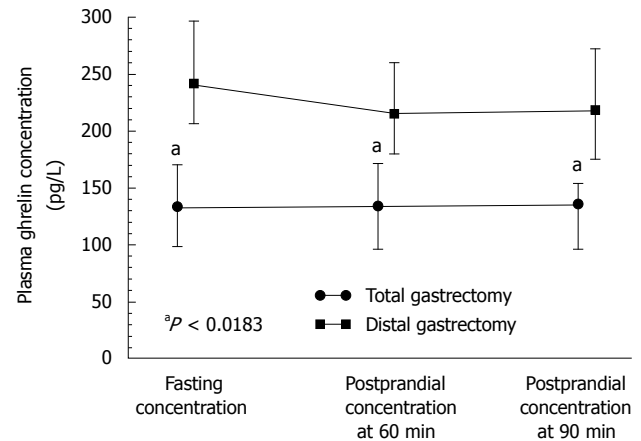


Figure 9 Plasma gastrin concentrations under fasting conditions and postprandially at 60 and 90 min after food intake in gastric cancer patients after total or distal gastrectomy. "a" indicates significant difference ($P < 0.05$) compared to values recorded before the surgery.

food intake. It is possible that gastric acid stimulated by the combined action of ghrelin and gastrin^[13-15] activates local gastric somatostatin release that might cause paracrine inhibition of secretory activity of Gr-cells, which decreases plasma ghrelin level, but this requires further experimental and clinical data.

The major purpose of the present study was to explain the role of ghrelin in gastric carcinogenesis associated with *H. pylori* infection^[16-18], the spread of GC to the proximal or distal portion the stomach, and impairment of the ghrelin-gastrin relationship. The location and extent of tumor infiltration combined with widespread multifocal atrophic gastritis (caused by *H. pylori* infection) was found to affect ghrelin and gastrin release, and subsequently, to impair the intricate relationship between these two hormones. Indeed, we found that GC patients secrete significantly less ghrelin under fasting conditions, with little tendency

of this hormone to decline postprandially, whereas gastrin release greatly increases. The reduction in fasting plasma ghrelin in GC appears to depend upon: (1) the extent and proliferation of cancer in the stomach; and (2) *H. pylori*-induced atrophic gastritis. As expected from our previous studies, plasma gastrin is enhanced in GC; probably due to its expression and release by cancer cells themselves^[17,18]. In fundic GC, the fasting and postprandial ghrelin levels were lower than those in with GC in distal stomach (Figures 3 and 5).

The most marked alteration in ghrelin/gastrin ratio was found in patients who underwent total, and to a lesser extent distal gastrectomy. The inverse relationship between ghrelin and gastrin disappeared and both hormones became markedly altered by total gastrectomy. This agrees with the results of our most recent study^[13], which has shown that GC associated with *H. pylori* infection results in a marked decrease in ghrelin in plasma and cancer tissue. In the present study, we found that plasma levels of ghrelin were lower in patients with GC and in those with previous GC who underwent surgery 4-5 years previously, compared with the control group. Huang *et al*^[19] have shown no correlation between preoperative levels of ghrelin and the proximal or distal location of GC. Similarly, Jeon *et al*^[9] have not found any marked difference in preoperative serum ghrelin concentrations in patients with GC scheduled for gastric resection (total, subtotal and proximal). However, their analysis of ghrelin levels before and after surgery (on day 10) showed a significant difference in plasma ghrelin between patients who underwent total and distal gastrectomy. In the group with total gastrectomy, similar to our study, the decrease in ghrelin after surgery was significantly greater, and reached a maximum of about 70% of the preoperative concentration. This confirms the important role of the stomach, particularly its proximal portion, as the main source of ghrelin secretion^[3,8,9].

In our study, the mean ghrelin concentrations tended to increase slightly but significantly after distal but not total gastrectomy. The mean concentrations of plasma ghrelin among GC patients depended on the type of resection, particularly on its extent, and most importantly, upon preservation of the part of the stomach responsible for the production of most ghrelin, that is, the fundus and corpus. In the total resection group, on postoperative day 10, taking into account the plasma ghrelin concentration, it can be concluded that, in some patients, other tissues begin to take over slow production of ghrelin. However, after distal resection, when the fundus of the stomach was preserved, the levels of ghrelin actually increased compared to preoperative values^[20].

The plasma level of ghrelin after surgery in the subsequent samples (fasting and postprandial) showed a significant difference between patients who underwent total and subtotal resection. Plasma ghrelin levels at 60 and 90 min postprandially were significantly higher in patients who underwent distal gastric resection, compared to the group treated by total resection. Furthermore, physiological regulation of ghrelin secretion and its plasma level were, however, at least in part preserved; being highest in the fasting

state, lowest in the hour after a meal, and intermediate at 90 min after a meal (Figures 3 and 5). Jeon *et al*^[9] have also analyzed plasma levels of ghrelin in patients who underwent various types of gastric resection. However, unlike in our study, patients with early GC or GC infiltrating < 2 cm were examined within the first 7 d after surgery. In patients after resection of the proximal stomach, postoperative ghrelin increased more slowly than in patients after fundus-preserving resection. They concluded that compensatory ghrelin production occurs in the preserved part of the stomach. According to their experience, preservation of the fundus is more important than the distal stomach for ghrelin production after gastrectomy. After complete resection of the stomach, ghrelin levels in serum fell to about 30% of those before surgery. They did not observe compensatory production of ghrelin by other tissues until day 7 after surgery^[9]. Also, Takachi *et al*^[21] have demonstrated a significant decrease in the concentration of ghrelin after total gastrectomy, which was up to 12% of preoperative levels (measured on days 3 and 7 after surgery). Very low levels of ghrelin were observed in this group in the long-term follow-up after surgery (mean: 41 mo). By contrast, in patients treated by distal gastrectomy, on postoperative day 3, the hormone levels decreased to 39%, and on day 7, increased back to 56% of preoperative levels. Different results have been presented by Kim *et al*^[22], who found that after distal gastric resection for GC, the number of ghrelin-producing cells failed to increase, which correlated well with low plasma ghrelin levels.

In our study, the concentration of plasma ghrelin in patients who had undergone total gastrectomy 4-5 years previously was significantly lower than that after distal gastrectomy, and lower again when compared to the control group. The fasting levels of ghrelin were also lower compared to those in GC patients before surgery. Plasma ghrelin level in this group of GC patients was at 26%-66% of the reference value in the healthy controls. Thus, in our study group, only partial compensation of ghrelin production by other tissues was observed after removal of the stomach. Hosoda *et al*^[23] have shown that, after complete gastric resection, serum ghrelin concentration tends to normalize gradually as a result of production by other tissues. Plasma ghrelin level in their three patients fell at 30 min after gastrectomy to 50% of the preoperative concentration. At 240 d after gastric resection, plasma ghrelin reached about two-thirds of the preoperative value in two patients and 100% in the other. However, Ariyasu *et al*^[31] have found that plasma ghrelin in patients at 1-8 years after total gastrectomy was comparable with the control group, with a concentration that was about 35% of the preoperative level. Also, the above-mentioned study of Takachi *et al*^[21] has demonstrated very low levels of ghrelin during long-term follow-up after total gastrectomy.

In our study, we analyzed gastrin as well. Basal plasma gastrin was found to be markedly elevated in our patients with GC and coexisting *H. pylori* infection, as compared to the control group. Patients with GC tended to have higher levels of gastrin (Figures 6-9), which can result from *H. pylori* infection with accompanying atrophy of the mucous

membrane, and increased production of the hormone by the tumor cells themselves^[17,18]. We noted, however, significant differences in plasma levels of gastrin before and after surgery only in patients who underwent total gastrectomy.

In summary, a significant decrease to about 70% of preoperative levels of ghrelin was observed in our patients after total gastrectomy, which confirms an important role of the stomach as the principal place of ghrelin expression. After distal resection of the stomach, a compensatory increase in the production of ghrelin in relation to the level before surgery was observed. At 4-5 years after gastrectomy, full compensatory production of ghrelin by other tissues was not demonstrated. Higher basal plasma gastrin levels in patients with GC accompanied by *H. pylori* infection, compared to the control group, were probably associated with increased production of the hormone by the tumor cells themselves.

ACKNOWLEDGMENTS

We thank Ms Sara Padol, a student at the Medical English School for Foreigners, who corrected the English in this paper and prepared it for publication. The project was supported from the funds of State Committee for Scientific Research for science in the years 2006-2008 as a research project no K/PBP/000012.

COMMENTS

Background

The major purpose of the present study was to explain the role of ghrelin and gastrin in gastric carcinogenesis associated with *Helicobacter pylori* (*H. pylori*) infection, the spread of gastric cancer (GC) into the proximal or distal stomach, and the profound impairment of the ghrelin-gastrin relationship.

Research frontiers

The location and extent of tumor infiltration combined with widespread multifocal atrophic gastritis (caused in this study by *H. pylori* infection) was found to affect ghrelin and gastrin release, and subsequently, to impair the intricate relationship between these two hormones. The authors found that GC patients express significantly less ghrelin under fasting conditions, with little tendency of this hormone to decline postprandially, whereas gastrin release remained strongly increased. The reduction in fasting plasma ghrelin in GC appears to depend upon: (1) extent and proliferation of the cancer in the stomach; and (2) *H. pylori*-induced atrophic gastritis. As expected from our previous studies, plasma gastrin was markedly enhanced in GC, probably due to its expression and release by cancer cells themselves. In fundic cancer, the fasting and postprandial ghrelin levels were lower than those in healthy controls; probably due to impairment by cancer and accompanying gastritis of the Gr-cells that are present mainly in the mucosal lining of the proximal stomach. In this study, the authors analyzed the plasma concentrations of ghrelin and gastrin in patients with advanced GC before and after total or subtotal gastrectomy. In addition, plasma concentrations of gastrin, ghrelin and gene expression of these hormonal peptides and growth hormone secretagogue receptor (GHS-R), were evaluated in intact mucosa and GC tissue associated with *H. pylori* infection.

Innovations and breakthroughs

A significant decrease to about 70% of preoperative levels of ghrelin observed in our patients after total gastrectomy confirms a crucial role of the stomach, particularly its proximal portion, as the principal place of ghrelin expression and release. After distal resection of the stomach, a compensatory increase in the production of ghrelin was clearly demonstrated. At 4-5 years after total gastrectomy, full compensatory takeover of ghrelin production by other tissues did not occur possibly due to the absence of the proximal stomach, which is the major source of ghrelin production. Higher basal plasma gastrin levels in patients with GC accompanied by *H. pylori* infection, compared to the control group, were

probably associated with increased production of the hormone by the tumor cells themselves.

Applications

Further study is required: (1) long-term observation of patients after resection of the stomach; (2) measurement of plasma ghrelin to establish whether compensation of hormone production by other tissues can be expected; and (3) consideration of the possible need for substitution of exogenous ghrelin or its active analogs in these patients.

Terminology

Ghrelin is a natural ligand for GHS-R. It was originally identified in rat gastric mucosa and shown to be expressed by endocrine Gr-cells. These ghrelin (Gr)-producing cells, represent about 20% of the total population of endocrine cells in the gastric mucosa. They are located in the lower parts of the oxyntic glands, and are present mostly in the proximal portion of the stomach (fundus and corpus). They are also the major source of hydrochloric acid, intrinsic factor, numerous growth factors, and pepsinogens, and therefore, they are important for digestive processes stimulated by the vagal nerves, gastrin/histamine, as well as by ghrelin itself. Ghrelin is a 28-amino acid peptide that acts as a stimulant for GH secretion by interacting with the receptor GHS-R. Characteristically, it is also the only circulatory gastrointestinal hormone that is known to be secreted by the empty (fasting) stomach to enhance food intake and maintain energy homeostasis following its central or peripheral administration. Ghrelin is currently considered to be the most powerful endogenous orexigenic (appetite stimulating) hormone, which results in a positive energy balance and subsequently in weight gain.

Peer review

This paper reports the results of studies on the relationship of ghrelin to gastrin in GC before and after gastrectomy. Using blood and biopsy specimens obtained from 70 patients who underwent endoscopy of the upper gastrointestinal tract, the authors demonstrated that, in healthy controls, plasma ghrelin reaches higher values under fasting conditions and falls postprandially, while gastrin level is low during fasting and shows a marked increase following food ingestion. Moreover, the decrease in fasting levels of ghrelin was observed in GC patients after total gastrectomy, but the level of plasma gastrin showed an increase. It is concluded, that the elevated plasma gastrin and suppression in fasting ghrelin in patients with GC suggests an intimate relationship between these two hormones in gastric carcinogenesis.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

***Bifidobacterium lactis* attenuates onset of inflammation in a murine model of colitis**

David Philippe, Laurent Favre, Francis Foata, Oskar Adolfsson, Genevieve Perruisseau-Carrier, Karine Vidal, Gloria Reuteler, Johanna Dayer-Schneider, Christoph Mueller, Stéphanie Blum

David Philippe, Laurent Favre, Francis Foata, Oskar Adolfsson, Genevieve Perruisseau-Carrier, Karine Vidal, Gloria Reuteler, Stéphanie Blum, Department of Nutrition and Health, Nestlé Research Centre, CH-1000 Lausanne 26, Switzerland
Johanna Dayer-Schneider, Christoph Mueller, Department of Pathology, University of Bern, CH-3010 Bern, Switzerland
Author contributions: Philippe D and Favre L wrote the manuscript and contributed equally to this work; Philippe D, Favre L, Adolfsson O, Vidal K, Mueller C, Foata F and Blum S designed the study and participated in data analysis; Foata F, Perruisseau-Carrier G, Dayer-Schneider J and Reuteler G performed research; all authors approved the final manuscript.

Correspondence to: David Philippe, PhD, Department of Nutrition and Health, Nestlé Research Centre, Vers-chez-les-Blanc, PO Box 44, CH-1000 Lausanne 26,

Switzerland. david.philippe@rdls.nestle.com

Telephone: +41-21-7858967 Fax: +41-21-7858544

Received: July 8, 2010 Revised: July 30, 2010

Accepted: August 7, 2010

Published online: January 28, 2011

forkhead box protein 3, a marker of regulatory T cells, was significantly up-regulated by *B. lactis*.

CONCLUSION: Daily oral administration of *B. lactis* was able to reduce inflammatory and T cells mediators and to promote regulatory T cells specific markers in a mouse model of colitis.

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Key words: Probiotics; *Bifidobacterium*; Colitis; Adoptive transfer model; Regulatory T cells; Inflammation; Mice

Peer reviewer: Kristin A Verbeke, Professor, Katholieke Universiteit Leuven, Pathophysiology, Section Gastroenterology, University Hospital Gasthuisberg, E462, Herestraat 49, Leuven, 3000, Belgium

Philippe D, Favre L, Foata F, Adolfsson O, Perruisseau-Carrier G, Vidal K, Reuteler G, Dayer-Schneider J, Mueller C, Blum S. *Bifidobacterium lactis* attenuates onset of inflammation in a murine model of colitis. *World J Gastroenterol* 2011; 17(4): 459-469 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/459.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.459>

Abstract

AIM: To assess the anti-inflammatory effect of the probiotic *Bifidobacterium lactis* (*B. lactis*) in an adoptive transfer model of colitis.

METHODS: Donor and recipient mice received either *B. lactis* or bacterial culture medium as control (deMan Rogosa Sharpe) in drinking water for one week prior to transfer of a mix of naive and regulatory T cells until sacrifice.

RESULTS: All recipient mice developed signs of colonic inflammation, but a significant reduction of weight loss was observed in *B. lactis*-fed recipient mice compared to control mice. Moreover, a trend toward a diminution of mucosal thickness and attenuated epithelial damage was revealed. Colonic expression of pro-inflammatory and T cell markers was significantly reduced in *B. lactis*-fed recipient mice compared to controls. Concomitantly,

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), referred to as inflammatory bowel diseases (IBDs), are chronic relapsing and remitting inflammatory diseases of the gastrointestinal tract. IBD affects people in the prime of their lives, with first diagnosis usually between the ages of 15 and 25 years, and due to the chronic nature of IBD, patients usually require lifelong treatment. More than one million people in the United States and more than four million people worldwide suffer from IBD. According to the current increase in affected people, IBD medical costs will reach approximately 2 billion euros by the end of 2010. The high cost and limited response to current thera-

pies prompt research and development of new treatment options.

IBD is thought to result from an inappropriate, overwhelming and ongoing activation of the mucosal immune system in genetically susceptible individuals driven by antigens originating from the microbiota of the gastrointestinal tract^[1]. Some of the immune-related characteristics of IBD include over-activity of effector lymphocytes, induction of pro-inflammatory cytokines, and failure of regulatory T cells (Tregs) to control inflammation^[2,3].

The crucial role of the intestinal microbiota in the induction and progress of disease has been validated in patients and in animal models of intestinal inflammation resembling IBD^[4,5]. Indeed, it was demonstrated that intestinal inflammation could not be induced under germ-free conditions^[6] and that antibiotic treatment attenuated the disease severity^[7]. Interestingly, the equilibrium of the intestinal microbiota appears to be perturbed in IBD as decreased levels of *Bifidobacterium* and *Lactobacillus* strains have been described in fecal samples, whereas raised counts of *Enterococcus* and *Bacteroides* species are found in inflamed mucosa of patients^[8]. Thus, as probiotics are known to have a strong homeostatic impact on the intestinal flora^[9], it is valuable to consider probiotics as alternative therapies for IBD. Hence, although conflicting reports exist, a number of studies report the benefits of probiotic therapy in the alleviation of IBD^[10-13]. For example, VSL#3, a mixture of four lactobacilli, three bifidobacteria and a streptococcus species, proved to be effective in inducing and maintaining remission in UC patients or in preventing pouchitis^[14-17].

While most clinical studies have relied on mixes of probiotic strains or on synbiotics to act on IBD, a few preclinical or *ex-vivo* studies have begun to reveal that the use of single probiotic strains, mainly consisting of *Lactobacillus* strains, can also be efficient in dampening inflammation^[18-21].

The present study reports on the beneficial effects of the use of a single *Bifidobacterium* strain, *Bifidobacterium lactis* (*B. lactis*), on intestinal inflammation in a murine model of T cell-mediated colitis. This well established Th1-type cytokine-mediated hyper-response model relies on the adoptive transfer of CD4⁺CD45RB^{high} naive T cells in immunodeficient mice^[22], such as RAG2^{-/-} mice which lack adaptive immunity^[23]. This cell transfer initiates colitis pathology akin to that of humans^[24], which develops as a result of the absence of suppressive regulatory cells in the recipient mice. Indeed, co-transfer of mature CD4⁺CD45RB^{low} T cells, a source of Tregs, reduces or even prevents colitis^[25]. In the present work, naive T cells were adoptively transferred together with a low proportion of Tregs. This particular setting, still permitting the induction of inflammation in recipient mice, provided an interesting tool allowing the simultaneous analysis of the impact of prophylactic *B. lactis* administration on both T cell partners involved in the induction (naive T cells) or regulation (Tregs) of IBD pathology.

MATERIALS AND METHODS

Animals

C57BL/6J mice (8- to 12-wk-old) were purchased from Harlan (Oxon, UK). Immunodeficient RAG2^{-/-} mice (8- to 12-wk-old)^[26] were used from a colony of RAG2^{-/-} mice maintained at the Institute for Labortierkunde, University of Zurich, Switzerland. The RAG2^{-/-} mouse colony was derived from a colony from Bern (Switzerland) by embryo transfer under gnotobiotic conditions and recolonized with an Altered Schaedler Flora^[27].

Preparation of *B. lactis* and experimental design

A freshly prepared solution containing *Bifidobacteria lactis* (*B. animalis* subsp. *lactis* NCC 2818) from the Nestlé Culture Collection (Nestlé Research Center, Lausanne, Switzerland) was used for this study. This strain was chosen according to *in vitro* anti-inflammatory properties (data not shown). Bacteria were grown for two passages under strictly anaerobic conditions in deMan Rogosa Sharpe (MRS) broth containing 0.05% cysteine (BD, Switzerland). After quantification of bacteria by serial dilution as described for fecal microbiota, 10% glycerol was added to the bacteria stock; aliquots were made and stored at -80°C until use. Frozen bacteria were added to drinking water each day of the study at 1×10^9 colony-forming units (CFU)/mL leading to a dose approximating 3×10^9 CFU/d per mouse. Donor and recipient mice were supplemented with either probiotic or MRS control solution (both containing 10% glycerol) according the study design (Figure 1). Note that as the settings of the present adoptive transfer model permit a focus on Tregs, probiotics were also given to donor mice in order to potentially stimulate these cells as soon as possible. Each group of recipient mice consisted of 5 mice.

Induction of colitis in RAG2^{-/-} mice by adoptive transfer

CD4⁺ T cells from mesenteric lymph nodes (MLNs) were isolated from C57BL/6 donor mice by negative depletion using MACS technology (Miltenyi Biotec, Germany). The negative fraction, enriched for CD4⁺ T cells, was stained using Fluorescein isothiocyanate-conjugated CD45RB antibody (mAb) (eBioscience, San Diego, USA) and Phycoerythrin-conjugated CD4 mAb (eBioscience, San Diego, USA). Subsequently, CD4⁺ T cells were sorted according to the expression of CD45RB on a FACS Aria (BD; Allschwil, Switzerland). Sorted CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} T cells were washed, resuspended at 1×10^6 cells/mL in sterile phosphate buffered saline and injected i.p. at the ratio of 9:1 respectively (2×10^5 total cells) into each of the 8- to 12-wk-old syngeneic RAG2^{-/-} recipient mice. This 9:1 ratio was chosen because it still allowed development of colitis despite the co-injection of regulatory cells. Body weight of recipient mice was measured every three days from day 0 to day 21, and then every other day until sacrifice (day 27). Body weights were recorded as percentage of initial body weight.

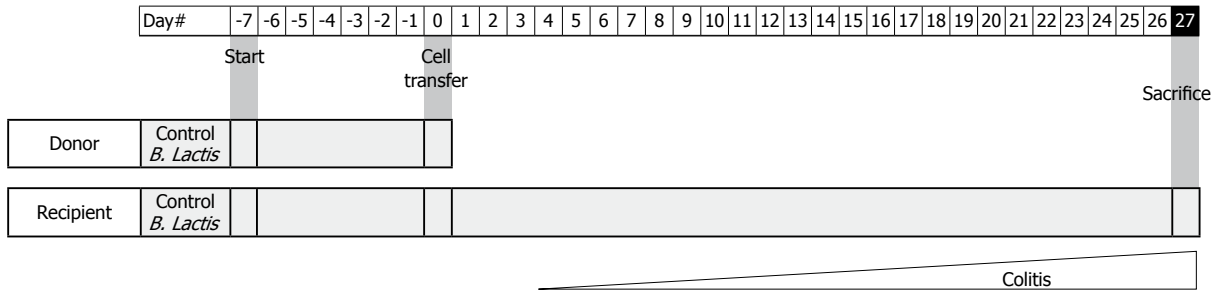


Figure 1 Experimental set up. Feeding of *Bifidobacterium lactis* (*B. lactis*)-fed donors and recipients mice were started on day -7 (D-7) until D0 for donor mice and until D27 for recipients. At D0, donor mice were sacrificed and cell transfer was performed in RAG⁺ recipient mice..

Sample collection

Fresh fecal samples (approximately 0.05 g) were collected at three time-points during the study: before initiation of *B. lactis* supplementation (day -7), at the day of T cell transfer (day 0), and at the end of the study (day 27). They were collected into 0.5 mL Ringer's solution, immediately homogenized and snap-frozen, then stored at -80°C until analysis. Blood samples were collected at the same time points as fecal samples and frozen at -80°C until needed. At the end of the experimental period, mice were sacrificed and colon was divided into three parts. The proximal and the distal parts were snap-frozen in liquid nitrogen for protein and mRNA expression analysis, respectively. A smaller sample of the middle colon was used for histopathological analysis.

Fecal microbiota

Fecal extracts were diluted in pre-reduced Ringer's solution (0.5% cysteine) to perform serial dilutions (10^{-2} to 10^{-6}) and then plated. Portions (100 μ L) of appropriate dilutions were plated onto selective or semi/selective media. *Bifidobacteria* were counted on tomato juice agar medium. Plates for the enumeration of *Bifidobacteria* colonies were incubated at 37°C in anaerobic conditions, in a jar containing Anaerocult A tablets (Merck, Germany), for 48 h. After incubation, each plate was examined for bacterial colonies. The detection-limit for the assessed bacteria dilutions was 10^3 . Bacterial counts were expressed as means log₁₀ CFU/g feces \pm SE. Detection of *B. lactis* was performed by polymerase chain reaction (PCR) using *B. lactis*-specific primers.

Histological assessment

Paraffin-embedded colonic tissue sections were scored as previously described^[28], with minor modifications. Briefly, specimens of the transverse colon of each animal were collected and fixed immediately in 4% buffered paraformaldehyde for 16-24 h for subsequent preparation of paraffin-embedded tissue blocks. Tissue sections were stained with hematoxylin/eosin for subsequent histopathological assessment. Each tissue section was independently evaluated by at least two trained pathologists according to a standard evaluation sheet in a blinded fashion. Histological assessment was performed using the scoring criteria displayed in Table 1. The range of histopathological scores was from 1 (no alteration) to 16 (most severe signs of colitis).

Table 1 Summary of assessed criteria to determine histological scoring

Criteria	Scoring
Infiltration of the colonic lamina propria	0-3
Loss of goblet cells	0-3
Crypt abscesses	0-3
Epithelial erosion	0-2
Hyperemia	0-2
Thickness of the colonic mucosa	1-3

Protein expression

Protein extraction and quantification: Colons were homogenized in RIPA (Radio Immuno-Precipitation Assay) buffer containing 50 mmol/L Tris base 50, 150 mmol/L NaCl, 2 mmol/L EDTA (Ethylene Diamine Tetraacetic Acid), 2 mmol/L EGTA (Ethylene Glycol Tetraacetic Acid), 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS (Sodium Dodecyl Sulfate), 50 mmol/L NaF, 200 μ mol/L Na₃VO₄, 0.1% β -mercaptoethanol, 500 μ mol/L AEBSF [4-(2-AminoEthyl) BenzeneSulfonyl Fluoride hydrochloride], 20 μ mol/L bestatin, 7 μ mol/L E-64, 11 μ mol/L leupeptin, 7.5 μ mol/L pepstatin A, and 0.4 μ mol/L aprotinin. The pH was adjusted to 7.2. The homogenate was then centrifuged at 10000 g for 10 min at 4°C to remove debris. Protein determination was carried out using a modified Lowry method, as described by the manufacturer (Bio-Rad, USA).

Enzyme-linked immunosorbent assay measurements:

Interleukin (IL)-6 and tumor necrosis factor (TNF)- α levels were measured in the colon protein extracts by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D Systems, England). To avoid interference of the protein lysis buffer with the ELISA reaction, four independent dilutions were performed. All samples were measured in technical duplicates and the concentration calculations were derived from appropriate standard curves.

Electrophoresis and Western blotting: 50 μ g of protein were separated by electrophoresis on a MOPS [3-(N-Morpholino)PropaneSulfonic acid] SDS running 4%-12% bis-tris gel (Invitrogen, USA). Proteins were then transferred to a nitrocellulose (NC) membrane by electroblotting

(30 V for 60 min). Western blot analysis was performed with antibodies against murine cyclooxygenase 2 (COX-2) (Cayman Chemicals, USA), p38 and phospho p38 (Cell Signaling Technology, USA), signal transducer and activator of transcription 3 (STAT-3) and phospho STAT-3 (Cell Signaling Technology, USA) and β -actin (Sigma, St. Louis, MO, USA). Secondary antibodies were from Molecular Probes (USA) or Jackson ImmunoResearch Laboratories (USA). Relative quantitation of bands was determined using the Scion Image Densitometry System (Scion Corp., USA), with all quantities normalized to expression levels of β -actin.

Immunohistochemistry for Ki-67 on formalin-fixed tissue sections: After dewaxing, tissue sections were pretreated in 10 mmol/L citrate buffer, pH 6.0, for 7 min at 1 bar/121°C. Pretreated tissue sections were subsequently incubated for 60 min with a primary rat-anti-mouse Ki-67 mAb (Clone: Tec-3; isotype: rat IgG2a; DakoCytomation, USA). After washing, sections were incubated with the biotinylated secondary reagent (rabbit-anti-rat Ig; absorbed with mouse Ig (DakoCytomation). Streptavidin-HRP complexes with 3,3'-diaminobenzidine as the chromogen were used for detection.

mRNA expression

RNA extraction of the homogenized tissues was performed using the NucleoSpin RNA II Kit (Macherey-Nagel, Germany). Total RNA was quantified using the Ribogreen RNA Quantitation Kit (Molecular Probes, USA), and the RNA quality was assessed by Agilent RNA 6000 Nano LabChip Kit (Agilent Technologies, USA). Total RNA (2 μ g) was reverse transcribed using MultiScribe reverse transcriptase (Applied Biosystems, USA). Real-time PCR was carried out using custom design Low Density Array (Applied Biosystems, X, USA). The quantitative PCR (qPCR) reactions were performed with 2 ng of cDNA on ABI PRISM 7900HT qPCR machine (Applied Biosystems, USA) piloted by SDS 2.2 software (Applied Biosystems, USA). Results of target mRNA are expressed as the number of specific copies for 10^6 GAPDH mRNA molecules in the same sample.

Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney *U* test. Differences were considered statistically significant when $P < 0.05$. All data are shown as median \pm interquartile-range (IQR) except for the body weight analysis.

RESULTS

Detection of *B. lactis* in fecal samples

In order to control the probiotic intake, detection of *B. lactis* in fecal samples was performed using PCR detection. No *B. lactis* was identified in the feces prior to administration of this strain in donor C57BL/6J and recipient RAG^{-/-} mice. All donor animals receiving the treatment were positive for *B. lactis* on the day of cell transfer (day 0,

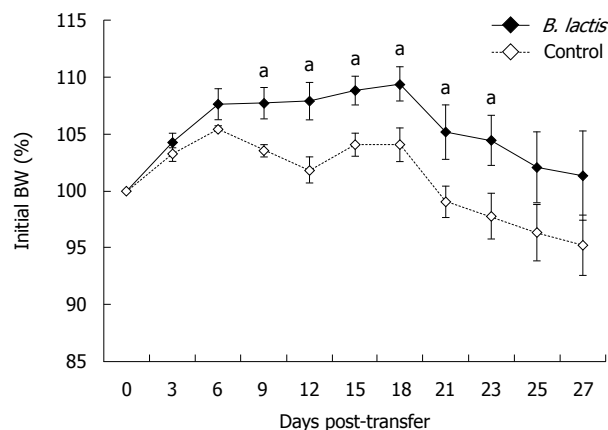


Figure 2 *Bifidobacterium lactis* feeding significantly delayed weight loss. Weight curves for control-fed and *Bifidobacterium lactis* (*B. lactis*)-fed recipient mice following adoptive T cell transfer. Every three days or every other day following T cell transfer, body weight (BW) of control-fed and *B. lactis*-fed recipient mice was recorded. Results are expressed as the mean \pm SE ($n = 5$ mice per group) and statistical significance is indicated ($^aP < 0.05$).

data not shown). In the recipient animals, supplemented with the probiotic strain, approximately 1×10^8 CFU of *B. Lactis*/g fecal content could be measured at day 0 and day 27 (sacrifice day).

Effect of *B. lactis* on body weight loss

As already described in other studies^[29,30], control recipients of CD4⁺ T cells started to lose body weight from day 9 after adoptive cell transfer. Thereafter they continuously lost weight and on the day of sacrifice (day 27), their body weight was lower than on the day of initial T cell transfer (Figure 2). In contrast, *B. lactis*-fed recipient mice showed a marked delay of onset of body weight loss, starting only at day 18. Thereafter *B. lactis*-fed recipients also continued to lose weight until the day of sacrifice (day 27). At the end of the experimental period, body weight of *B. lactis*-fed recipients of CD4 T cells was comparable to the initial body weight at the time of colitis induction by adoptive T cell transfer (Figure 2).

Effect of *B. lactis* on colon histopathology

Histopathology scores, ranging from 0 (no colitis) to 16 (most severe colitis) (Table 1), were based on the analysis of 6 criteria as described in the methodology section. At sacrifice, the total colitis scores of *B. lactis*-treated and -untreated recipient groups were not significantly different (data not shown). When each of the histopathology criteria was assessed individually, differences between the two groups of recipients were nonetheless observed at the level of mucosal thickness; *B. lactis*-treated recipient mice showing a tendency for an attenuated mucosal thickening compared to control recipient mice (Figure 3B), resulting in a lower colitis score as shown in Figure 3A.

Beyond criteria used for histopathology scoring, epithelial proliferation is considered to be strongly associated with a gastrointestinal inflammatory status^[31]. In order to evaluate these criteria, paraffin-embedded colon sections were stained for Ki67 expression by immunohistochemical

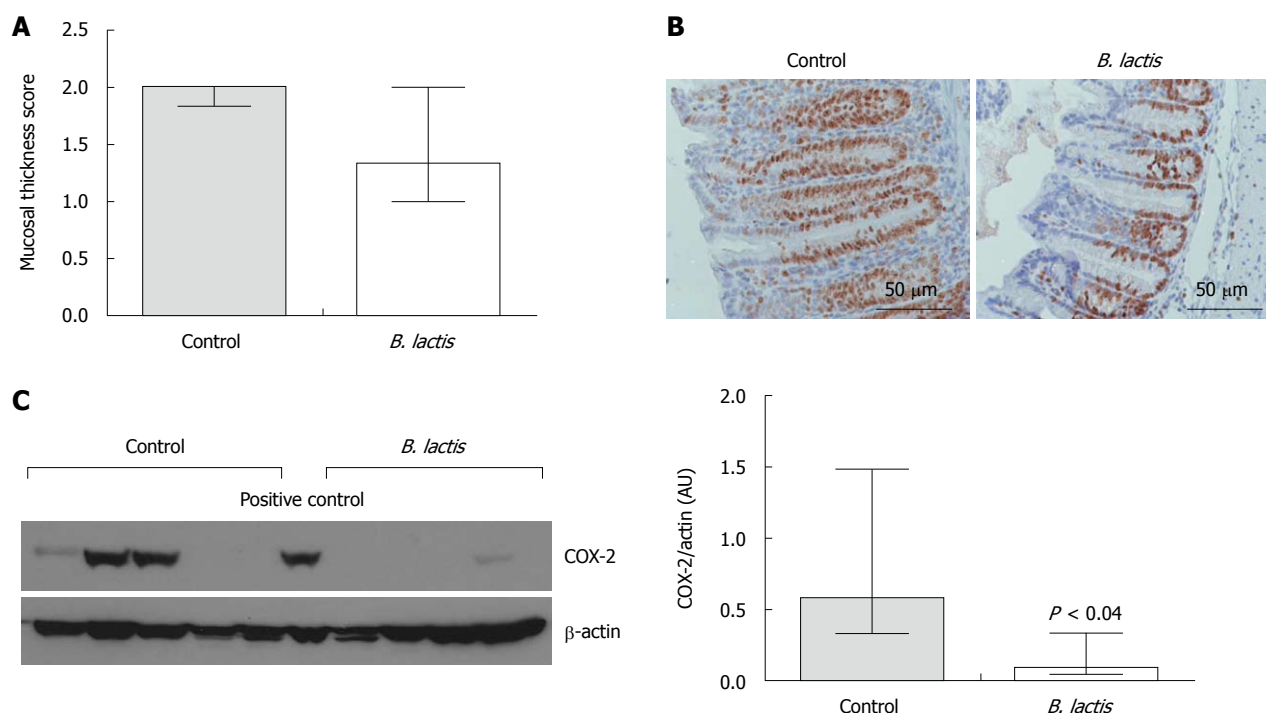


Figure 3 Histological assessment of colon inflammation for control- and *Bifidobacterium lactis*-fed recipient mice following adoptive T cell transfer. A: Mucosal thickness was assessed using a specific scored graduated from 0-3. Results are expressed as the mean \pm interquartile-range (IQR) ($n = 5$ mice per group); B: Assessment by immunostaining of the proliferation marker Ki67 (proliferating cells are characterised by the brown nuclear staining). Representative examples of longitudinal slices from colons of control colitic (left panel) and *Bifidobacterium lactis* (*B. lactis*)-fed colitic (right panel) mice are shown; C: Expression of cyclooxygenase 2 (COX-2) protein in colon tissue samples from colitic mice was quantified by Western blotting analysis (left panel). Individual expressions and relative densitometric quantification of the bands are presented (right panel). Results are expressed as the mean \pm IQR ($n = 5$ mice per group) and statistical significances is indicated.

labeling reaction to determine the proportion of proliferating cells in the colonic epithelium in colitic mice, treated or not with *B. lactis* (Figure 3B). Colonic epithelium from all recipient mice showed a high proliferation rate in the lower two-thirds of the colonic crypts (Figure 3B), whereas proliferation was only restricted to crypts in healthy controls (data not shown). Nevertheless, epithelial hyper-proliferation appeared markedly reduced in *B. lactis*-treated recipient mice (Figure 3B).

Effect of *B. lactis* on colonic pro-inflammatory markers

The effect of *B. lactis* feeding on the expression of well known proinflammatory markers, such as COX-2, IL-6 or TNF- α , in the colon of recipient mice was assessed. COX-2 is an enzyme known to be strongly induced in intestinal epithelial cells upon inflammatory conditions. Upon disease induction and in absence of treatment (control recipient group), the protein expression of COX-2 was strongly detected in two out of five samples and slightly in a third one (Figure 3C). Conversely, in the *B. lactis*-fed group, only one out of five samples had slight COX-2 protein expression (Figure 3C). Relative densitometric quantification of the bands clearly revealed a significant decrease of COX-2 expression in the *B. lactis*-fed group compared to control mice (Figure 3C).

Feeding with *B. lactis* also resulted in a significant decrease of IL-6 and TNF- α protein production in the colon of recipient mice compared to the non-treated recipient mice (Figure 4A and C). Accordingly, phosphorylation of the transcription factors STAT-3 and p38, associated

with the signaling pathways of these two cytokines, was also diminished by *B. lactis* feeding (Figure 4B and D).

Effect of *B. lactis* on dendritic cell markers

It is now well established that co-stimulatory interactions between antigen-presenting cells and cells of the adaptive immune system, such as the CD40/CD40 ligand (CD40L) and OX40/OX40 ligand (OX40L) (CD134/C134L) pathways, play a crucial role in colitis induction and severity of disease^[32,33]. mRNA expressions of these four molecules were thus assessed in colon tissue samples of recipient mice. Whereas they were all induced in recipient mice in comparison to healthy controls (Figure 5A-D), *B. lactis* feeding significantly down-regulated the expression levels of CD40L and OX40/OX40L when compared to non-treated colitic controls (Figure 5B-D). Expression of CD40 only showed a tendency to be down-regulated by *B. lactis* feeding.

Effect of *B. lactis* on colonic T cell markers

Colitis induction in the transfer model of colitis critically depends on the expansion and preferential differentiation of transferred T cells into Th1 T cells. Hence, T cell-related gene transcripts interferon (IFN)- γ , CD3 γ and T-bet were measured in colonic tissue samples in both groups of recipients. All of these T-cell transcripts, highly expressed in colitic animals when compared to healthy control mice, were significantly decreased in *B. lactis*-fed mice (Figure 6A-C). In order to gain insight into regulatory functions of T cells, mRNA expression of forkhead

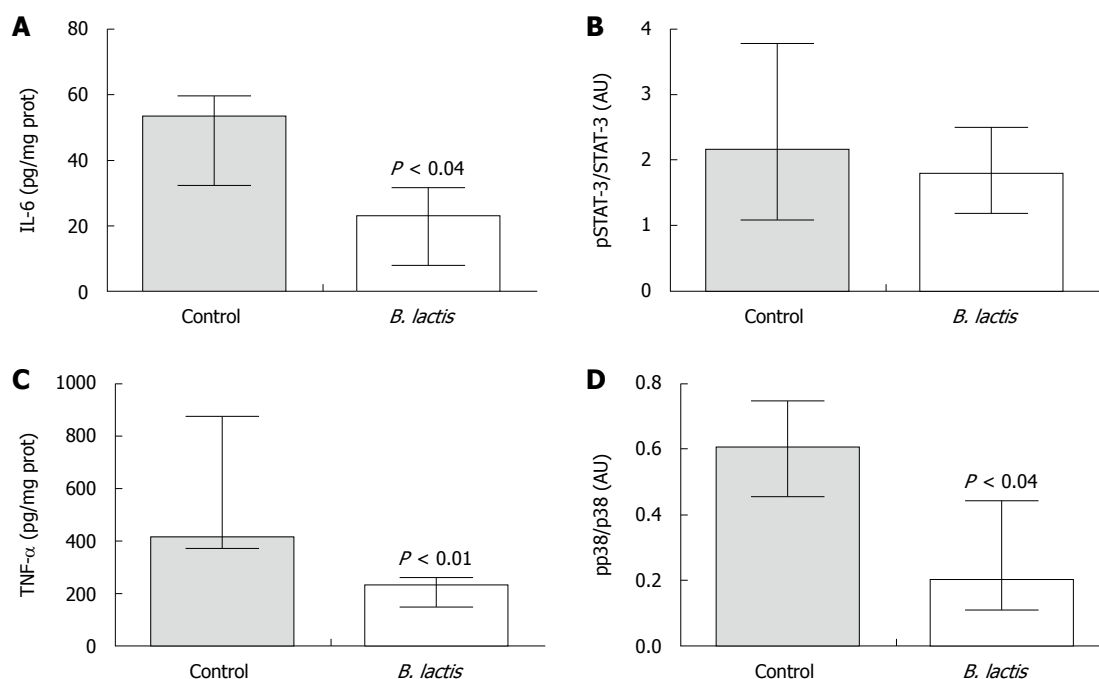


Figure 4 *Bifidobacterium lactis* feeding significantly diminished protein expression and phosphorylation status of pro-inflammatory markers in colon of recipient mice following adoptive T cell transfer. Expression of interleukin-6 (IL-6) (A) and tumor necrosis factor- α (TNF- α) (C) protein was measured by enzyme-linked immunosorbent assay in colon tissue samples from colitic recipients. The ratios of phosphorylated signal transducer and activator of transcription-3 (pSTAT-3) vs STAT-3 (B) and of pp38 vs p38 (D) were assessed by Western blotting analysis in colon tissue samples of colitic mice. Relative densitometric quantifications of the Western blotting bands are presented. Results are expressed as the mean \pm interquartile-range (A and C, $n = 5$; B, $n = 3$; and D, $n = 4$ mice per group) and statistical significance is indicated. *B. lactis*: *Bifidobacterium lactis*.

box protein 3 (Foxp3) in colon samples was also quantified and showed that Treg cells were significantly potentiated by feeding with the probiotics, as a 9.2-fold increase of the expression of Foxp3 was observed in *B. lactis*-fed mice when compared to control colitic mice (Figure 6D).

DISCUSSION

The exact etiology of chronic IBD is still unknown but seems complex and multifactorial. From human studies and animal models of experimental colitis, increasing evidence has been generated that the resident intestinal flora play a critical role in the development of the intestinal inflammation in genetically susceptible individuals. Indeed, on given genetic backgrounds, such as in IL-2- and IL-10-knockout mice or in HLA-B27 transgenic rats, as well as in the present colitis model, colitis develops when animals are raised under specific pathogen-free conditions, but almost no disease can be observed under germ-free conditions^[34-36]. With regard to the importance of the quality of the microflora in colitis, clinical and preclinical observations have demonstrated beneficial effects of probiotic microorganisms in the treatment of IBD in humans^[11,12,14,15] and in experimental models of colitis^[20,37-39].

While most reports in the literature describe effects of mixes of probiotic strains or of synbiotics, only a few papers have investigated the beneficial potential of the use of single probiotic strains, and moreover of *Bifidobacterium* probiotic strains, to alleviate intestinal inflammation. As a preclinical model of IBD, the CD4⁺CD45RB^{high} naive T cell transfer model of colitis^[29,30] was exploited in this

study. This widely used model of colitis allows the study of the Th1 cell-mediated immune events leading, without treatment, to an irreversible colonic inflammation characterized by a massive influx of mononuclear cells into the colonic mucosa, an elevated level of pro-inflammatory cytokines, appearance of crypt abscesses and epithelial cell erosions^[40]. Noteworthy, as for experimental models using genetically modified animals, the composition of the intestinal flora is also clearly known to affect the kinetics and the severity of the colitis in this particular model^[41]. Moreover, in the present study, the fact that CD4⁺CD45RB^{low} T cells were also transferred to recipient mice permitted us to also study the role that Tregs play in suppressing or limiting the onset and/or regulation of inflammation. Thus, for all the above mentioned reasons, this is to our knowledge one of the few studies that shows the preventive effects of a *Bifidobacterium* strain on the development of IBD in the CD4⁺CD45RB^{high} T cell reconstituted RAG-2 deficient mouse model with insights on Tregs.

Hence, it was demonstrated in the present study that *B. lactis* actually possesses *in vivo* immuno-modulatory properties and was able to improve the inflammatory status of treated mice. Indeed, it was revealed that supplementation of mice with *B. lactis*, one week before and during onset of colitis, resulted in a diminished colitis-induced weight loss (Figure 2), a reduction of mucosal thickness (Figure 3), a decreased expression of pro-inflammatory cytokines and related transcription factors (Figure 4), a diminution of T cell infiltration (Figure 5) and an increase of regulatory T cell markers (Figure 6) when compared to non-supplemented control animals.

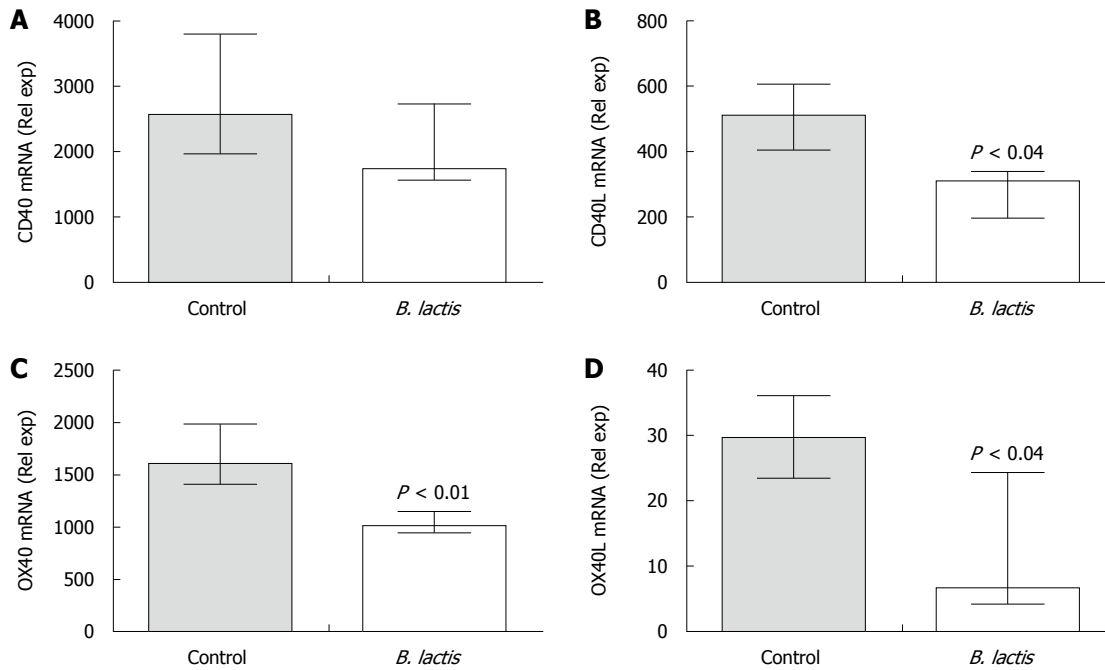


Figure 5 *Bifidobacterium lactis* feeding diminished the expression of mRNA coding for antigen-presenting cells and T cell costimulatory molecules in colon of recipient mice following adoptive T cell transfer. The expressions of mRNA coding for CD40 (A), CD40 ligand (CD40L) (B), OX40 (C) and OX40 ligand (OX40L) (D) were assessed in colon samples of colitic and healthy mice by real-time polymerase chain reaction using the low density array technology. Results are expressed as the mean ± interquartile-range ($n = 5$ mice per group) and statistical significance is indicated. *B. lactis*: *Bifidobacterium lactis*.

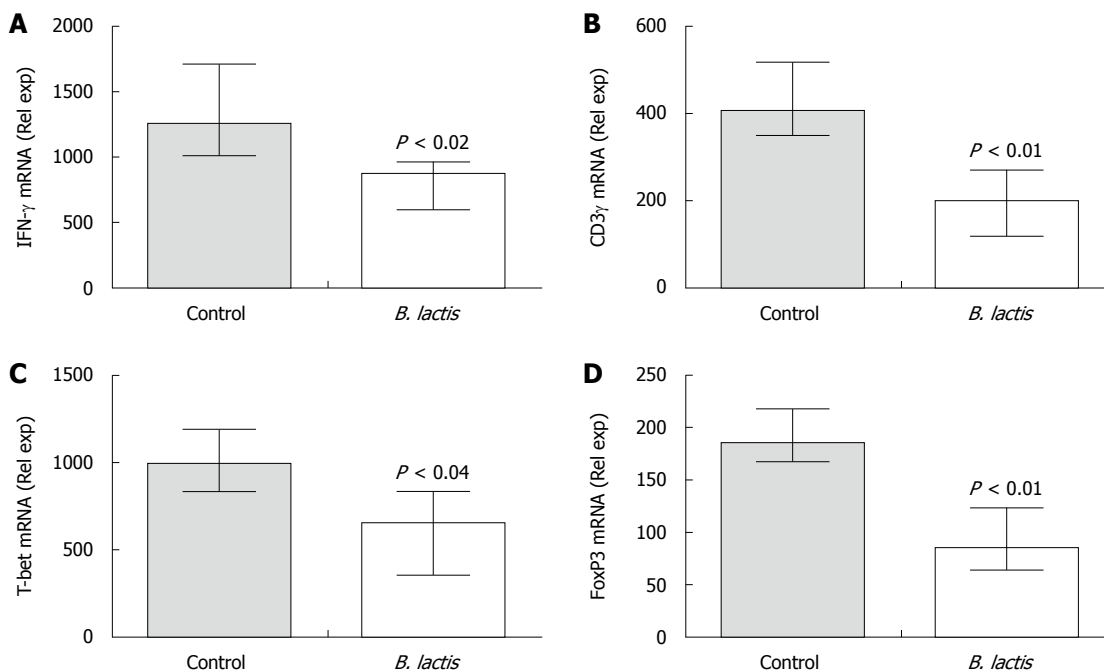


Figure 6 *Bifidobacterium lactis* feeding significantly diminished mRNA expression of Th1 cell markers and significantly increased mRNA expression of a Treg marker in colon of recipient mice following adoptive T cell transfer. The expressions of mRNA coding for interferon- γ (IFN- γ) (A), CD3 γ (B), T-bet (C) and FoxP3 (D) were assessed in colon samples of recipient mice by real-time polymerase chain reaction using the low density array technology. Results are expressed as the mean ± interquartile-range ($n = 5$ mice per group) and statistical significance is indicated. *B. lactis*: *Bifidobacterium lactis*.

Follow-up analysis of the weight of mice showed that *B. lactis* treatment significantly delayed the body weight loss observed in control recipient mice (Figure 2), revealing a major impact of the *B. lactis* supplementation on the overall metabolism of treated mice. After some gain of weight due to normal growth of the mice, weight loss was already

visible at day 9 in the control recipient mice whereas this only started at day 18 in the *B. lactis*-fed mice. In this latter group, no real weight loss was even observed at the end of the study, the mice having reached their starting weight. Whether this weight loss would continue under the starting weight should be tested in future studies.

Concerning histopathological analyses of the colons at sacrifice, even if the benefit observed regarding weight loss was not visible on total score, among all criteria investigated mucosal thickness was particularly improved by probiotic supplementation, suggesting a diminution of cellular infiltration in the mucosa (Figure 3A).

In line with this reduction of colonic thickness, a strong decrease of the reparative proliferative activity of colonic epithelial cells, assessed by immunostaining using the proliferative marker Ki-67, was observed in the mice fed with *B. lactis*. Indeed, colonic inflammation induces strong epithelial erosion due to uncontrolled apoptosis^[42]. This increased level of apoptosis, which contributes significantly to the IBD pathology^[11], is presumably due in part to a strong increase of inflammatory cytokines such as TNF and IFN- γ as observed in our study; cytokines known to be able to induce apoptosis directly by suppressing anti-apoptotic signals in the epithelium^[43,44]. Hence, since in our experimental setting mice were sacrificed at a late time point relative to onset of inflammation, reparative proliferation was the more relevant parameter to be investigated here rather than apoptosis.

The role of pro-inflammatory cytokines in IBD pathology has been firmly established^[45]. Dysregulation of the intestinal immune system both at humoral and cellular level constitutes an important element in the multifactorial pathogenesis of IBD. A strongly elevated expression of pro-inflammatory mediators, most notably IL-6 and TNF- α , has been identified in IBD patients and experimental models of colitis in mice^[46]. In cases of acute inflammation, IL-6 synthesized mainly by macrophages first binds to the IL-6 receptor (IL-6R); this complex then associates with gp130, inducing dimerization and the initiation of signaling through STAT-3. In the present study, the decrease of colonic IL-6 expression and STAT-3 phosphorylation induced by *B. lactis* strongly suggest that the probiotic could act, at least in part, on the IL-6 trans-signaling pathway and could by this process dampen the inflammation. Indeed, previous studies performed in both animal models and humans clearly described that abrogation of IL-6 pathway activation was associated with an improvement of colonic inflammation^[47-49].

Moreover, analysis of p38 phosphorylation has confirmed the beneficial impact of *B. lactis* feeding on inflammation. Indeed, p38 kinase regulates the production of key inflammatory mediators, including TNF- α or IL-1 β . In addition, p38 also acts downstream of cytokines such as TNF- α , mediating some of their effects^[50]. Hence, the significant diminution of p38 phosphorylation observed in this study upon *B. lactis* feeding (Figure 4) revealed the broad range of potential effects of the probiotic treatment on colitis.

B. lactis feeding also modulated COX-2 protein expression in the colon of mice (Figure 3C). COX-2, an enzyme responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane), has been shown to be specifically induced in epithelial cells under IBD conditions^[51]. Among the prostanoids related to COX-2 activity,

prostaglandins represent one of the most important components of mucosal defence in the small intestine and colon. The weight of evidence collected so far suggests that prostaglandins derived from COX-2 are important in promoting the healing of mucosal injury, in protecting against bacterial invasion, and in down-regulating the mucosal immune system. Suppression of COX-2 in a setting of gastrointestinal inflammation and ulceration has been shown in experimental models to result in impaired healing and exacerbation of inflammation-mediated injury^[52-55]. Hence, at the intestinal level, expression of COX-2 is a natural response of the organism to prevent tissue damage due to inflammation and is sustained by this inflammation. In this way, COX-2 expression represents a good marker of the actual disease activity^[56]. In the present study, the observed attenuated COX-2 expression upon *B. lactis* feeding strongly indicates an anti-inflammatory effect of this probiotic bacterium.

Colitis, in the T-cell adoptive transfer model, is accompanied by the accumulation of dendritic cells (DCs) in the MLN as well as locally in the colon^[57]. DCs in the MLN express an activated phenotype with increased expression of CD40 and the TNF-like molecule OX40L^[58]. Indeed, activated T cells express the cell-surface costimulatory molecules CD40L and OX40. CD40L binds to CD40 on antigen-presenting cells (APCs) inducing OX40L expression, and leading to the transmission of further activatory signals to both the T cell and the APC^[59,60]. The CD40-CD40L and OX40L-OX40 pathways play functional roles in the inflammatory response in this model, as blockade of either pathway inhibits colitis^[58,61,62]. The overall decrease in these four costimulatory partners observed upon *B. lactis* feeding (Figure 5) thus revealed a diminished activity of the immune system in sustaining the adaptive inflammatory reactions. The diminution in the number of T cells present in the colonic mucosa of *B. lactis*-treated mice compared to control animals, as revealed by the measurement of the relative expression of CD3 γ mRNA (Figure 6B), might be considered as a partial consequence of this dampening of the costimulatory activity.

Beyond the intensity of the DC-T cell interaction, *B. lactis* feeding could interfere with the outcome of the interaction in the mucosa. Indeed, *B. lactis* was also able to significantly decrease T-bet and IFN- γ mRNA expression compared to untreated animals (Figure 6A and C). IFN- γ is the hallmark Th1 cytokine and T-bet is a critical factor for both the initiation and perpetuation of Th1-mediated colitis. Indeed, in another type of adoptive transfer model, T-bet-deficient CD4⁺CD62L⁺ T cells failed to induce Th1-mediated colitis in immunodeficient hosts, whereas T-bet-overexpressing CD4⁺CD62L⁺ T cells induced a more rapid onset of colitis^[63]. The observed diminution of the IFN- γ mRNA expression is fully in line with the decrease of T-bet, revealing a functional impact on activated T cells by *B. lactis*.

Development of colitis following transfer of CD4⁺CD45RB^{high} T cells into immunodeficient recipients can be modulated or even abrogated by cotransfer of cells from the antigen-experienced CD4⁺CD45RB^{low} population^[24], a potential source of Tregs. In the present study, as a small

proportion of such cells have been actually cotransferred, we performed a quantification of mRNA expression for Foxp3 in colon samples of recipient mice. This marker has been identified as being necessary for both the development and function of Tregs^[64-66]. It appeared that *B. lactis* feeding significantly potentiated the presence of these particular cells in the inflamed mucosa as treated mice showed a strong augmentation of Foxp3 mRNA expression compared to control mice (Figure 6D) whereas total number of T cells decreased in a converse fashion (Figure 6B). Moreover, while the dampened expression of all analyzed T cell-related proinflammatory markers might be a direct consequence of the diminution in T cell number in the colonic mucosa of *B. lactis*-fed animals, the fact that Foxp3 mRNA expression was up-regulated indicates a strongly increased prevalence of Tregs in the inflamed tissues. This increase of Treg cells may be one of the key players in the alleviation of the colitis observed in this study.

Despite the fact that mechanisms of action remain unclear, two different options could be hypothesized to explain the effect of probiotics on the Tregs. The classical one will be that probiotics in the colon generated antigenic peptides or molecules able to inhibit or modulate DC activation responsible for uncontrolled T cell proliferation which leads to colitis development, as supported by the diminished expression of the CD40-CD40L and OX40L-OX40 costimulatory partners. More recently, a new hypothesis could be proposed to explain the probiotic effect, involving the toll-like receptor (TLR) pathway. Indeed, it has been revealed that TLR molecules that recognize a vast range of microbial products, thought to be only restricted to cells of innate immunity, are also expressed by Tregs^[67]. Interestingly, a recent study demonstrated a role of the TLR2 pathway in the control of expansion and function of Tregs^[68]. Such a direct effect of TLR ligands on Tregs thus opens a new way to consider the impact of probiotics in the regulation of colitis. Future investigations on the impact of *B. lactis* on Tregs will provide interesting pieces of information regarding how and when probiotics exert their effect, as the present study does not allow discrimination as to whether Tregs are potentiated already in donor mice or only in recipient mice upon induction of inflammation.

In conclusion, it was shown in the present study that *B. lactis* is a *Bifidobacterium* probiotic strain able to provide anti-inflammatory properties in an adoptive cell transfer model of IBD. Mechanisms of actions are not completely elucidated and need further investigations, but a clear effect on Tregs may be suggested as a key influence.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Mark Waldron (Nestlé Research Center, Lausanne, Switzerland) for critically reading the manuscript.

COMMENTS

Background

Ulcerative colitis and Crohn's disease, commonly known as inflammatory

bowel disease (IBD), affect 0.5%-1% of the Western world's population and are increasing in the developing countries. IBD is a chronic disease that requires lifelong treatment. Many patients do not respond or do not comply well with the recent medications. This, in addition to the high cost of these approaches, urges the scientific community to develop new therapeutic approaches.

Research frontiers

Many probiotics have been identified as a powerful strategy to reduce intestinal inflammation in preclinical and human trials. However, despite a high number of publications there are very few explanations concerning their mechanism of action.

Innovations and breakthroughs

Using one of the most valuable murine models of colitis, we have highlighted the fact that a single *Bifidobacterium* probiotic strain was able to prevent intestinal inflammation by increasing the number of regulatory T cells in the gut.

Applications

Through this publication we would like to emphasize that *Bifidobacterium lactis* (NCC 2818) could be considered as a good auxiliary to current treatment used to reduce intestinal inflammation in IBD patients.

Terminology

According to the World Health Organization definition, probiotics are live microorganisms which when consumed in adequate amounts confer health benefits to the host.

Peer review

In the manuscript, the authors evaluated the anti-inflammatory properties of a *B. lactis* strain in a colitis mouse model. The experiments were carefully designed and the manuscript is easy to read.

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S- Editor Sun H L- Editor Logan S E- Editor Lin YP

A potential oncogenic role of the commonly observed E2F5 overexpression in hepatocellular carcinoma

Yuzhu Jiang, Seon-Hee Yim, Hai-Dong Xu, Seung-Hyun Jung, So Young Yang, Hae-Jin Hu, Chan-Kwon Jung, Yeun-Jun Chung

Yuzhu Jiang, Hai-Dong Xu, Seung-Hyun Jung, So Young Yang, Hae-Jin Hu, Yeun-Jun Chung, Integrated Research Center for Genome Polymorphism and Department of Microbiology, School of Medicine, The Catholic University of Korea, Socho-gu, Seoul 137-701, South Korea

Seon-Hee Yim, Integrated Research Center for Genome Polymorphism and Department of Medical Humanities and Social Sciences, School of Medicine, The Catholic University of Korea, Socho-gu, Seoul 137-701, South Korea

Chan-Kwon Jung, Hospital Pathology, Seoul St Mary's Hospital, Socho-gu, Seoul 137-701, South Korea

Author contributions: Chung YJ and Jiang Y designed the research; Jiang Y, Xu HD, Jung SH and Yang SY performed the experiments; Jiang Y, Yim SH and Hu HJ analyzed the data; Jung CK contributed reagents/analytic tools; Chung YJ, Jiang Y and Yim SH wrote the paper.

Supported by A grant of the Korea Healthcare technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A092258) and by FG08-11-06 of the 21C Frontier Functional Human Genome Project from the Ministry of Education, Science and Technology

Correspondence to: Dr. Yeun-Jun Chung, Integrated Research Center for Genome Polymorphism and Department of Microbiology, School of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, South Korea. yejun@catholic.ac.kr

Telephone: +82-2-22587343 Fax: +82-2-5370572

Received: September 16, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: January 28, 2011

Abstract

AIM: To explore the expression pattern of E2F5 in primary hepatocellular carcinomas (HCCs) and elucidate the roles of E2F5 in hepatocarcinogenesis.

METHODS: E2F5 expression was analyzed in 120 primary HCCs and 29 normal liver tissues by immunohistochemistry analysis. E2F5-small interfering RNA was transfected into HepG2, an E2F5-overexpressed HCC cell line. After E2F5 knockdown, cell growth capacity

and migrating potential were examined.

RESULTS: E2F5 was significantly overexpressed in primary HCCs compared with normal liver tissues ($P = 0.008$). The E2F5-silenced cells showed significantly reduced proliferation ($P = 0.004$). On the colony formation and soft agar assays, the number of colonies was significantly reduced in E2F5-silenced cells ($P = 0.004$ and $P = 0.009$, respectively). E2F5 knockdown resulted in the accumulation of G0/G1 phase cells and a reduction of S phase cells. The number of migrating/invasive cells was also reduced after E2F5 knockdown ($P = 0.021$).

CONCLUSION: To our knowledge, this is the first evidence that E2F5 is commonly overexpressed in primary HCC and that E2F5 knockdown significantly repressed the growth of HCC cells.

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Key words: E2F5; E2F family; Hepatocellular carcinoma; Oncogene; Small interfering RNA

Peer reviewer: Dr. Anna Chiara Piscaglia, MD, Specialist in Gastroenterology and Digestive Endoscopy, GI and Liver Stem Cell Research Group (GILSteR), Department of Internal Medicine - Digestive Endoscopy Unit, Gemelli Hospital - Catholic University of Rome, Largo A. Gemelli, 8 - 00168 Rome, Italy

Jiang Y, Yim SH, Xu HD, Jung SH, Yang SY, Hu HJ, Jung CK, Chung YJ. A potential oncogenic role of the commonly observed E2F5 overexpression in hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(4): 470-477 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/470.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.470>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer and the third leading cause of cancer death worldwide^[1]. More than half a million cases are newly diagnosed every year, and a similar number of patients die each year. Given that the overall incidence of HCC is still rising and the prognosis remains poor, it is important to develop effective diagnostic and therapeutic modalities based on the biological knowledge of hepatocarcinogenesis^[2,3].

Our group previously explored the profiles of chromosomal alterations in primary HCC by performing a genome-wide, microarray-based, comparative genomic hybridization analysis and found that the 8q21.2 locus harboring the *E2F5* gene was recurrently amplified in HCC^[4]. *E2F5* is a member of the E2F transcription factor family that binds to the promoters of the target genes involved in cell cycle control and that consequently regulates the expression of these target genes^[5]. The E2F family, located in the downstream of the growth factor signaling cascades, plays a central role in cell growth and proliferation through regulating the genes involved in cell cycle progression^[6]. Therefore, it is possible for the members of the E2F family to become involved in oncogenesis. The members of the E2F family are divided into activator (E2F1-E2F3) and repressor (E2F4-E2F8) subclasses^[5]. Overexpression of the E2F activators has been reported to induce uncontrolled proliferation of cells in diverse human cancers such as breast, ovarian, lung and gastrointestinal cancers^[7-10]. Although the E2F repressors are expected to behave as tumor suppressors, a substantial amount of evidence instead indicates the possibility that some E2F repressors may have oncogenic effects in tumorigenesis^[5].

The overexpression or amplification of the *E2F5* gene has been reported in various solid tumors such as breast, colon, ovarian cancers and osteogenic sarcoma^[11-17]. When the *E2F5* gene was co-transfected with DP-1 and Ras into rat kidney cells, the number of transformed foci increased^[11]. However, there has been no report on the expression profile of *E2F5* in human HCC and its biological roles in hepatocarcinogenesis.

In this study, we explored the expression profiles of *E2F5* in primary human HCC and its biological effects by performing knockdown of the *E2F5* using specific small interfering RNA (siRNA) in a human HCC cell line.

MATERIALS AND METHODS

Tissue microarray

For screening *E2F5* expression in primary HCC, HCC tissue microarrays were prepared. Formalin-fixed, paraffin-embedded tissue blocks from 120 HCC patients were identified from the archives of the Department of Hospital Pathology under the approval of the Institutional Review Board of the Catholic University of Korea, College of Medicine (CUMC06U014). Tissue microarrays were constructed using a manual tissue arrayer (Quick-Ray Manual Tissue Microarrayer, Unimta Co., Ltd., Seoul, Korea). Hematoxylin-eosin stained sections from each sample were examined and then tumor cell-rich areas were selected for use in tissue microarrays. A microarray con-

sisting of 29 samples of normal liver tissue was also constructed. The tissue array paraffin block was then cut into 5- μ m paraffin sections for immunohistochemistry (IHC) analysis.

HCC cell line

HepG2 was obtained from ATCC (Manassas, VA, USA) and maintained in DMEM (Gibco BLR, Gaithersburg, MD, USA) supplemented with 10% FBS at 37°C containing 5% CO₂. As controls, THLE-2 and THLE-3 (human normal liver epithelial cell lines) were purchased from ATCC (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and 25 mmol/L HEPES.

IHC

E2F5 immunostaining was performed using tissue microarray slides. Briefly, the tissue sections were deparaffinized and quenched with 3% hydrogen peroxide for 10 min. Antigen retrieval was then conducted using 0.01 mol/L citrate buffer (pH 6.0) by heating the sample in a microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at a controlled final temperature of 121°C for 15 min. The monoclonal mouse anti-*E2F5* antibody (220D1a, Abcam, Cambridge, UK) was then diluted 1:50 in Dako Antibody Diluent (Dako, Carpinteria, CA, USA) with background-reducing components, after which it was incubated overnight at 4°C and then detected using the Envision plus System (Dako, Carpinteria, CA, USA). The immunoreaction was developed by treating the samples with diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min, and hematoxylin counterstaining was applied.

Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using oligo-(dT) primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with a mixture containing cDNA, 1 \times SYBR Green *Thr* polymerase mixture (FINNZYMES, Espoo, Finland), 0.5 \times ROX, and primers using Mx3000P QPCR (Stratagene, La Jolla, CA, USA). The thermal cycling included one cycle at 95°C for 10 min, followed by 45 cycles of 95°C at 10 s, 55°C for 30 s and 72°C for 30 s. Relative quantification was performed by the $\Delta\Delta$ Ct method as described elsewhere^[4]. *E2F5* specific primers for qRT-PCR were as follows; 5'-TCAGGACCTATCCATGTGCTGCTT-3' for forward and 5'-TCAGAGACATGTTGCTCAGGCAGA-3' for reverse. The *GAPDH* gene was used as an internal control and its specific primers were as follows; 5'-GCGGGGCTCTCCAGAACATCAT-3' for forward and 5'-CCAGCCCCAGCGTCAAAGGTG-3' for reverse.

Transfection of *E2F5* siRNAs

Three types of *E2F5*-specific siRNAs were purchased (Invitrogen, Carlsbad, CA, USA), whose sequences were

as follows: UUAUAAGCAGCACAUGGAUAGGUCCG-GACCUAUCCAUGUGCUGCUUAAUAA for siE2F5-1; AAUUAAGUUGUAGUCAUCUGCCGCGCG-GCAGAUGACUACAACUUUAAUU for siE2F5-2; AUGAUAUCUCCACUAAUAGAUCUGCAG-GAUCUAUUAGUGGAGAUUAUCAU for siE2F5-3. The siE2F5-1, -2, and -3 target exons were 6, 7 and 8, respectively. To estimate the sequence-specific effectiveness of the E2F5 siRNAs, we used a negative control siRNA (siNEG) (Invitrogen, Carlsbad, CA, USA) that has no significant homology with any known sequences in the human genome. In total, 100 nmol/L of each siRNA was transfected onto 6×10^5 HepG2 cells in a 100 mm culture dish using lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Forty-eight hours following the transfection, the cells were harvested and E2F5 expression was measured by qRT-PCR and Western blotting.

Western blotting

Transfected cells were harvested and lysed in cell lysis buffer (50 mmol/L NaF, 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 2 mmol/L EDTA, 0.1% Triton X-100) with protease inhibitor. The resulting cellular proteins (30 µg per lane) were electrophoresed in a 10% SDS-polyacrylamide gel and separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat dried milk in TBST (20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20, pH 7.5), and then incubated overnight with anti-E2F5 (Abcam, Cambridge, UK) and anti α -tubulin antibodies (Sigma, St. Louis, MI, USA) at 4°C according to the manufacturer's instructions. After washing with TBST, the PVDF membrane was incubated with diluted HRP-conjugated anti-rabbit IgG for 1 h at room temperature. The blots were detected using an enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Braunschweig, Germany). The expression of α -tubulin was used as a control.

Cell proliferation assay

Growth of HepG2 cells was determined using the cell proliferation reagent WST-1 (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Optical density was read at 450 nm at various time points. Three independent experiments were carried out.

Invasion and migration assay

Invasion of HepG2 cells was assayed using the 24-well format transwell chambers (Becton Dickinson Labware, Franklin Dickinson, NJ, USA) of 8 µm-pore size. siRNA transfected cells were added onto the Matrigel (BD Biosciences, San Jose, CA, USA) coated filters in 200 µL of serum-free RPMI1640 (2×10^4 cells/filter). In the lower chambers, 500 µL of RPMI1640 media containing 10% FBS was added. After 20-h incubation at 37°C in the 5% CO₂ incubator, cells were stained with 0.5% crystal violet in 20% methanol and then examined under the micro-

scope. Cells on the top surface of the membrane were removed by wiping with a cotton swab. The numbers of cells were counted in five microscopic fields ($\times 40$). The migration assay was carried out in the same manner but using filters without Matrigel coating. Two independent experiments were performed for each assay.

Colony formation assay

Forty-eight hours after transfection with siRNA, 1×10^4 HepG2 cells were seeded in 100-mm culture dishes. Two weeks later, cultured cells were stained with 0.5% crystal violet and the number of colonies was counted. Three independent experiments were carried out for each assay.

Soft agar assay

To explore the effect of E2F5 on anchorage independent growth of HepG2 cells, a soft agar assay was performed. HepG2 cells were suspended in RPMI1640 containing 0.35% low melting agarose, and plated onto solidified 0.6% agarose containing RPMI1640 in six-well culture plates at a density of 1×10^5 cells per dish. After incubating for 2 wk at 37°C in the 5% CO₂ incubator, the number of colonies was counted. Two independent experiments were carried out for each assay.

Cell cycle analysis

Forty-eight hours after transfection with siRNA, the adherent cells were detached by trypsin treatment, washed twice with PBS, and then exposed to 70% ethanol on ice for 2 h. After washing, cells were incubated with 5 mg/mL propidium iodide and 50 mg/mL RNase-A in PBS for 15 min at 37°C. A flow activated cell sorter (FACS) analysis was carried out using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) with CELLQUEST software. A total of 10000 events were measured per run.

Statistical analysis

The statistical analysis was performed using the SPSS statistical software (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by the Mann-Whitney test for continuous variables and by the χ^2 or Fisher's exact test for categorical variables. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression of E2F5 in primary human HCC

We examined the E2F5 protein expression level in the 120 primary HCCs and 29 normal liver tissues by tissue microarray-based IHC. E2F5 expression was mostly localized in the cytoplasm with occasional nuclear staining. Cytoplasmic staining was considered a positive result. We graded the E2F5 staining intensity from 0 (negative) to 3 (strongly positive) (Figure 1A-D). E2F5 expression was absent in all the normal liver tissues tested, while positive in 22 (18.3%) of 120 HCCs (*P* = 0.008); 17 cases of grade 1, 4 cases of grade 2, and one case of grade 3. Although E2F5 intensities showed a trend of positive correlation

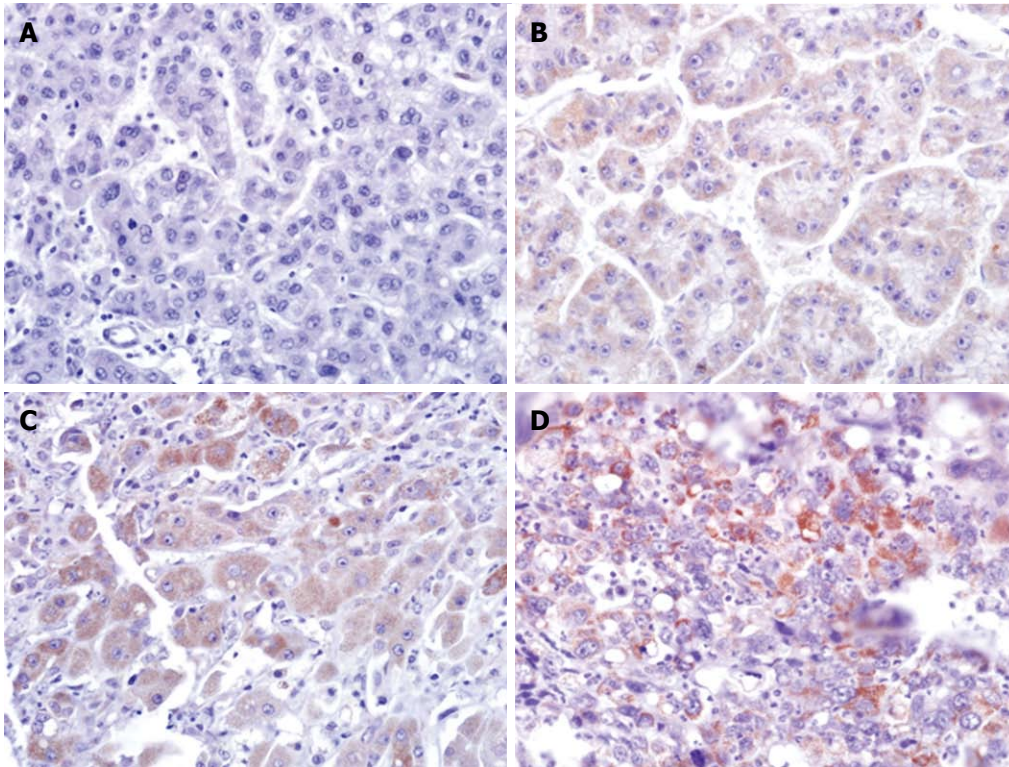


Figure 1 Expression pattern of E2F5 in primary hepatocellular carcinoma by tissue microarray based immunohistochemistry. Cytoplasmic staining was considered E2F5 expression positive. E2F5 expression was absent in all normal liver tissues tested. A: No E2F5 expression; B-D: Grade 1 to 3 E2F5 expression, respectively ($\times 400$).

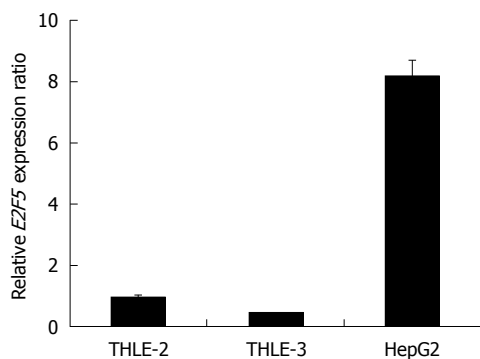


Figure 2 E2F5 RNA expressions of HepG2 and normal liver cell lines (THLE-2 and -3). E2F5 gene specific reverse transcriptase polymerase chain reaction was performed for HepG2, THLE-2 and THLE-3 as described in methods. In HepG2 cell line, E2F5 RNA expression was up-regulated more than 8 fold compared with that of the normal liver cells.

with tumor grades, it was not statistically significant (data not shown).

Repression of E2F5 expression by siRNA transfection

To assess the biological effects of E2F5 overexpression in hepatocarcinogenesis, we used E2F5 gene specific siRNA to disrupt its expression in the HepG2 liver cancer cell line. Before knockdown, we assessed the expression level of E2F5 in the HepG2 cells by qRT-PCR and confirmed that the E2F5 expression was up-regulated more than 8-fold compared with that of the normal liver

cells (Figure 2). Before transfection, we optimized the amount of lipofectamine to ensure the maximal transfection efficiency and minimal cellular toxicity (data not shown). Then we introduced three E2F5 gene specific siRNA constructs (siE2F5-1, -2, and -3) and siNEG into the HepG2 cells. The effect of each siRNA on the E2F5 expression level was examined by qRT-PCR and Western blotting analysis. Of the three siRNAs examined, siE2F5-1 and siE2F5-3 were shown to efficiently repress the RNA expression level (approximately 90% reduction) by qRT-PCR (Figure 3A). In the Western blotting analysis, both siE2F5-1 and siE2F5-3 also efficiently repressed the E2F5 expression, but the latter was slightly better than the former (Figure 3B). Therefore, all the downstream functional analyses were performed using siE2F5-3 (hereafter called siE2F5).

Effect of E2F5 knockdown on HepG2 cell growth

To explore the effect of E2F5 on HCC cell growth, we performed proliferation and colony formation assays. In the proliferation assay, the siE2F5 transfected cells showed reduced proliferation compared with that of the siNEG transfected cells by over 75% ($P = 0.004$, Figure 4A). In the colony formation assay, the number of colonies from the siE2F5 treated cells [median 119, interquartile range (IQR) 116-124] was significantly smaller than that from the siNEG treated cells (median 243, IQR 228-267, $P = 0.004$) (Figure 4B). Next, we examined the E2F5 knockdown effect on the anchorage independent growth of HCC

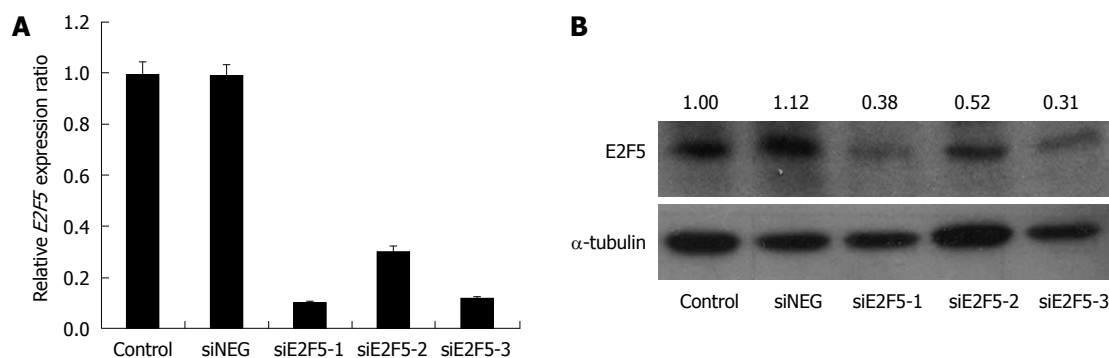


Figure 3 Small interfering RNA-mediated silencing of *E2F5* gene expression in HepG2 cells. A: After siE2F5 transfection into the HepG2 cells, *E2F5* expression was measured by real time quantitative reverse transcriptase polymerase chain reaction. Human *GAPDH* gene was used as an internal control. The X axis represents samples and the Y axis relative *E2F5* expression ratios (siE2F5 or siNEG/control); B: *E2F5* expression in Western blotting analysis was measured by densitometry. Alpha-tubulin was used as an internal control. *E2F5* specific signal intensities of siE2F5-treated cells are profoundly repressed compared with siNEG-treated cells. Control: HepG2 cells without transfection; siNEG: Negative oligonucleotide (siNEG) transfected HepG2.

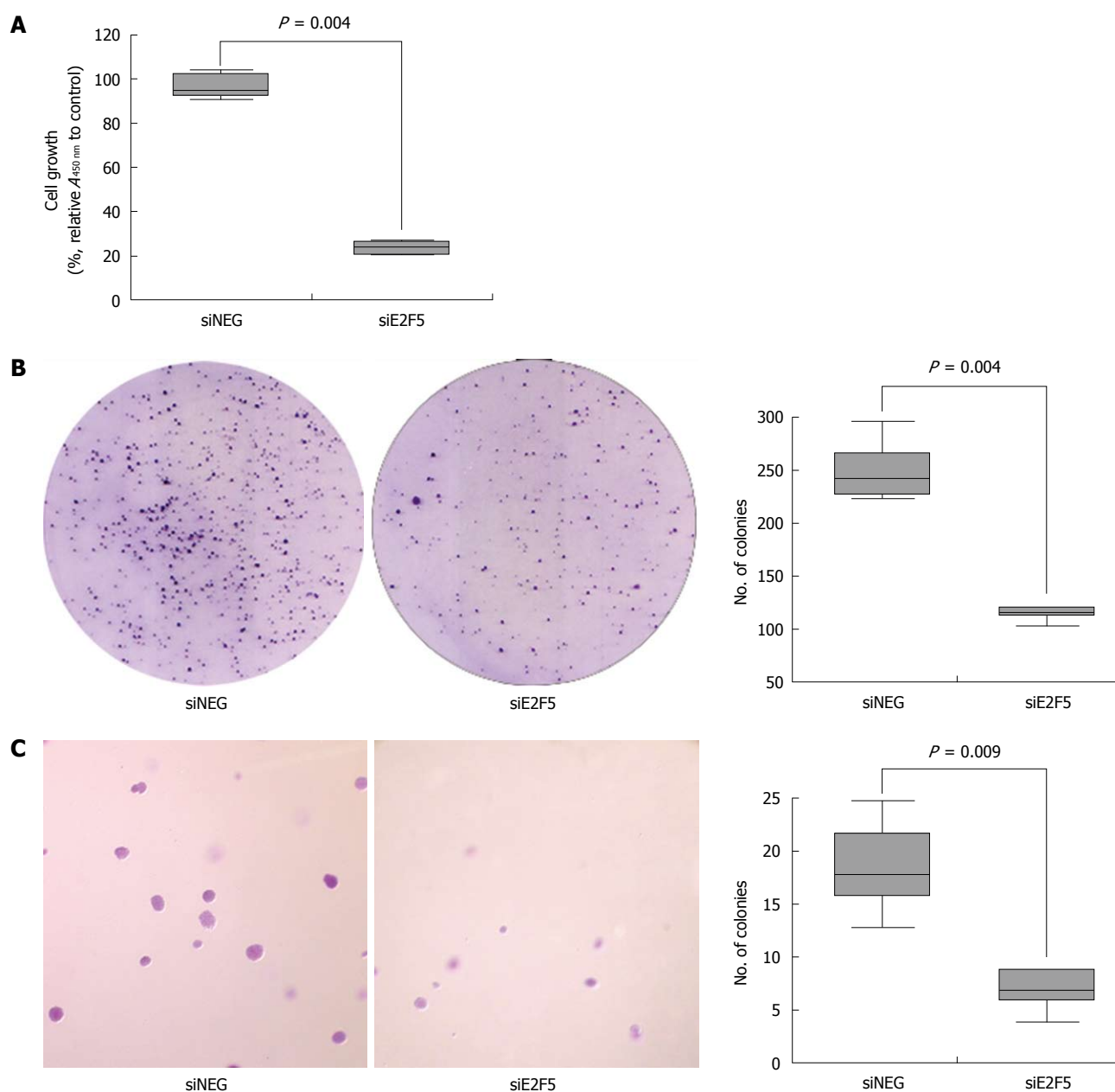


Figure 4 Effects of *E2F5* knockdown on HepG2 cell growth. Proliferation assay (A), colony formation assay (B), and soft agar assay (C) consistently demonstrated the inhibition of HepG2 cell growth by *E2F5*-specific small interfering RNA transfection ($\times 40$).

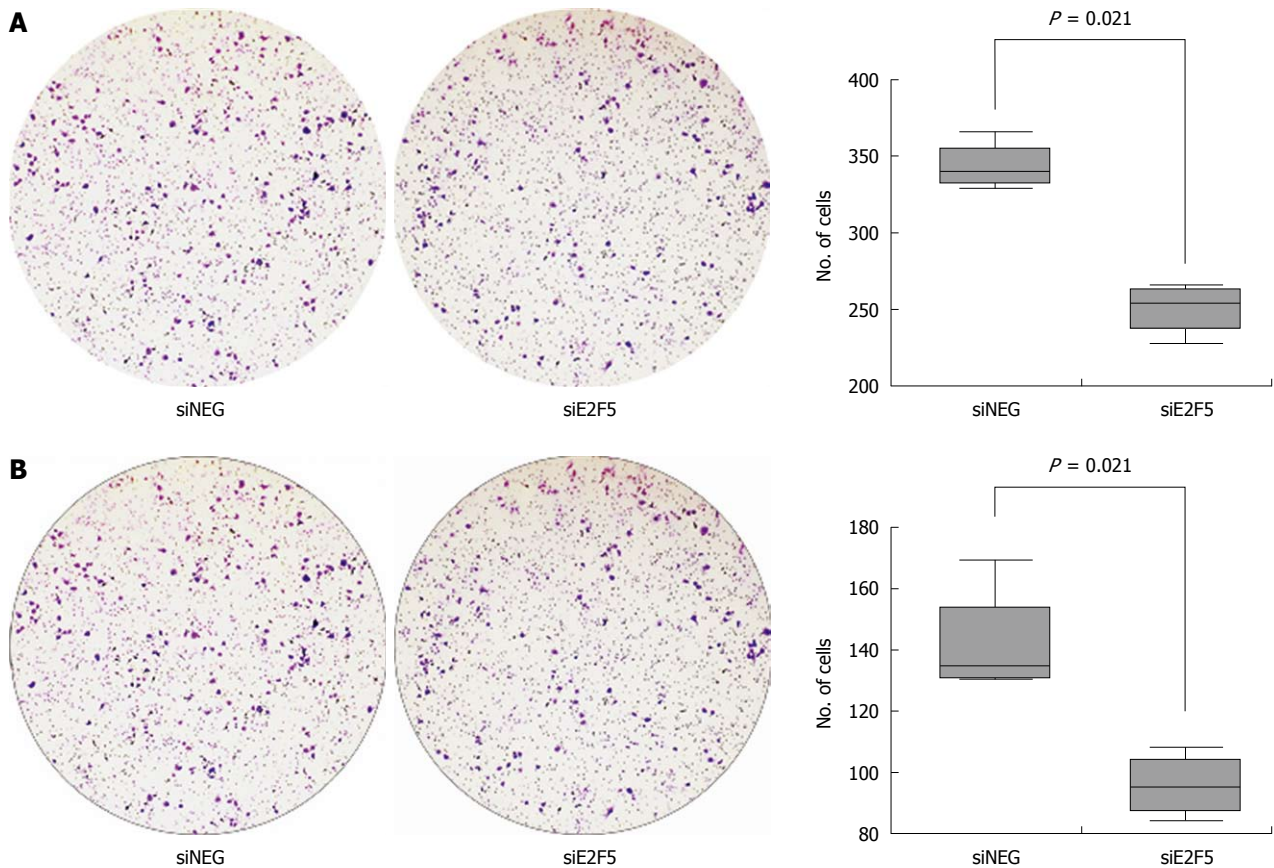


Figure 5 Effects of E2F5 knockdown on migration and invasion of HepG2 cells. A: The number of migrating colonies in siE2F5 treated cells was significantly lower than that in siNEG transfected cells on Matrigel uncoated transwell membrane ($P = 0.021$); B: The number of invading colonies in siE2F5 treated cells was significantly lower than that in siNEG transfected cells into Matrigel coated transwell membrane ($P = 0.021$).

cells using the soft agar assay. The number of anchorage-independent colonies of the siE2F5 treated cells (median 7, IQR 6-9) was significantly reduced as compared with that of the siNEG treated cells (median 18, IQR 16-22, $P = 0.009$) (Figure 4C).

Effect of E2F5 knockdown on HepG2 cell migration and invasion

The number of migrating cells was significantly reduced by E2F5 knockdown (median 339.5, IQR 331.5-354.5 in the siNEG treated cells *vs* median 253.5, IQR 236.5-263, $P = 0.021$). The number of invading cells was also significantly reduced (median 134.5, IQR 130.5-153.5 *vs* median 95, IQR 87-104, $P = 0.021$) (Figure 5).

Effect of E2F5 siRNA on cell cycle progression in HepG2 cells

To explore the mechanisms behind the anti-proliferative effect in the siE2F5 transfected cells, we observed the cell cycle profile of the HepG2 cells by performing FACS analysis after siE2F5 transfection. E2F5 knockdown resulted in the accumulation of G0/G1 phase cells and the reduction of S phase cells compared with that of the siNEG transfected cells. The proportion of G1-phase cells in the siE2F5 treated cells was 76.5%, while it was 71.7% in the siNEG treated cells. In contrast, the

proportion of S phase cells in the siE2F5 treated cells (5.2%) was much lower than that in the siNEG treated cells (11.9%) (Figure 6).

DISCUSSION

Amplification of the *E2F5* gene has been frequently observed in diverse human cancers^[11,12,16]. We previously reported that the 8q21.2 locus, which harbors the *E2F5* gene, was recurrently amplified in primary human HCC^[4]. These data suggest the oncogenic potential of *E2F5* amplification. However, the E2F repressors (E2F4-E2F8) among the E2F family members have been reported to show paradoxical behaviors in tumorigenesis^[5]. In *Rb*^{+/-} *E2f4*^{-/-} mice, *E2f4* loss suppressed the development of pituitary and thyroid tumors, which suggested its oncogenic role in these tissues^[18]. This phenomenon was also observed in *Rb*^{-/-} *E2f4*^{-/-} chimeric mice, where *E2F4* loss delayed the development and reduced the incidence of pituitary tumors^[19]. However, the same *Rb*^{-/-} *E2f4*^{-/-} chimeras developed ganglionic neuroendocrine neoplasms and urothelial transitional carcinomas, which indicates a tumor suppressive role for E2F repressors^[19]. In 3T3 mouse fibroblasts, artificially overexpressed *E2F4* and *E2F5* inhibited malignant transformation, while overexpressed *E2F2* and *E2F3a* showed an oncogenic capacity^[20]. Thus, E2F

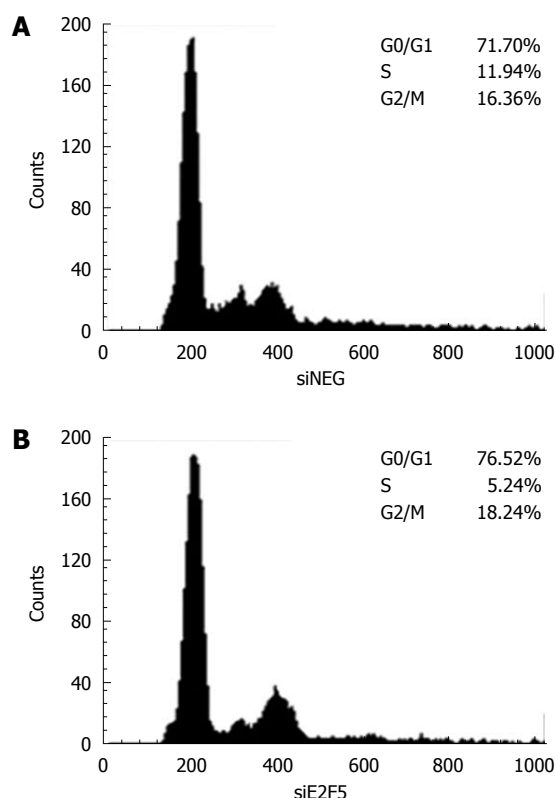


Figure 6 Effect of siE2F5 treatment on HepG2 cell cycle progression. After siE2F5 transfection into the HepG2 cells, cell cycle profile was examined by flow cytometry analysis. DNA histogram shows the accumulation of G0/G1-phase cells in siE2F5 transfected HepG2 cells compared with the siNEG transfected cells.

repressors may play an oncogenic or tumor suppressor role in a tissue specific manner^[5].

In this study, we explored the biological consequences of E2F5 overexpression in hepatocarcinogenesis and the involved molecular mechanisms. To begin with, we examined the expression of E2F5 protein in 120 primary HCCs and 29 normal liver tissues by tissue microarray based IHC and found that 18.3% of HCCs were positive, while none of the normal liver tissues were positive ($P = 0.008$). Although there has been no report on E2F5 overexpression in HCC, E2F5 overexpression has been reported in ovarian and breast cancers^[11-15]. E2F5 overexpression was observed in both early and advanced stage ovarian cancer, and this suggested the potential involvement of E2F5 in the pathogenesis of ovarian cancer^[13,15]. In breast cancer, E2F5 overexpression was also significantly associated with an ER/PR/HER2 triple negative status and with a worse clinical outcome^[12]. In this study, we did not find a significant correlation between tumor grade and E2F5 expression, but the correlation cannot be excluded because the sample size may not have been large enough. Associations between E2F5 expression and other phenotypes were not able to be assessed due to the limited clinical information of the primary HCC used in this study.

We knocked down the E2F5 expression using E2F5 gene specific siRNA transfection to explore the biological

effects of E2F5 overexpression in hepatocarcinogenesis. We found that E2F5 knockdown profoundly repressed the growth of HCC cells compared with the untransfected cells. The repression of HepG2 cell growth after E2F5 knockdown was simultaneously observed in the proliferation, colony formation and soft agar assays. These data can be related to the results of Polanowska *et al.*^[11], where the amplification and subsequent overexpression of the E2F5 gene promoted cell transformation together with other oncogenes in human breast tumors. Our results as well as the previous observations suggested the involvement of E2F5 gene amplification and overexpression in the tumorigenesis of various cancers including HCC.

It is widely recognized that the E2F family, including E2F5, is involved in tumorigenic processes through deregulation of cell cycle progression^[5,21]. In order to understand the nature of the siE2F5-mediated growth inhibition, we analyzed the cell cycle of the HCC cell line by FACS analysis after E2F5 knockdown. Consistent with our prediction, treatment with siE2F5 seemed to arrest the cell cycle at the G0/G1 phase. In a previous study with human WI38 fibroblasts, the E2F5 mRNA expression was maximal in the mid-G1 phase before the E2F1 expression was detectable, which suggests the contribution of E2F5 to the regulation of early G1 events, including the G0/G1 transition^[22]. The extent of cell cycle arrest was relatively small considering the prominent growth suppression induced by knockdown of E2F5 in this study. This may indicate the existence of additional effects of unknown factors added to E2F5 overexpression in HCC tumorigenesis. Migration and invasion capacities of HepG2 cells were also significantly inhibited by E2F5 knockdown. However, we did not explore whether E2F5 was directly involved in the migration- or invasion-related pathways or if the inhibitory effect was due to repression of HepG2 cell proliferation. Further functional studies are required to fully understand the oncogenic roles of E2F5 in HCC. The E2F5-interacting molecules also need to be identified to properly understand the behaviors of HCC.

To our knowledge, this is the first evidence that E2F5 is commonly overexpressed in primary human HCC and that E2F5 knockdown profoundly repressed the growth of HCC cells. The overexpression of E2F5 may induce uncontrollable cell cycle progression in liver cells and eventually contribute to cancer transformation by working together with other carcinogenic factors. This study will help to understand hepatocarcinogenesis mechanisms and to define therapeutic targets of early HCC.

COMMENTS

Background

Although the incidence of hepatocellular carcinoma (HCC) is still high and the prognosis remains poor, the molecular mechanisms of HCC are largely unknown. To develop effective diagnostic and therapeutic modalities, it is important to understand the mechanisms underlying hepatocarcinogenesis.

Research frontiers

E2F5 is a member of the E2F transcription factor family, and plays a key role

in cell growth and proliferation. Overexpression of E2F5 has been reported in various human cancers, but not in liver cancer, and its biological implication is largely unknown. In this study, the authors investigated the expression profile of E2F5 in primary HCCs and explored the biological effects of E2F5 overexpression by knockdown of the gene.

Innovations and breakthroughs

This is the first evidence that E2F5 is relatively overexpressed in primary human HCC. Knockdown of E2F5 expression suppressed growth, invasion, and migration of HCC cell lines. Overexpression of E2F5 has been found to induce uncontrollable cell cycle progression, which may contribute to hepatocarcinogenesis.

Applications

Repressed proliferation of E2F5 silenced cells suggests that blocking this molecule could be used to decrease the growth of cancer cells in HCC patients. Expression of E2F5 can also be used as a diagnostic or prognostic marker in HCC.

Peer review

This is an interesting experimental study regarding the potential role of the transcription factor E2F5 in hepatocarcinogenesis.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

Blocking NF- κ B nuclear translocation leads to p53-related autophagy activation and cell apoptosis

Bao-Song Zhu, Chun-Gen Xing, Fang Lin, Xiao-Qing Fan, Kui Zhao, Zheng-Hong Qin

Bao-Song Zhu, Chun-Gen Xing, Kui Zhao, Department of General Surgery, The Second Affiliated Hospital, Soochow University, Suzhou 215006, Jiangsu Province, China

Fang Lin, Zheng-Hong Qin, Laboratory of Aging and Nervous Diseases, Soochow University School of Medicine, Suzhou 215123, Jiangsu Province, China

Xiao-Qing Fan, Department of General Surgery, The Second Hospital Of Jiaying City, Jiaying 314000, Jiangsu Province, China

Author contributions: Xing CG, Zhu BS and Qin ZH designed the research; Xing CG, Zhu BS, Fan XQ, Lin F and Qin ZH wrote the paper; Zhao K collected and analyzed data; Zhu BS selected the color figures in the paper; all authors contributed to the intellectual context and approved the final version.

Supported by Health Foundation of Jiangsu Province (H20 0719), the Higher Education Foundation of Jiangsu Province (08KJB320014), the Natural Science Foundation of Jiangsu Province (BK2008168), Suzhou High-Level Talents Project (2008-11) and the Science, Education and Health Foundation of Soochow City (SWKQ00814)

Correspondence to: Chun-Gen Xing, Professor, Department of General Surgery, The Second Affiliated Hospital, Soochow University, Suzhou 215006, Jiangsu Province, China. xingcg@126.com

Telephone: +86-512-67784107 Fax: +86-512-67784107

Received: August 21, 2010 Revised: October 20, 2010

Accepted: October 27, 2010

Published online: January 28, 2011

pression of light chain 3 (LC3). Mitochondrial membrane potential was measured using the fluorescent probe JC-1. Western blotting analysis were used to determine the expression of proteins involved in apoptosis and autophagy including p53, p53 upregulated modulator of apoptosis (PUMA), damage-regulated autophagy modulator (DRAM), LC3 and Beclin 1. We detected the effects of p53-mediated autophagy activation on the apoptosis of SGC7901 cells with the p53 inhibitor pifithrin- α .

RESULTS: The viability of SGC7901 cells was inhibited after SN50 treatment. Inductions in the expression of apoptotic protein p53 and PUMA as well as autophagic protein DRAM, LC3 and Beclin 1 were detected with Western blotting analysis. SN50-treated cells exhibited punctuate microtubule-associated protein 1 LC3 in immunoreactivity and MDC-labeled vesicles increased after treatment of SN50 by MDC staining. Collapse of mitochondrial membrane potential $\Delta\psi$ were detected for 6 to 24 h after SN50 treatment. SN50-induced increases in PUMA, DRAM, LC3 and Beclin 1 and cell death were blocked by the p53 specific inhibitor pifithrin- α .

CONCLUSION: The anti-tumor activity of NF- κ B inhibitors is associated with p53-mediated activation of autophagy.

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Key words: Nuclear factor- κ B; SN50; Autophagy; P53; Cell apoptosis

Peer reviewer: Dr. Hui-Kang Liu, PhD, Assistant Research Fellow, National Research Institute of Chinese Medicine, 155-1, Li-nung street section 2, Taipei 112, Taiwan, China

Zhu BS, Xing CG, Lin F, Fan XQ, Zhao K, Qin ZH. Blocking NF- κ B nuclear translocation leads to p53-related autophagy activation and cell apoptosis. *World J Gastroenterol* 2011; 17(4): 478-487 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/478.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.478>

Abstract

AIM: To investigate the anti-tumor effects of nuclear factor- κ B (NF- κ B) inhibitor SN50 and related mechanisms of SGC7901 human gastric carcinoma cells.

METHODS: MTT assay was used to determine the cytotoxic effects of SN50 in gastric cancer cell line SGC7901. Hoechst 33258 staining was used to detect apoptosis morphological changes after SN50 treatment. Activation of autophagy was monitored with monodansylcadaverine (MDC) staining after SN50 treatment. Immunofluorescence staining was used to detect the ex-

INTRODUCTION

Nuclear factor- κ B (NF- κ B) is an ubiquitously expressed family of Rel-related transcription factors^[1]. Typically, in unstimulated cells, NF- κ B is sequestered in the cytoplasm by binding to inhibitory κ B proteins (I κ B). In response to a variety of stimuli, such as inflammatory cytokines, oncogenes, and viruses, the proteasome-dependent degradation of I κ B allows the translocation of NF- κ B to the nucleus, where it binds to the promoter region of target genes involved in the control of different cellular responses, including apoptosis^[2-4]. In many cancer cells, the constitutive activation of NF- κ B activity lowers cell sensitivity to apoptotic stimuli and consequently favors neoplastic cell survival^[5].

The mammalian NF- κ B family contains 5 members: p50/p105 (NF- κ B1), p52/p100 (NF- κ B2), c-Rel, RelB, and p65 (RelA). These proteins are characterized by their Rel homology domains, which control DNA binding, dimerization and interactions with inhibitory factors known as I κ B proteins^[4,6]. NF- κ B is first discovered and studied as a major activator of immune and inflammatory function *via* its ability to induce expression of genes encoding cytokines, cytokine receptors, and cell-adhesion molecules^[4,7]. NF- κ B recently has been found to be linked to the control of cell growth and oncogenesis. The role of NF- κ B in cancer appears to be complex, but is likely to involve the ability of this transcription factor to control programmed cell death (PCD) and cell-cycle progression, and possibly cell differentiation, angiogenesis and cell migration. It has been reported that NF- κ B is activated in cancer cells by several chemotherapies and by radiation, and that in many cases this response inhibits the radiotherapy- and chemotherapy-induced cell death^[8].

Recent studies have suggested that there are three types of PCD: apoptosis (PCD I), autophagic cell death (PCD II) and necrosis (PCD III)^[9]. Autophagy is a genetically programmed, evolutionarily conserved process that degrades the long-living cellular proteins and organelles. Autophagy is important in normal development and response to changing environmental stimuli and, in addition to its role in cancer, and in numerous diseases, including bacterial and viral infections, neurodegenerative disorders, and cardiovascular diseases^[10]. Autophagy involves the formation of a double-membrane vesicle, which encapsulates cytoplasm and organelles and fuses with lysosomes, thus degrading the contents of the vesicle. The formation of the double-membrane vesicle is a complex process involving 16 autophagy-related proteins (Atg proteins). Two ubiquitin-like conjugation systems are involved in autophagy. These systems produce modified complexes of autophagic regulators (Atg8-PE and Atg5-Atg12-Atg16) that may determine the formation and size of the autophagosome. Nucleation, expansion, uncoating, and completion of the autophagosome then occur, priming it to fuse with lysosomes^[11].

The term "autophagic cell death" describes a form of programmed cell death morphologically distinct from apoptosis and presumed to result from excessive levels of cellular autophagy^[12]. In classical apoptosis, or type I programmed cell death, there is early collapse of

cytoskeletal elements but preservation of organelles until late in the process. In contrast, in autophagic, or type II, programmed cell death, there is early degradation of organelles but preservation of cytoskeletal elements until late stages. Whereas apoptotic cell death is caspase-dependent and characterized by internucleosomal DNA cleavage, caspase activation and DNA fragmentation occur very late (if at all) in autophagic cell death^[13]. In contrast with necrosis, both apoptotic and autophagic cell death are characterized by the lack of a tissue inflammatory response. The mitochondrion may integrate cell death signals and autophagy activation. Mitochondria generate apoptotic signals but can be removed by autophagy when they are damaged; therefore, mitochondria represents a nexus at which autophagy may interact with apoptosis pathways^[14].

The mutual regulation of NF- κ B and autophagy has been reported^[15]. Autophagy degrades nuclear shuttle protein-interacting kinase (NIK) and I κ B kinase (IKK), and inhibits NF- κ B activation, while NF- κ B depresses autophagy^[16]. We predict that activation of autophagy by blocking NF- κ B may contribute to the anti-tumor actions of NF- κ B inhibitors. We examined the effects of the nuclear import inhibitor SN50 on the activation of apoptosis and autophagy and the contribution of autophagy to the cytotoxic effects of SN50 in gastric cancer cell line SGC7901. The results showed that p53-dependent activation of apoptotic and autophagic pathways was induced by blocking the NF- κ B nuclear transport, and autophagic activation contributed to SN50-induced death of cancer cells.

MATERIALS AND METHODS

Reagents

SGC7901 gastric cancer cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China); RPMI1640 medium from Gibco (Rockville, MD, USA); fetal bovine serum from Hangzhou Sijiqing Biological Engineering Material Co., Ltd., (Hangzhou, China); L-glutamine, MTT from Sigma (St Louis, MO, USA); antibodies against p53 (1:500), p65 (1:500), PUMA (1:500), LC3 (1:200), Beclin1 (1:700) from Cell Signaling Technology (Beverly, MA, USA); and antibodies against DRAM (1:700) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Drug preparation

SN50 (BIOMOL, Plymouth Meeting, PA, USA) were diluted in distilled sterilization water to create a stock solution that was stored according to the manufacturer's suggestions. Pifithrin- α (Pft- α , Cell Signaling Technology, Beverly, MA, USA) was diluted in DMSO to create a stock solution that was stored according to the manufacturer's instructions. The final concentration of the SN50 solution used in the experiments was 18 μ mol/L, and that of Pft- α was 30 μ mol/L. This concentration of SN50 was selected on the basis of our pilot experiments on SGC7901 cells, and the concentration of Pft- α was selected following the manufacturer's suggestions.

Cell culture and viability assay

SGC7901 cells were maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum, 0.03% L-glutamine and incubated in a 5% CO₂ atmosphere at 37°C. Cells in a mid-log phase were used in experiments. Cell viability was assessed by MTT assay. To determine the time-course of response of SGC7901 cells to SN50, SGC7901 cells were plated into 96-well microplates (7×10^4 cells/well), and SN50 (18 μ mol/L) was added to the culture medium and cell viability was assessed with MTT assay 24, 48 and 72 h after drug treatment. MTT (Sigma, St Louis, MO, USA) solution was added to the culture medium (500 mg/L as a final concentration) for 4 h before the end of treatment, and the reaction was stopped by addition of 10% acidified SDS 100 μ L. The absorbance value (A) at 570 nm was read using an automatic multiwell spectrophotometer (Bio-Rad, Richmond, CA, USA). The percentage of cell death was calculated as follows: cell death (%) = $(1 - A \text{ of experiment well} / A \text{ of positive control well}) \times 100\%$.

Detection of mitochondrial potential ($\Delta\psi$)

Mitochondrial $\Delta\psi$ was determined using the KeyGEN Mitochondrial Membrane Sensor Kit (KeyGEN, Nanjing, China). The mitosensor dye aggregates in the mitochondria of healthy cells and emits red fluorescence against a green monomeric cytoplasmic background staining. However, in cells with a collapsed mitochondrial $\Delta\psi$, the dye cannot accumulate in the mitochondria and remains as monomers throughout the cells with green fluorescence^[17]. Briefly, SGC7901 cells were incubated with SN50 in 24-well plates for indicated times and then pelleted, washed with PBS, and resuspended in 0.5 mL of diluted mitosensor reagent (1 μ mol/mL in incubation buffer). After the cells were incubated with mitosensor reagent for 20 min, 0.2 mL incubation buffer was added and the cells were centrifuged and resuspended in 40 μ L incubation buffer. Finally, the cells were washed and resuspended in 1 mL phosphate buffered solution (PBS) for flow cytometric analysis.

Visualization of monodansylcadaverine-labeled vacuoles

Exponentially growing cells were plated onto 24-chamber culture slides, cultured for 24 h and incubated with the drug in 10% FCS/RPMI1640 for 6, 12 and 24 h. Autophagic vacuoles were labeled with monodansylcadaverine (MDC)^[18] (Sigma, St Louis, MO, USA) by incubating cells with 0.001 mmol/L MDC in RPMI1640 at 37°C for 10 min. After incubation, cells were washed three times with phosphate buffered solution (PBS) and immediately analyzed with a fluorescence microscope (Nikon Eclipse TE 300, Japan) equipped with a filter system (V-2A excitation filter: 380-420 nm, barrier filter: 450 nm). Images were captured with a CCD camera and imported into Photoshop.

Immunofluorescence staining LC3

SGC7901 cells were seeded onto 24-chamber culture slides and treated with SN50 (18 μ mol/L). After fixation in methanol for 10 min and blocked with a buffer containing 1% BSA 0.1% Triton X-100 for 1 h, cells were incubated

with either primary antibody against LC3 from Cell Signaling Technology (Beverly, MA, USA) diluted at 1:200 with PBS containing 1% bovine serum albumin (BSA) at 4°C overnight, and then incubated for 1 h with 1:500 secondary fluorescence conjugated antibodies (Sigma) to visualize the binding sites of the primary antibody under laser confocal microscope (Leica, Germany).

Hoechst 33258 staining

After treatment, cell cultures were washed twice with PBS and incubated with 2 μ mol/L Hoechst 33258 (Beyotime, Nantong, China) for 1 h in dark at 37°C. After washing thrice with PBS, the cells were viewed under a fluorescence microscope (Nikon, Tokyo, Japan) equipped with a UV filter. The images were recorded on a computer with a digital camera (DXM 1200, Nikon) attached to the microscope, and the images were processed by computer. The Hoechst reagent was taken up by the nuclei of the cells, and apoptotic cells exhibited a bright blue fluorescence.

Total cell and nuclear protein extraction and Western blotting analysis

For extraction of total cell proteins, cells were washed with pre-cooled PBS and subsequently lysed in pre-cooled RIPA lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L dithiothreitol (DTT), 0.25% sodium deoxycholate, 0.1% NP-40] containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 50 mmol/L sodium pyrophosphate, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, and protease inhibitors cocktail. Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4°C. For nuclear protein extraction, the Nuclear Protein Extraction Kit (KeyGEN, Nanjing, China) was used according to the manufacturer's instructions. Protein extraction from SGC7901 gastric cancer cells was performed as previously described. Protein concentration was determined with a Bradford protein assay kit. Proteins were resolved on 8.5% polyacrylamide gels and subsequently transferred onto nitrocellulose membranes. For immunoblotting, nitrocellulose membranes were incubated with specific antibodies recognizing target proteins overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody (1:3000) for 1 h at room temperature and subsequently analyzed by enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) and visualized by autoradiography. Protein β -actin (1:5000; Sigma) was used as loading controls.

Statistical analysis

All data were presented as mean \pm SD. Statistical analysis was carried out by ANOVA followed by a Dennett's test, and $P < 0.05$ was considered statistically significant.

RESULTS**Blocking NF- κ B nuclear translocation activates autophagy and induces cell apoptosis**

To confirm the blockade of NF- κ B p65 nuclear trans-

location by SN50, we prepared nuclear proteins from in SGC7901 cells treated with SN50 (18 μ mol/L) for 6-24 h and the nuclear p65 protein levels were measured with Western blotting analysis. The results showed that SN50 lowered the nuclear p65 levels in SGC7901 cells, suggesting that NF- κ B activity can be inhibited by SN50 (Figure 1A). The autofluorescence substance MDC has been recently shown to be a marker for late autophagic vacuoles (L-AVs) but not endosomes. To determine if SN50 treatment increases the formation of autophagosomes, SGC7901 cells were treated with SN50 and then incubated with MDC. MDC was trapped in acidic, membrane-rich organelles and also exhibited increased fluorescence quantum yield in response to the compacted lipid bilayers present in L-AVs^[19]. When cells are viewed under a fluorescence microscope, autophagic vacuoles (AVs) stained with MDC appeared as distinct dot-like structures distributed in the cytoplasm or localizing in the perinuclear regions. This study found that there was an increase in the number of MDC-labeled vesicles after treatment of SN50 for 6-24 h (Figure 1B).

Microtubule-associated protein 1 light chain 3 (LC3), the mammalian ontology of Atg8, targets to the autophagosomal membranes in an Atg5-dependent manner and remains there even after Atg12-Atg5 dissociates. LC3 is considered to be the only credible marker of the autophagosome in mammalian cells^[20]. The present study used immunofluorescence to detect the expression and localization of LC3. The results showed that SN50 induced punctuate distribution of LC3 immunoreactivity, and the formation of autophagosomes was enhanced by SN50 (Figure 1C). There are two forms of LC3, LC3-I and LC3-II. During formation of autophagosomes, cytoplasmic form LC3-I is cleaved and liquefied to give rise to membranous form LC3-II. To determine if SN50 increases the production of LC3-II, Western blotting analysis was used to detect the protein levels of LC3-I and LC3-II. The results showed that the levels of LC3, particularly LC3-II, increased, leading to an increased ratio of LC3-II/LC3-I after SN50 treatment (Figure 1D). Beclin 1 is an autophagy regulator and plays an important role in tumorigenesis and autophagic activation. Similar increases in Beclin 1 proteins were also detected after SN50 treatment (Figure 1E). Treatment with 18 μ mol/L SN50 for 6, 12 and 24 h in SGC7901 cells produced intense Hoechst-positive staining for condensed nuclei indicative of apoptosis. Significant increase in Hoechst staining was observed along with apoptosis when cells were treated with 18 μ mol/L SN50. The result indicated that SN50 activated autophagy and induced cell apoptosis (Figure 1F).

Blocking NF- κ B nuclear translocation induces p53 and its target proteins

Inhibition of NF- κ B has anti-tumor effects. To confirm if SN50 induced expression of pro-apoptotic proteins in SGC7901 cells, Western blotting analysis were used to detect the expression of p53 and its target proteins. The analyses revealed a robust increase in p53 protein levels in SGC7901 cells 6 h after SN50 treatment (Figure 2A).

PUMA is a p53 target protein involved in p53-mediated apoptosis. To determine if SN50 increases the expression of PUMA, protein levels of PUMA in SGC7901 cells were detected with Western blotting analysis. The results showed that PUMA was significantly increased after the treatment with SN50 (Figure 2B). Crichton *et al.*^[21] recently identified a new p53 target gene damage-regulated autophagic modulator (DRAM). DRAM is a lysosomal protein with six membrane-spanning regions. Its exogenous expression leads to the accumulation of autophagosomes. The previous studies found that DRAM is required for p53-induced apoptosis and DRAM-dependent autophagy acts upstream of cytochrome c release from mitochondria^[21,22]. The present study indicated that SN50 treatment also resulted in a significant increase in DRAM protein levels in SGC7901 cells (Figure 2C).

P53 inhibitor blocks SN50-induced autophagic activation and cell apoptosis

Pifithrin- α (Pft- α) was used as a specific inhibitor of signaling by the tumor suppressor protein p53^[23]. To determine the contribution of p53 to SN50-induced expression of pro-apoptotic and autophagic proteins and apoptosis of SGC7901 cells, the cells were pre-treated with the p53 specific inhibitor Pft- α 6 h before the addition of SN50. The results showed that when p53 was inhibited, the induction of PUMA and DRAM was inhibited (Figure 3A and B). Similarly, pft- α significantly decreased the SN50-induced upregulation of LC3-II and Beclin 1 in SGC7901 cells (Figure 3C and D).

SN50 inhibited SGC7901 viability in a time-dependent fashion. MTT assays revealed that after 24 h of treatment, the rate of inhibition had reached 25.31% \pm 4.13% at the highest dose of 18 μ mol/L used. When the incubation time was prolonged to 72 h, the inhibition rate rose up to 44.79% \pm 1.65% and after 48 h of treatment the rate was about 34.19% \pm 2.06% (Figure 3E). To evaluate the contribution of p53 to SN50-induced death of SGC7901 cells, the cells were pre-treated with the p53 specific inhibitor Pft- α 6 h before SN50. As shown in Figure 3F, Pft- α significantly attenuated the inhibitory effects of SN50 in a time-dependent manner. Mitochondria plays a central role in regulating cell death and survival. Diverse proapoptotic stimuli act on mitochondria, triggering mitochondrial membrane potential collapse, cytochrome c release and caspase activation. We detected collapse of mitochondrial membrane potential $\Delta\psi$ as early as 6 h after SN50 treatment. This change reached a peak 24 h after SN50 treatment (Figure 3G).

DISCUSSION

NF- κ B signaling pathways play critical roles in a variety of physiological and pathological processes. One function of NF- κ B is to promote cell survival through induction of target genes, whose products inhibit components of the apoptotic machinery in normal and cancerous cells. NF- κ B can also prevent programmed necrosis by inducing genes encoding antioxidant proteins. Regardless of mechanism,

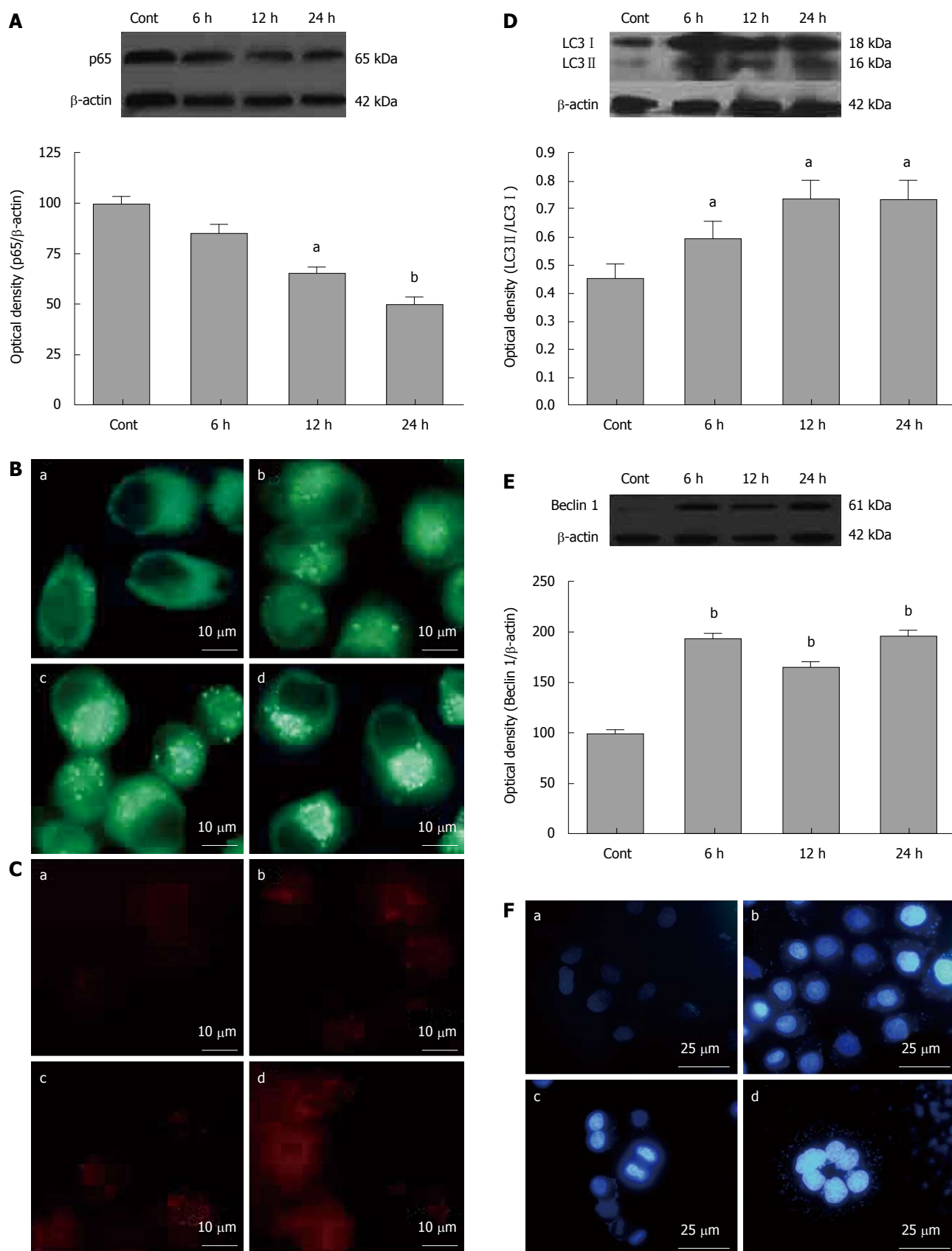


Figure 1 Blocking nuclear factor- κ B nuclear translocation activates autophagy and induces cell apoptosis. **A:** Western blotting analysis of the nuclear p65 in the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the nuclear protein levels of p65 after SN50 treatment for 6–24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) at various time points and were harvested for nuclear p65 protein levels. It indicates that SN50 may down-regulate the expression of nuclear p65. Values were given as mean \pm SE. Statistical comparisons were carried out with Dennett's test. ^a $P < 0.05$ and ^b $P < 0.01$ vs control ($n = 3$); **B:** Autophagy was activated after SN50 treatment. SGC7901 cells were incubated with SN50 (18 μ mol/L) and stained with MDC (100 μ mol/L). Fluorescence particles indicate L-AVs. (a) control, (b) 6 h, (c) 12 h, (d) 24 h after SN50 treatment ($\times 1000$) ($n = 3$); **C:** MAP1 LC3 expression and location in SGC7901 cells after treatment with SN50. Cells were treated

with SN50 (18 μ mol/L) for 6 h (b), 12 h (c) and 24 h (d), and observed under immunofluorescence microscope. (a) Control ($\times 1000$) ($n = 3$). SN50 increased the punctuate distribution of LC3 from 6 to 24 h; D: Western blotting analysis of the LC3 in the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the protein levels of LC3 after SN50 treatment for 6-24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) and were harvested for total proteins. It indicates that SN50 may up-regulate the expression of LC3 I and LC3 II. SN50 increased the ratio of LC3 II/LC3 I. Values were given as mean \pm SE. Statistical comparisons were carried out with Dennett's test. $^aP < 0.05$ vs control ($n = 3$); E: Western blotting analysis of the Beclin 1 in the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the protein levels of Beclin 1 after SN50 treatment for 6-24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) and were harvested for total proteins. It indicates that SN50 may up-regulate the expression of Beclin 1. Values were given as mean \pm SD. Statistical comparisons were carried out with Dennett's test. $^bP < 0.01$ vs control ($n = 3$); F: Hoechst 33258 staining showed apoptosis was induced after SN50 (18 μ mol/L) treatment. SGC7901 cells were incubated with SN50 (18 μ mol/L) and stained with Hoechst 33258 (10 mmol/L). Fluorescence particles showed apoptosis. (A) control, (B) 6 h, (C) 12 h, (D) 24 h after SN50 treatment ($\times 400$) ($n = 3$).

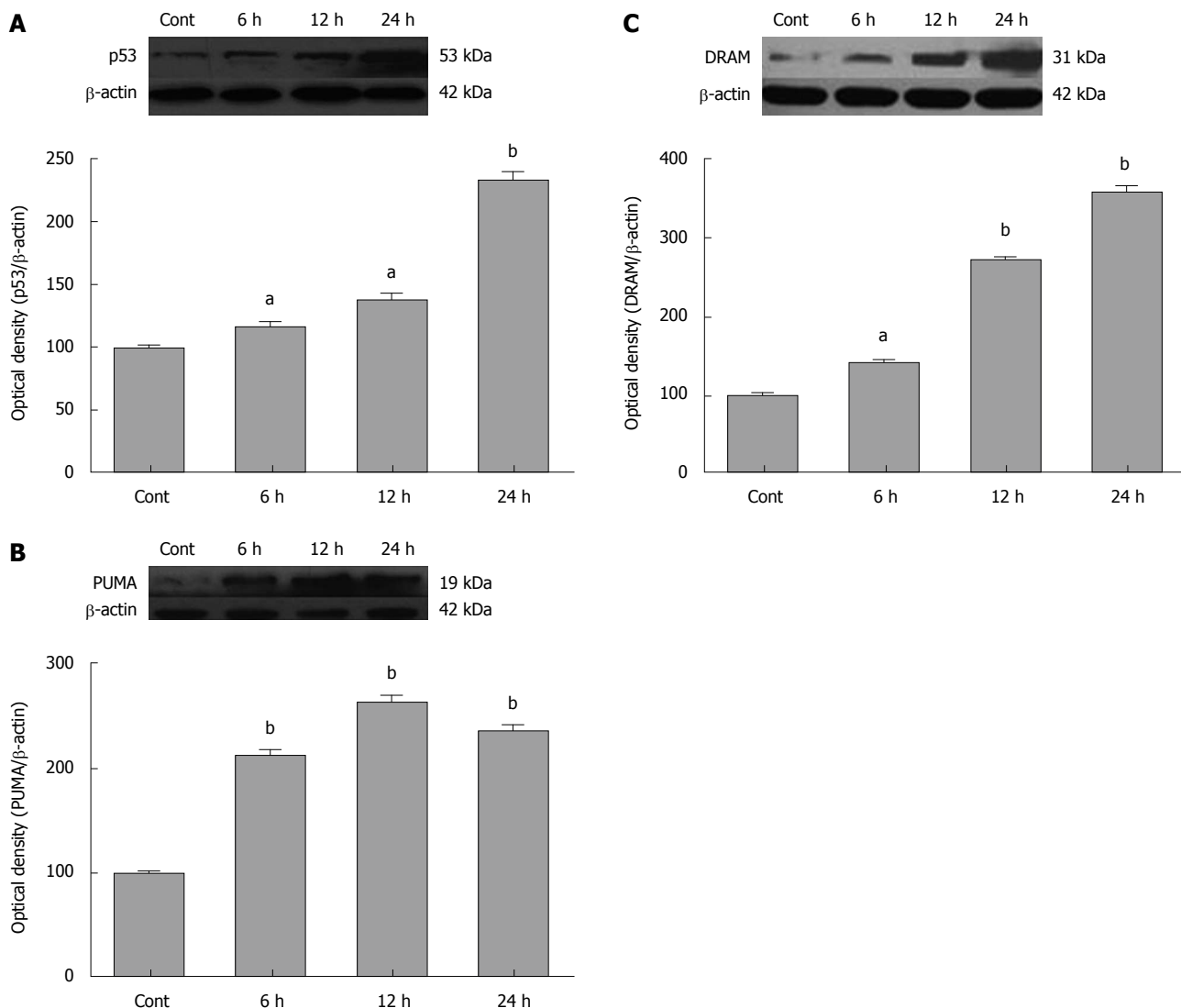
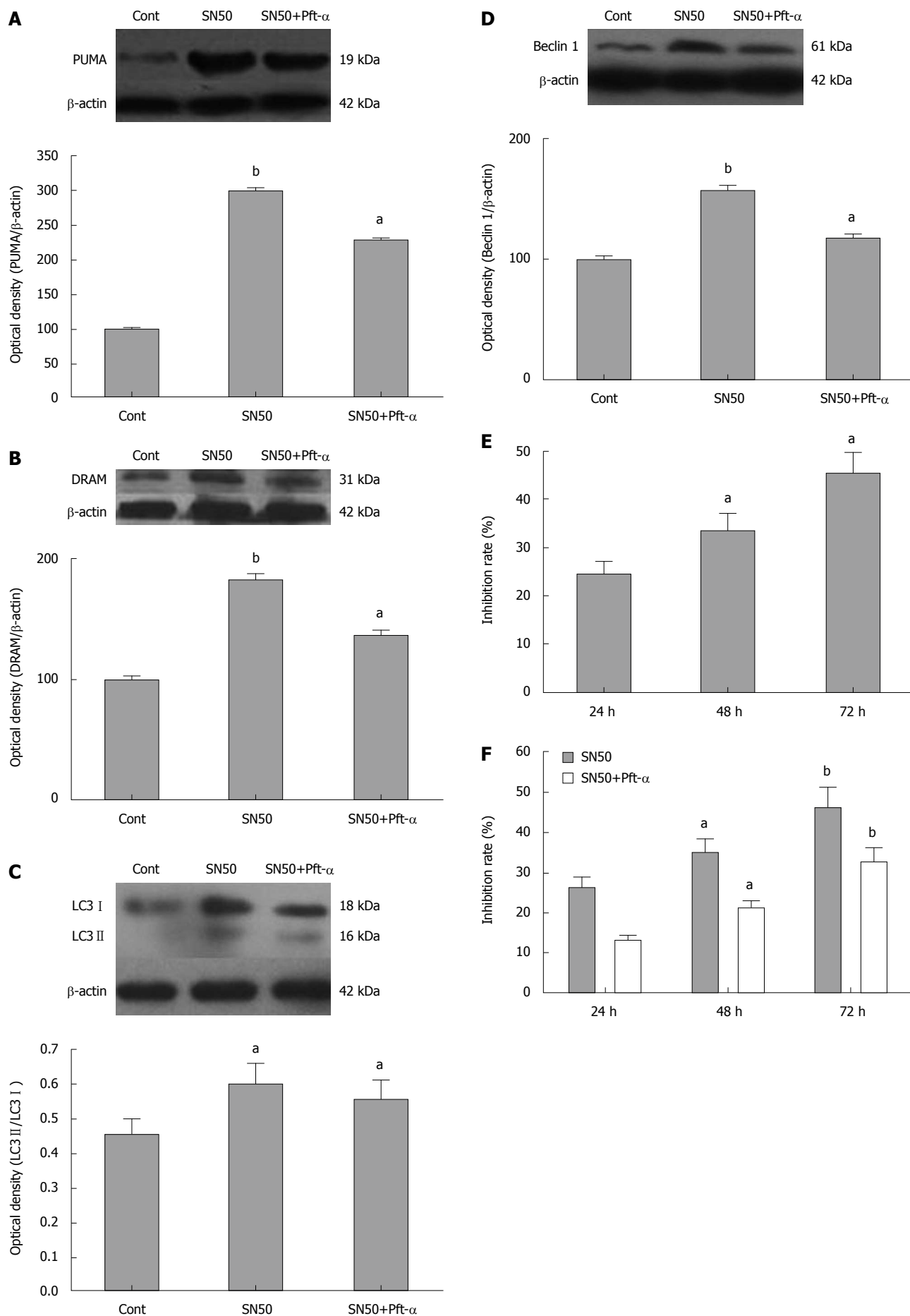


Figure 2 Blocking nuclear factor- κ B nuclear translocation induces p53 and its target proteins. A: Western blotting analysis of p53 of the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the protein levels of p53 after SN50 treatment for 6-24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) and were harvested for total proteins. It indicates that SN50 may up-regulate the expression of p53. Values were given as mean \pm SD. Statistical comparisons were carried out with Dennett's test. $^aP < 0.05$ and $^bP < 0.01$ vs control ($n = 3$); B: Western blotting analysis of P53 upregulated modulator of apoptosis (PUMA) of the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the protein levels of PUMA after SN50 treatment for 6-24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) and were harvested for total proteins. It indicates that SN50 may up-regulate the expression of PUMA. Values were given as mean \pm SD. Statistical comparisons were carried out with Dennett's test. $^bP < 0.01$ vs control ($n = 3$); C: Western blotting analysis of damage-regulated autophagy modulator (DRAM) of the control and SN50-treated SGC7901 cells. Western blotting analysis was used to detect the protein levels of DRAM after SN50 treatment for 6-24 h. SGC7901 cells were treated with SN50 (18 μ mol/L) and were harvested for total proteins. It indicates that SN50 may up-regulate the expression of DRAM. Values were given as mean \pm SD. Statistical comparisons were carried out with Dennett's test. $^aP < 0.05$ and $^bP < 0.01$ vs control ($n = 3$).

many cancer cells, of either epithelial or hematopoietic origin, use NF- κ B to achieve resistance to anticancer drugs and radiation. Hence, inhibition of NF- κ B activation offers a strategy for treatment of different malignancies and can induce apoptosis in gastric cancer SGC7901 cells.

NF- κ B plays an important role in proliferation and survival of tumor cells. NF- κ B binding sites have been identified in the promoter region of cyclin D1. NF- κ B promotes cyclin D1 expression and cell cycle progression^[24,25]. NF- κ B activity has been shown to inhibit activa-



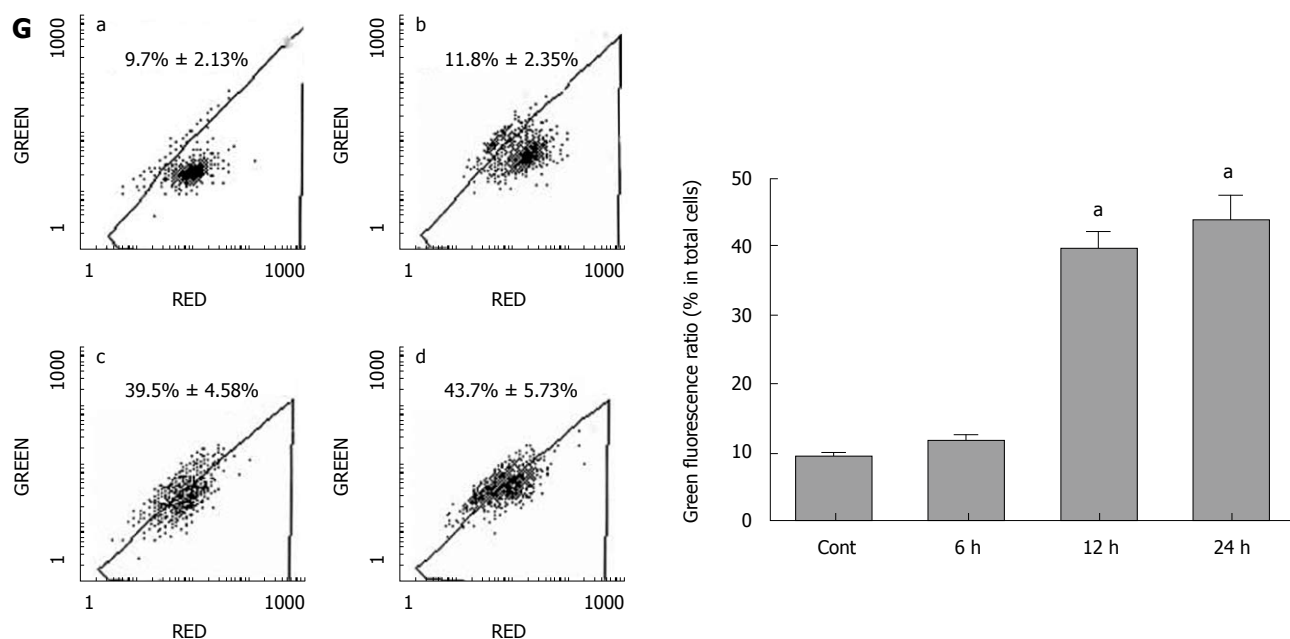


Figure 3 P53 inhibitor blocks SN50-induced autophagy activation and cell apoptosis. A: Effects of p53 inhibitor Pifithrin (Pft)- α on SN50-induced P53 upregulated modulator of apoptosis (PUMA) expression of SGC7901 cells. SGC7901 cells were pretreated with Pft- α (30 μ mol/L) 6 h before SN50 (18 μ mol/L). Protein expression was evaluated by Western blotting at 24 h. Results were presented as mean \pm SD of three independent experiments. ^a P < 0.05 and ^b P < 0.01 vs control group; B: Effects of p53 inhibitor Pft- α on SN50-induced damage-regulated autophagy modulator (DRAM) expression of SGC7901 cells. SGC7901 cells were pretreated with Pft- α (30 μ mol/L) 6 h before SN50 (18 μ mol/L). Protein expression was evaluated by Western blotting at 24 h. Results were presented as mean \pm SD of three independent experiments. ^a P < 0.05 and ^b P < 0.01 vs control group; C: Effects of p53 inhibitor Pft- α on SN50-induced LC3 expression of SGC7901 cells. SGC7901 cells were pretreated with Pft- α (30 μ mol/L) 6 h before SN50 (18 μ mol/L). Protein expression was evaluated by Western blotting at 24 h. Results were presented as mean \pm SD of three independent experiments. ^a P < 0.05 vs control group; D: Effects of p53 inhibitor Pft- α on SN50-induced Beclin 1 expression of SGC7901 cells. SGC7901 cells were pretreated with Pft- α (30 μ mol/L) 6 h before SN50 (18 μ mol/L). Protein expression was evaluated by Western blotting at 24 h. Results were presented as mean \pm SD of three independent experiments. ^a P < 0.05 and ^b P < 0.01 vs control; E: Reduced viability of SGC7901 cells after SN50 treatment. SGC7901 cells (7×10^4 cells/mL) were cultured with 18 μ mol/L of SN50 and cell viability was analyzed by MTT assay. MTT assays revealed that the inhibition rate reached 25.31% \pm 4.13%, 34.19% \pm 2.06%, 44.79% \pm 1.65% after treatment of SN50 (18 μ mol/L) 24, 48 and 72 h. Values were given as mean \pm SD of three independent experiments. ^a P < 0.05 and vs control; F: P53 inhibitor may attenuate the inhibitory effects of SN50-induced death of SGC7901 cells. SGC7901 cells were pretreated with Pft- α (30 μ mol/L) 6 h before SN50 treatment (18 μ mol/L). Cell viability was evaluated by MTT assay at 24, 48 and 72 h. Results were presented as mean \pm SD of three independent experiments. ^a P < 0.05 vs control, ^b P < 0.01 compared with SN50 alone treated group; G: Flow cytometric analysis of mitochondria membrane potential in the control and SN50-treated SGC7901 cells. Cells were treated with SN50 (18 μ mol/L) for 6, 12 and 24 h and were stained with JC-1 (5 μ mol/L) for 30 min. Values were given as mean \pm SD. Statistical analysis was carried out with ANOVA followed by Dunnett's *t* test. ^a P < 0.05 vs control.

tion of caspase-8, thus inhibiting the apoptosis initiation^[26]. Activation of NF- κ B confers resistance of tumor cells to radiochemotherapy-induced cytotoxicity^[27,28]. In contrast, various NF- κ B inhibitors inhibit tumor cell growth and induce cell death through apoptotic mechanisms^[29].

The tumor suppressor p53 plays a central role in sensing various genotoxic stresses. Our results showed that blocking NF- κ B with SN50 induced expression of p53 remarkably. P53 downstream point such as the apoptosis gene PUMA was up-regulated after the treatment of SN50. P53 is known to play an important role in apoptosis by regulating expression of pro-apoptotic proteins. PUMA is one of p53 target genes involved in apoptosis. The activation of PUMA by DNA damage is dependent on p53 and is mediated by the direct binding of p53 to the PUMA promoter region^[30]. PUMA plays an essential role in p53-dependent and -independent apoptosis induced by a variety of stimuli^[31,32]. Here, we demonstrated that the inhibitor of NF- κ B p65 nuclear import, SN50, significantly up-regulated the levels of PUMA, indicating that apoptosis may be triggered by SN50. In supporting this notion, we found that mitochondria membrane potential was collapsed after SN50 treatment. Mitochondria plays a

central role in regulating cell death and survival. Diverse proapoptotic stimuli act on mitochondria, triggering mitochondrial membrane potential collapse, cytochrome c release and caspase activation. The mitochondrial permeability transition (MPT) represents an important event initiating apoptotic cell death.

Increasing evidence suggests that autophagy plays an important role in tumor cell growth, differentiation and response to anti-tumor drugs^[33]. Many classical anti-tumor drugs have been found to exert their cytotoxic actions by autophagic mechanisms^[34-36]. It has been suggested that autophagic death may play a role in both physiological and pathological cell death. This issue has been addressed by some recent reviews^[32,37]. In the present study, inhibitor of NF- κ B p65 nuclear import with SN50 resulted in a significant increase in the levels of DRAM, a newly identified p53 target gene involved in autophagy activation and cell death^[21]. We also found that SN50 increased the expression of LC3, Beclin 1, particularly the production of LC3-II. LC3 is an autophagosomal ortholog of yeast Atg8. LC3 has been best characterized as an autophagosomal marker in mammalian autophagy, and the levels of LC3 may also reflect the levels of autophagy^[38]. Beclin 1

is the mammalian orthologue of the yeast ATG6-Vps30 gene. It can complement the defect in autophagy present in ATG6^{-/-} yeast strains and stimulate autophagy when overexpressed in mammalian cells^[13]. Beclin 1 is monoallelically deleted in human breast and ovarian cancers and is expressed at reduced levels in those tumors^[13,39]. The present results suggest that autophagy is induced by SN50 and its activation may contribute to anti-tumor effects of NF- κ B inhibitors.

The evidence has shown that p53 is not only involved in apoptosis but also in autophagy. The present study investigated if p53 signaling plays an essential role in autophagy activation and cell death induced by NF- κ B inhibition. P53 signaling was blocked with a specific inhibitor pft- α , the compound has been shown to selectively inhibit p53 activity and p53-mediated apoptosis *in vitro* and *in vivo*^[40]. The results demonstrated that pft- α blocked SN50-induced increases in PUMA, LC3-II and Beclin 1. Moreover, SN50-induced cell death was significantly attenuated by pft- α . These data suggest that p53 mediates activation of apoptosis and autophagy and cell death following blockade of NF- κ B. This study shed new lights on elucidating molecular mechanisms of anti-tumor actions of NF- κ B inhibitors.

In summary, the present study revealed that a new mechanism associated with NF- κ B inhibition triggered impairment of cell proliferation and induction of apoptosis of cancer cells. Blocking NF- κ B increases expression of p53, induces pro-apoptotic and autophagic proteins. P53 contributes to NF- κ B inhibitor-induced apoptosis of cancer cells by activating autophagic mechanisms. Further investigation of the relationship between autophagy activation and anti-tumor effects of NF- κ B inhibitors will unveil new strategies for tumor therapy.

COMMENTS

Background

Nuclear factor- κ B (NF- κ B) signaling pathways play critical roles in a variety of physiological and pathological processes. The authors predicted that activation of autophagy by blocking NF- κ B may contribute to the anti-tumor actions of NF- κ B inhibitors.

Research frontiers

SN50 is a specific inhibitor of NF- κ B p65. The anti-tumor activity of SN50 might be related to the induction of apoptosis of tumor cells, but the precise mechanism of its anti-tumor activity is not well understood.

Innovations and breakthroughs

Blocking NF- κ B increases the expression of p53, and induces pro-apoptotic and autophagic proteins. P53 contributes to the NF- κ B inhibitor-induced apoptosis of cancer cells through both apoptotic and autophagic mechanisms. Further investigation of the relationship between autophagy activation and anti-tumor effects of NF- κ B inhibitors will unveil new strategies for tumor therapy.

Applications

Blocking NF- κ B increases expression of p53, induces pro-apoptotic and autophagic proteins. P53 contributes to NF- κ B inhibitor-induced apoptosis of cancer cells by activating autophagic mechanisms. And it will provide new idea for tumor treatment.

Terminology

NF- κ B: The NF- κ B comprises a family of transcription factors involved in the regulation of a wide variety of biological responses. SN50: SN50 is a kind of NF- κ B p65 nuclear translocation inhibitor. Autophagy: Autophagy is a general term for the degradation of cytoplasmic components within lysosomes. There are three

types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, and the term "autophagy" usually indicates macroautophagy.

Peer review

The authors examined the effects of the nuclear import inhibitor SN50 on activation of apoptosis and autophagy and the contribution of autophagy to cytotoxic effects of SN50 in gastric cancer cell line SGC7901. The results showed that blocking NF- κ B nuclear transport leads to p53-dependent activation of apoptotic and autophagic pathways, and autophagy activation contributes to SN50-induced death of cancer cells.

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S- Editor Sun H L- Editor Ma JY E- Editor Ma WH

Association between *EGF* +61A/G polymorphism and gastric cancer in Caucasians

Ana Paula Araújo, Bruno M Costa, Ana L Pinto-Correia, Maria Fragoso, Paula Ferreira, Mário Dinis-Ribeiro, Sandra Costa, Rui M Reis, Rui Medeiros

Ana Paula Araújo, Ana L Pinto-Correia, Paula Ferreira, Rui Medeiros, Molecular Oncology Group, Portuguese Institute of Oncology of Porto, 4200-072 Porto, Portugal
Bruno M Costa, Sandra Costa, Rui M Reis, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, 4704-553 Braga, Portugal

Maria Fragoso, Oncology Department, Portuguese Institute of Oncology of Porto, 4200-072 Porto, Portugal

Mário Dinis-Ribeiro, Gastroenterology Department, Portuguese Institute of Oncology of Porto, Portugal; and Cintesis/Faculty of Medicine University of Porto, 4200-072 Porto, Portugal

Rui Medeiros, Centro de Estudos em Biomedicina, Health Sciences Faculty-Fernando Pessoa University, 4200-150 Porto, Portugal

Rui Medeiros, Instituto de Ciencias Biomédicas de Abel Salazar, Abel Salazar Institute for the Biomedical Sciences, 4099-003 Porto, Portugal

Author contributions: Araújo AP, Costa BM, Costa S performed the research; Araújo AP and Medeiros R wrote the paper; Fragoso M and Ferreira P identified and diagnosed the patients; Medeiros R and Dinis-Ribeiro M designed, supervised and participated in the editing of the manuscript; all authors read and approved the final manuscript.

Supported by The Portuguese League Against Cancer (Liga Portuguesa Contra o Cancro-Núcleo Regional do Norte) and AstraZeneca Foundation

Correspondence to: Rui Medeiros, Professor, Molecular Oncology Group, Portuguese Institute of Oncology of Porto, Edifício Laboratórios-PISO 4, Rua. Dr Ant. Bernardino Almeida, 4200-072 Porto, Portugal. ruimedei@ipporto.min-saude.pt
Telephone: +351-22-5084000 Fax: +351-22-5084001

Received: January 26, 2010 Revised: April 30, 2010
Accepted: May 7, 2010

Published online: January 28, 2011

type *EGF* +61 in 207 patients with gastric lesions (162 patients with gastric adenocarcinomas, 45 with atrophy or intestinal metaplasia) and 984 controls. All subjects were Caucasian.

RESULTS: Genotype distribution was 23.5% for GG and 76.5% for GA/AA in the control group, 18.4% for GG and 68.6% for GA/AA in the entire group with gastric lesions and 17.9% for GG and 82.1% for GA/AA in the group with gastric adenocarcinoma. No statistically significant associations were found between *EGF* +61 variants and risk for developing gastric cancer [odds ratios (OR) = 1.41, 95% confidence intervals (CI): 0.90-2.21, $P = 0.116$]. However, the stratification of individuals by gender revealed that males carrying A alleles (*EGF* +61A/G or AA) had an increased risk for developing gastric cancer as compared to GG homozygous males (OR = 1.55, 95% CI: 1.05-2.28, $P = 0.021$).

CONCLUSION: In summary, we found that males who were A carriers for *EGF* +61 had an increased risk for developing gastric cancer. This result may be explained by the suggestion that women secrete less gastric acid than men.

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Key words: Epidermal growth factor polymorphism; Epidermal growth factor receptor; Gastric cancer

Peer reviewers: Ralph Graeser, PhD, Group Leader, Molecular and Cellular Biology, ProQinase GmbH, Breisacher Str. 117, Freiburg, 79106, Germany; Raquel Almeida, MS, Institute of Molecular Pathology and Immunology, University of Porto, Rua Dr Roberto Frias s/n, Porto 4200, Portugal; Guida Portela-Gomes, MD, PhD, Professor, Faculty of Medicine, University of Lisbon, Rua Domingos Sequeira-128, Estoril 2765-525, Portugal

Araújo AP, Costa BM, Pinto-Correia AL, Fragoso M, Ferreira P, Dinis-Ribeiro M, Costa S, Reis RM, Medeiros R. Association between *EGF* +61A/G polymorphism and gastric cancer in Cau-

Abstract

AIM: To investigate the association between epidermal growth factor (*EGF*) +61A/G polymorphism and susceptibility to gastric cancer, through a cross-sectional study.

METHODS: Polymerase chain reaction restriction fragment length polymorphism analyses were used to geno-

casians. *World J Gastroenterol* 2011; 17(4): 488-492 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/488.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.488>

INTRODUCTION

Growth factors activate the complex processes of cellular signalling, promoting cell changes^[1,2]. They are “positive signals” in the cell and are regulated by “negative signals” which control amplitude and duration^[2]. The balance between these signals is all-important in cell homeostasis^[2]. Epidermal growth factor (EGF) performs a key role in promoting cell survival^[3]. After binding to its receptor (EGFR), it induces a signalling cascade that culminates in change of gene transcription^[3-5]. EGFR signalling is not only important in cellular proliferation but also contributes to several other cellular processes involved in cancer progression, including angiogenesis, metastatic spread, and inhibition of apoptosis^[6].

EGFR (also known as ErbB1) belongs to the family of ErbB (from avian erythroblastic leukaemia viral oncogene homologue) receptors which are involved in the development of several human cancers^[7]. The increase in EGFR signalling may be caused by overexpression of EGFR, increased concentration of ligand(s) (through autocrine/paracrine processes), the presence of aberrant receptors due to gene alteration, and alterations in molecules that control receptor signalling output^[6,8]. EGFR and ErbB-2 are frequently overexpressed in gastric carcinomas^[9].

EGF gene has a polymorphism in position +61 which consists of the substitution of adenine (A) for guanine (G). AA genotype carriers have lower levels of EGF expression than individuals with the GG or AG genotypes^[10]. Ethnic differences in the distribution of the EGF gene have been reported, and several studies have been carried out regarding the role of EGF genotypes in susceptibility to gastric cancer in Asian populations and in other organs.

In this cross-sectional study we analysed the association between this EGF polymorphism and the risk for gastric cancer in a high incidence Caucasian population.

MATERIALS AND METHODS

Subjects

This cross-sectional study was performed in 1191 individuals, including 207 patients histologically diagnosed with gastric lesions followed at the Portuguese Institute of Oncology-Porto (IPO-P), and a control group of 984 individuals without cancer disease history, all from the northern region of Portugal. All individuals provided informed consent according to the Declaration of Helsinki, and the patients in both groups were of Caucasian ethnicity.

Patients were further divided according to the type of lesions at histopathological diagnosis following multiple endoscopic biopsies. Patients included those who displayed lesions such as high-grade dysplasia and intestinal-type invasive gastric adenocarcinoma ($n = 162$) and patients with non-dysplastic lesions associated with gastric

adenocarcinoma such as atrophy or intestinal metaplasia ($n = 45$), who had received standardized follow-up since 2001. The group of patients with gastric adenocarcinoma included 73 females and 89 males (55%) with a median age at diagnosis of 54 years (mean 54.3 years, standard deviation 11.8 years), and the group of patients with atrophy or intestinal metaplasia included 25 females and 20 males (44%) with a median age at diagnosis of 59 years (mean 59.7 years, standard deviation 10.8 years).

The control subjects included 524 females and 460 males (46.7%) randomly recruited from the Blood Donor Bank of IPO-P and Hospital de S. Marcos, Braga, and had no current or history of neoplastic disease. The median age was 45 years (mean 46.2 years, standard deviation 11.1 years).

DNA was extracted from peripheral blood samples obtained by a standard venipuncture technique using EDTA-containing tubes, as previously described in studies from our group^[11,12].

EGF +61A/G genotype analysis

The +61A/G polymorphism was genotyped by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis, as previously described^[10]. Briefly, amplification was carried out in a 50 μ L reaction mixture containing: 1 \times Taq Buffer, 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTPs, 0.3 μ mol/L of each primer and 1U Taq DNA polymerase. The cycling conditions were: 95°C for 5 min, followed by 35 amplification cycles at 94°C for 60 s, 55°C for 60 s and 72°C for 60 s, followed by one elongation step at 72°C for 5 min. A 242 base pair (bp) fragment was amplified using primers: F-5'TGTCACCTAAAGGAAAGGAGGT3' and R-5'TTCACAGAGTTTAACAGCCC3'. The A61G variation was identified with the restriction enzyme *Afu I*. Two units of restriction enzyme were added to 10 μ L of PCR products in a final volume of 15 μ L. The incubation was performed at 37°C overnight. The products were separated on 3% agarose gels with 0.5% ethidium bromide and photographed under UV illumination.

After destruction of the recognition site by the restriction enzyme, the A allele produced 4 fragments: 15, 34, 91 and 102 bp, while the G allele produced 3 fragments: 15, 34 and 193 bp. In the gel only fragments 91, 102 and 193 bp were visible.

Statistical analysis

The computer software SPSS for Windows (version 15.0) and Epi Info (version 6.04) were used for all statistical analyses. The χ^2 test was used to compare differences between categorical variables, and verify that the observed allele distribution in the control group was in Hardy-Weinberg equilibrium. A 5% level of significance was used in the analysis. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess the relationship between the polymorphic variants and gastric lesions.

RESULTS

The frequencies of EGF genotypes in the gastric lesions

Table 1 Associations between *EGF* +61A/G variants and clinicopathological parameters in patients with gastric lesions¹ *n* (%)

	Cases	Controls	OR (95% CI)	<i>P</i>
Gastric lesions ¹ (<i>n</i> = 207)				
Genotypes				
GG	38 (18.4)	231 (23.5)	1	
GA	104 (50.2)	449 (45.6)	1.41 (0.92-2.15)	0.096
AA	65 (31.4)	304 (30.9)	1.30 (0.82-2.06)	0.237
GA + AA	169 (68.6)	753 (76.5)	1.36 (0.92-2.04)	0.109
Atrophy or intestinal metaplasia (<i>n</i> = 45)				
Genotypes				
GG	9 (20.0)	231 (23.5)	1	
GA	20 (44.4)	449 (45.6)	1.44 (0.49-2.76)	0.743
AA	16 (35.6)	304 (30.9)	1.35 (0.55-3.37)	0.478
GA + AA	36 (80.0)	753 (76.5)	1.23 (0.56-2.78)	0.590
Gastric adenocarcinoma (<i>n</i> = 162)				
Genotypes				
GG	29 (17.9)	231 (23.5)	1	
GA	84 (51.9)	449 (45.6)	1.49 (0.93-2.40)	0.082
AA	49 (30.2)	304 (30.9)	1.28 (0.77-2.16)	0.317
GA + AA	133 (82.1)	753 (76.5)	1.41 (0.90-2.21)	0.116

¹Gastric lesions: atrophy or intestinal metaplasia and gastric adenocarcinoma. OR: Odds ratios; CI: Confidence intervals.

group and the control group are presented in Table 1. The frequencies were 23.5% for GG and 76.5% for GA/AA in the control group, 18.4% for GG and 68.6% for GA/AA in the entire group with gastric lesions and 17.9% for GG and 82.1% for GA/AA in the group with gastric adenocarcinoma. As shown in Table 1, no increased risk of developing non-tumor gastric lesions ($P = 0.109$) or gastric adenocarcinoma ($P = 0.116$) was found for individuals who carried the A allele.

When adjustments were made for allele genotype and sex, we found that males who were A carriers had an increased risk of developing gastric cancer in comparison to females (OR = 1.55, 95% CI: 1.05-2.28, $P = 0.021$) (Table 2).

No other associations were found for the other characteristics tested.

DISCUSSION

The EGF protein is a growth factor that activates signal transduction pathways promoting proliferation, migration and differentiation^[13]. In particular, EGF is involved in regulating proliferation of mucosal cells in the gastrointestinal tract, stimulating mucus production, and inhibiting gastric secretion^[14,15]. However, in gastric cancer, EGF displays oncogenic properties^[16,17], and its overexpression is correlated with deep invasion, advanced malignancy stage, and poor patient prognosis^[18].

In this cross-sectional study, we analysed the association between a functional polymorphism of the *EGF* gene (+61 A/G) and the risk for developing gastric cancer. Our data showed a statistically significant increased risk for developing gastric cancer in males who were A carriers (OR = 1.55, 95% CI: 1.05-2.28, $P = 0.021$); however, no statistically significant differences were found

Table 2 Associations between *EGF* +61A/G variants and gender

		Controls	Cases	OR (95%CI)	<i>P</i>
GG	M	117	14	1	
	F	114	15	1.04 (0.45-2.41)	0.913
AA/AG	M	343	75	1	
	F	410	58	0.65 (0.44-0.95)	0.021

OR: Odds ratios; CI: Confidence intervals.

when the entire cancer group was considered (male and female gastric adenocarcinoma patients, $P = 0.116$).

Salomon *et al*^[9] reported that EGFR is overexpressed in 33% of gastric adenocarcinomas, compared to only 3.8% in the early stages of gastric carcinoma development or in non-malignant specimens. Nevertheless, EGFR expression was more frequent in well-differentiated advanced stage adenocarcinomas, and EGFR immunoreactivity was significantly higher in tumor stages III and IV as well as in metastatic carcinomas^[9]. EGF and EGFR are expressed at a frequency of 42% and 41%, respectively, in poorly differentiated gastric carcinomas, and most frequently in tumors greater than 6 cm in size^[9]. Although EGF and EGFR are associated with poor prognosis, less than half of gastric tumors have expression or overexpression of these proteins.

In our study, the most interesting result was the increased risk for gastric cancer in male patients. A sexual dimorphism in gastric acid secretion has been reported, with females secreting less gastric acid (approximately 40%) than males^[19]. The mechanisms mediating this difference are unknown, but a role for oestrogens has been suggested which may inhibit gastric acid secretion through two oestrogen receptor (ER) subtypes present in the stomach^[19]. EGF decreases FSH (follicle-stimulating hormone) and particularly inhibits the expression of hormones produced in the ovary (oestrogen and progesterone), acting in the evolution of ovarian follicles^[20-26]. Therefore, in females with a lower expression of EGF (A carriers) we may consider that stomach cells may receive less information to proliferate. Nevertheless, in G carriers (with greater expression of EGF), the differences between genders are not significant, and according to our previous studies it is proposed that EGFR expression may be lower in G carriers, because EGF is involved in internalization of EGFR^[27,28]. However, more work is required to elucidate the correct mechanism.

Others studies regarding the association between gastric cancer and *EGF* polymorphism in Asian populations have been reported^[29-31]. Hamai *et al*^[29] and Jin *et al*^[30] associated A carriers with a lower risk of developing gastric cancer. However, Goto *et al*^[31] did not find any differences between this polymorphism and gastric cancer. When analyzing the frequency of *EGF* +61 genotypes among these reports, one can observe that Asians present a significant difference in comparison to Caucasians (our study), namely in AA and GG genotypes (Table 3). In our results, the frequency of EGF genotypes in the control

Table 3 Genotype distribution of *EGF* +61A/G polymorphism in different control populations reported in different case-control studies *n* (%)

	<i>n</i>	Genotype		
		AA	AG	GG
Asia				
Jin <i>et al</i> ^[30]	660	57 (8.6)	289 (43.8)	314 (47.6)
Goto <i>et al</i> ^[31]	454	47 (10.4)	188 (41.8)	215 (47.8)
Hamai <i>et al</i> ^[29]	230	25 (10.9)	97 (42.1)	108 (47.0)
Europe				
Portugal (our data)	984	304 (30.9)	449 (45.6)	231 (23.5)
Shahbazi <i>et al</i> ^[10]	99	32 (32.3)	47 (47.5)	20 (20.2)
McCarron <i>et al</i> ^[32]	310	121 (39.0)	131 (42.3)	58 (18.7)
Costa <i>et al</i> ^[34]	570	173 (30.3)	266 (46.7)	131 (23.0)
Australia				
James <i>et al</i> ^[33]	2646	883 (33.4)	1317 (49.8)	446 (16.9)

population was in agreement with other published studies with Caucasians populations^[10,32-34]. Two recent meta-analyses have discussed the role of *EGF* polymorphism in susceptibility to cancer^[35,36]. Future studies may confirm these results with adjustment for non-genetic putative risk factors for gastric cancer (ex: smoking, alcohol consumption, social class or *H. pylori*).

In conclusion, we found that female patients who were A carriers of *EGF* +61A/G had a decreased risk of developing gastric cancer. Furthermore, it has been suggested that women secrete less gastric acid than men^[19], which is consistent with our hypothesis that different effects of *EGF* +61A/G variants may occur in males and females in relation to gastric cancer risk.

COMMENTS

Background

Epidermal growth factor (EGF) performs a key role in promoting cell survival. After binding to its receptor (EGFR), it induces a signalling cascade that culminates in change of gene transcription. EGFR signalling is not only important in cellular proliferation but also contributes to several other cellular processes involved in cancer progression, including angiogenesis, metastatic spread, and inhibition of apoptosis. EGFR (also known as ErbB1) belongs to the family of ErbB (from avian erythroblastic leukaemia viral oncogene homologue) receptors which are involved in the development of several human cancers. *EGF* gene has a polymorphism in position +61 which consists of the substitution of adenine (A) for guanine (G). AA genotype carriers have lower levels of EGF expression than individuals with the GG or AG genotypes. In this cross-sectional study, the authors analysed the associations between this *EGF* polymorphism and the risk for gastric cancer in a high incidence Caucasian population.

Research frontiers

Although several gene loci have been associated with susceptibility to gastric cancer, the aetiology of gastric cancer is still unknown. The current study is the first to assess the impact of *EGF* gene polymorphisms and disease susceptibility in gastric cancer in a southern European Caucasian population.

Innovations and breakthroughs

It is important to investigate the genetic variation in susceptibility to gastric cancer and to identify markers that will facilitate identification of individuals at risk of developing this disease. The results suggest that the stratification of individuals by gender revealed that males carrying A alleles (*EGF* +61A/G or AA) have an increased risk of developing gastric cancer as compared to GG homozygous males.

Applications

The results of this study will help us to further understand the genetic determinants of gastric cancer.

Peer review

This population based case-control study that adds to the existing evidence for a role of *EGF*/EGFR in pathogenesis of gastric carcinoma. The validity of the control population may be mildly contentious. The findings raise questions regarding the role of gender and sex hormones in gastric carcinoma. This is a small study with somewhat limited results, however it is well done, with a good sample size and with a good interpretation for the results observed. It can be relevant to the field.

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S- Editor Wang JL L- Editor Webster JR E- Editor Ma WH

Long-term outcome of chronic hepatitis C patients with sustained virological response to peginterferon plus ribavirin

María Trapero-Marugán, Jorge Mendoza, María Chaparro, Leticia González-Moreno, José Andrés Moreno-Monteagudo, María Jesús Borque, Ricardo Moreno-Otero

María Trapero-Marugán, Jorge Mendoza, María Chaparro, Leticia González-Moreno, José Andrés Moreno-Monteagudo, María Jesús Borque, Ricardo Moreno-Otero, Gastroenterology-Hepatology Department, University Hospital La Princesa and Princesa Research Institute, Autonomous University of Madrid, and CIBERehd (Centro de Investigación Biomédica en Red en Enfermedades Hepáticas y Digestivas), 28006 Madrid, Spain

Author contributions: Trapero-Marugán M and Moreno-Otero R contributed equally to this work; Trapero-Marugán M and Moreno-Otero R designed the research, analyzed the data, and wrote the paper; Trapero-Marugán M, Mendoza J, Chaparro M, González-Moreno L, Moreno-Monteagudo JA, Borque MJ, Moreno-Otero R performed the research.

Supported by Instituto de Investigación La Princesa and CIBERehd from Instituto de Salud Carlos III, Madrid, Spain

Correspondence to: Dr. María Trapero-Marugán, Gastroenterology-Hepatology Department, University Hospital La Princesa and Princesa Research Institute, Autonomous University of Madrid, and CIBERehd, C/Diego de León, 62, Third Floor, 28006 Madrid, Spain. mtraperomarugan@hotmail.com

Telephone: +34-91-3093911 Fax: +34-91-4022299

Received: May 22, 2010 Revised: October 7, 2010

Accepted: October 14, 2010

Published online: January 28, 2011

Abstract

AIM: To assess the clinical, biochemical and virological long-term outcome in chronic hepatitis C (CHC) patients with a sustained virological response (SVR) after peginterferon (PEG-IFN) plus ribavirin combination therapy.

METHODS: One hundred and fifty three patients with a SVR after treatment with PEG-IFN plus ribavirin were included in a 5-year follow-up study in a single Spanish center, based on standard clinical practice. Clinical anamnesis, biochemical analysis, hepatitis C virus RNA and alpha-fetoprotein measurement, ultrasonography and transient elastography were performed annually.

RESULTS: The mean follow-up period of the 153 patients was 76 ± 13 mo after they obtained a SVR. Five

patients (3.26%) presented with cirrhosis before treatment and 116 (75.8%) had genotype 1. No patient showed evidence of hepatic decompensation. One patient (0.65%) developed a hepatocellular carcinoma at month 30 after achieving SVR. There were no virological relapses during this follow-up period. Persistently elevated alanine aminotransferase was found in only one patient (0.65%). At the end of the 5-year follow-up, the mean value of transient elastography was 7 ± 4.3 kPa (F1). There were no deaths and no other tumors.

CONCLUSION: The long-term outcome of 153 CHC patients with SVR to PEG-IFN plus ribavirin was good. No evidence of a virological relapse was seen. One patient (0.65%) developed a hepatocellular carcinoma.

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Key words: Chronic hepatitis C; Peginterferon; Ribavirin; Sustained virological response; Long-term effects

Peer reviewer: Chifumi Sato, Professor, Department of Analytical Health Science, Tokyo Medical and Dental University, Graduate School of Health Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo 1138519, Japan

Trapero-Marugán M, Mendoza J, Chaparro M, González-Moreno L, Moreno-Monteagudo JA, Borque MJ, Moreno-Otero R. Long-term outcome of chronic hepatitis C patients with sustained virological response to peginterferon plus ribavirin. *World J Gastroenterol* 2011; 17(4): 493-498 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/493.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.493>

INTRODUCTION

The combination of peginterferon (PEG-IFN) plus ribavirin is the current standard of care for naïve chronic hepatitis C (CHC) patients, achieving a high sustained virological

response (SVR) rate^[1-4]. The long-term benefits of CHC combination therapy have been well established in several studies, but the results are limited by differing patient populations or treatment regimens and, importantly, a brief duration of follow-up^[5]; in addition, occult hepatitis infection by hepatitis C virus (HCV) has been proposed^[6]. The conclusions of long-term studies^[7-14] may be compromised by the small number of patients, and most studies using IFN monotherapy or IFN combined with ribavirin, while only 4 studies used PEG-IFN plus ribavirin combination therapy^[15-18].

We conducted an open-label cohort study in a single center in Spain from January 2000 to December 2009, and included patients with a SVR after antiviral combination therapy with PEG-IFN plus ribavirin achieved between 2000 and 2003, with a mean follow-up greater than 5 years. Our major aim was to assess the clinical, biochemical and virological outcomes, and the durability of the SVR.

MATERIALS AND METHODS

Study design

A total cohort of 303 CHC consecutive patients (18-65 years) treated with PEG-IFN plus ribavirin in 2000-2003 were included in this cohort study. All patients attended the Hepatology Unit, Hospital Universitario de La Princesa (a tertiary university care centre), CIBERehd, Madrid, Spain.

Patients

Eligible patients were those who achieved a SVR after PEG-IFN plus ribavirin for 24 or 48 wk (in genotypes 2/3 or genotypes 1/4, respectively), defined as negativization of HCV-RNA at the end of treatment and after 6 mo of follow-up. Criteria for exclusion were: alcohol or intravenous drug abuse; liver diseases not related to HCV infection (autoimmune, metabolic or toxicity by drugs); decompensated liver disease; coinfection with HBV or human immunodeficiency virus; and pregnancy.

All patients received the standard of care combination therapy: 60 (39%) patients had been treated with PEG-IFN α -2a (Pegasys, Roche) plus ribavirin (1-1.2 g/d) and 93 (61%) patients with PEG-IFN α -2b (Pegintron, Schering-Plough) plus ribavirin (1-1.2 g/d). Patients with genotypes 1 and 4 were treated for 48 wk and patients with genotypes 2 or 3 were treated for 24 wk^[19].

Clinical, biochemical and virological evaluation

We obtained data on patients' sex and age, treatment, virological data (genotype, baseline HCV-RNA), biochemical data [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (GGT), α -fetoprotein (AFP)], and length of follow-up. Pre-treatment liver biopsy and transient elastography analysis during the follow-up was obtained in some patients taking into account that this study was performed as routine clinical practice.

Patients were evaluated as outpatients at sequential annual clinical visits (1, 2, 3, 4, 5 years after the completion of antiviral therapy and 6 mo of follow-up). Blood tests

with hematological, biochemical and virological assays were performed at the basal visit and thereafter. Serum HCV-RNA levels (IU/mL) were determined with Cobas Amplicor Hepatitis C Monitor Test (v2.0 Roche Diagnostics) and reverse transcription-polymerase chain reaction (RT-PCR), with a limit of detection < 50-100 IU/mL. HCV genotyping was performed by a reverse-hybridization line probe assay (INNO-LiPA HCV; Innogenetics, Zwijndrecht, Belgium).

Liver decompensation or failure was defined if a patient showed any of these findings: bleeding varices, hepatic encephalopathy, jaundice, or ascites. Patients were classified pretreatment by liver biopsy, and the establishment of cirrhosis was done using the METAVIR score for fibrosis stage. In addition, transient elastography was performed during follow-up after achieving the SVR. Hepatocellular carcinoma (HCC) was diagnosed if 2 coincident imaging techniques [ultrasonography, computed tomography (CT) or magnetic resonance imaging] showed a focal lesion larger than 2 cm with arterial hypervascularization or if one imaging technique showed a focal lesion larger than 2 cm with arterial hypervascularization in the presence of an AFP level > 400 ng/mL.

Statistical analysis

Quantitative variables are expressed as mean \pm SD. Qualitative variables are expressed as percentage with range. The Student *t*-test with Welch's and Fisher's correction, the chi-squared test, the Mann-Whitney *U* test and the Kruskal Wallis test were used for continuous or discrete variables as appropriate. Logistic regression was used to analyze if baseline factors could be associated with SVR. The Kaplan-Meier method was used to determine the rate of HCC occurrence. A value of *P* < 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS version 15.0.

RESULTS

Characteristics of patients

Among the 303 CHC patients, a total of 150 were excluded as they did not achieve a SVR. Those 153 patients with a SVR after treatment with PEG-IFN plus ribavirin (weight based) were included, with a mean age of 49 ± 9 years. There were 82 males (53.6%). Genotypes of HCV were distributed as follows: 116 genotype 1 (75.8%), one genotype 2 (0.6%), 32 genotype 3 (21%) and 4 patients with genotype 4 (2.6%). The baseline characteristics of patients with a SVR are shown in Table 1. One hundred and thirty patients (85%) were followed up for 5 or more after SVR. The median duration of follow-up was 76 ± 13 mo (range, 54-90) after the end of treatment, i.e., after achievement of a SVR was established.

Clinical outcomes

At the end of the follow-up, all patients were alive. Of 153 sustained responders, 5 patients had cirrhosis (F4) and 8 patients had F3 stage fibrosis before the start of the treatment, as determined by the METAVIR score. No

Table 1 Characteristics of 153 patients with a sustained virological response after peginterferon plus ribavirin combination treatment

	<i>n</i> (%)
Age (mean \pm SD, yr)	47 \pm 9
Sex	
Female	71 (46.4)
Male	82 (53.6)
Genotypes	
1	116 (75.8)
2	1 (0.6)
3	32 (21)
4	4 (2.6)
Stage of fibrosis (before therapy)	
F1-2	140 (91.6)
F3	8 (5.2)
F4	5 (3.2)
Fibrosis by FibroScan® (mean \pm SD, kPa)	7 \pm 4.3
Type of PEG-IFN	
α -2a	60 (39)
α -2b	93 (61)
Follow-up [range (mean \pm SD), mo]	54-90 (76 \pm 13)
Patients follow-up	
5 yr	130 (85)
4 yr	23 (15)

PEG-IFN: Peginterferon.

patient with a SVR developed signs of liver failure during the follow-up. These patients were hepatitis B surface antigen negative and HCV-RNA negative, and no other risk factors for liver disease were reported.

One patient, with cirrhosis on pre-treatment biopsy and genotype 1b, developed a HCC at 30 mo of follow-up. It was assessed by ultrasonography, AFP level and CT scan, was 3.5 cm in size and located sub-diaphragmatically at segment VIII. The patient was still negative for serum HCV-RNA at the time of HCC diagnosis, and at last follow-up, HCV-RNA remained undetectable. The patient received an orthotopic liver transplant. Given that 5 patients had cirrhosis pre-treatment and one developed HCC, this represents a 20% risk of HCC after SVR in individuals with pre-treatment cirrhosis. The incidence of HCC in this cohort of 153 SVR patients after a mean of 76 \pm 13 mo was 1/153 (0.65%).

Biochemical outcomes

All 153 SVR patients had at least 2 biochemical evaluations, and 123 (80.4%) had 5 or more years of laboratory data after achieving the SVR. There were significant reductions in ALT, AST, GGT and ALP levels between the samples collected pre-treatment and samples after the end of treatment, as shown in Table 2. However, there were no statistically significant differences ($P > 0.05$) between the first and last samples collected during the follow-up, in mean ALT (20 \pm 9 IU/L), AST (20 \pm 5 IU/L), AST/ALT ratio (1 \pm 0.5), ALP (70 \pm 19 IU/L), GGT (25 \pm 20 IU/L), and AFP (3 \pm 1 ng/mL) values.

Virological outcomes

Out of 153 patients, 138 (90.2%) had at least 4 serum

Table 2 Biochemical values pre-treatment and post-treatment (last sample of follow-up)

	Pre-treatment	Post-treatment	<i>P</i>
AST (IU/L)	73 \pm 70	20 \pm 5	< 0.001
ALT (IU/L)	138 \pm 178	20 \pm 9	< 0.001
ALP (IU/L)	70 \pm 19	98 \pm 55	< 0.001
GGT (IU/L)	51 \pm 46	25 \pm 20	< 0.001
AFP (ng/mL)	4.9 \pm 4	3 \pm 1	NS

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ -glutamyl transpeptidase; AFP: α -fetoprotein; NS: Not significant.

HCV-RNA tests via RT-PCR. The mean number of samples tested per patient was 4 or 5 (range, 2-8). No patient had detectable HCV-RNA in serum *via* RT-PCR on any sample.

Liver fibrosis analysis

Liver biopsy and transient elastography evaluation were obtained only in a limited number of patients. Fifty four patients (45%) had pre-treatment liver biopsies. Eighty of these (53%) had a Fibroscan® analysis at least once during the follow-up with a mean number of 4 (range, 2-8) explorations. Mean time between 2 Fibroscan® analyses was 4 \pm 0.8 years. The technique was very well tolerated by all patients, without side effects. Results were obtained in all patients. The mean value of transient elastography after 4-5 years of follow-up was 7 \pm 4.3 kPa. One patient (0.8%) had a decrease in fibrosis stage from liver biopsy F3 to F1 by transient elastography. No progression of fibrosis was seen in any patient.

DISCUSSION

We assessed the long-term clinical, biochemical and virological outcomes of 153 patients with CHC who achieved a SVR after PEG-IFN plus ribavirin combination therapy. This study showed that a SVR is associated with permanent undetectable HCV-RNA in serum during a long-term follow-up. Nevertheless, it is difficult to say whether the treatment *per se* is important or whether a SVR is important because no controls were included. A late relapse of at least 0.8% after 4-5 years of follow-up has been reported^[7-11], however, introduction of more sensitive PCR methods may contribute to reduce this late-relapse rate.

There are some studies of the long-term clinical outcome of CHC patients with a SVR^[8,11-16,20-27] but the majority analyzed patients treated with recombinant IFN as monotherapy or in combination with ribavirin. At present, only 4 studies have enrolled patients treated with PEG-IFN plus ribavirin as shown in Table 3: in the study by Veldt *et al*^[15], 83 patients were analyzed; Chavalitthamrong *et al*^[16] studied 78 patients; George *et al*^[14] recently published the results of a long-term study of SVR patients including only 4 patients (3%) treated with PEG-IFN plus ribavirin; and Giannini *et al*^[18] included 231 patients treated with PEG-IFN plus ribavirin, but only 33.3% were genotype 1.

Table 3 Comparative results of other studies analyzing long term outcomes in chronic hepatitis C patients who achieved a sustained virological response *n* (%)

Study	IFN- α 2b alone or IFN- α 2b + RBV	PEG-IFN + RBV	Genotype 1	Genotype non-1	No data of genotype
George <i>et al</i> ^[14]	146 (97)	4 (3)	75 (53)	66 (47)	9
Veldt <i>et al</i> ^[15]	55 (39)	83 (59)	56 (39)	86 (61)	-
Chavalitdhamrong <i>et al</i> ^[16]	93 (54.4)	78 (45.6)	48 (28.1)	113 (66.1)	10
Giannini <i>et al</i> ^[18]	0	231 (100)	77 (33.3)	154 (66.7)	-
The current study	0	153 (100)	116 (75.8)	37 (24.2)	-

PEG-IFN: Peginterferon; RBV: Ribavirin.

It is noteworthy that we included only patients treated with PEG-IFN plus ribavirin with a high proportion being genotype 1 (75.8%), in contrast to other studies (Chavalitdhamrong *et al*^[16], where genotype 3 represented 62%; George *et al*^[14], genotype 1 represented 47%; and Giannini *et al*^[18] genotype 1 represented 66.6%).

Overall, our study showed that clinical events were rare in this population, indicating that SVR patients have an excellent prognosis, similar to previous studies^[8,9,11,14-16,20,21,25-29]. No patient developed decompensated liver disease. There were 5 (4.2%) patients with cirrhosis pre-treatment in this study. None of the patients with advanced fibrosis (F3) pre-treatment progressed to cirrhosis. Similar findings have been reported with PEG-IFN^[10,14,25]. In contrast, Pradat *et al*^[27] found that cirrhosis developed in 2 of 87 patients who were followed for at least 5 years after a SVR.

One patient with pre-treatment cirrhosis developed a HCC that represented a rate of 0.8%. This patient had no other risk factors such as obesity, alcohol intake or diabetes. This is a similar rate of HCC as reported in other studies^[12,15,20,21,23,24,26,30]. Veldt *et al*^[21] reported that 3/142 patients (2%) with a SVR and F3-F4 stage pre-treatment developed HCC during follow-up. Nevertheless, Japanese authors^[31,32] have reported a HCC rate of 0.02%-0.5% per year, slightly lower than our study. These data confirm that the risk of late development of HCC after a SVR is a real problem, and we must continue the follow-up of these patients for a long time. It is also important to take into account that HCV-RNA remains undetectable when HCC appears. Scientists speculate about the possibility of hepatocarcinogenesis, despite null replication of HCV, by other pathways^[29,33,34].

It is well-known that most patients with a SVR normalize their serum ALT, AST and total bilirubin, unless another liver disease is present^[8,9,11,24]. We found the same results in our study: 99% of patients had normal AST and ALT levels during the entire period of follow-up. The patient with HCC had persistently normal serum ALT values. Only one patient had elevated ALT and AST levels during follow-up period: a woman with fibromyalgia and relevant consumption of non-steroidal anti-inflammatory drugs. Nevertheless, ALP values were increased after treatment, but remained within the normal range (< 100 U/L). There is no explanation for this finding.

Limitations of our study are that not all patients had a period of follow-up greater than 5 years and, importantly, that analysis of outcomes of fibrosis (stability, improve-

ment or progression) are of limited value as no paired pre-treatment and post-treatment biopsies were analyzed from each patient. However, a large European study^[21] clearly demonstrated that the 5-year survival rate of patients achieving a SVR was similar to the overall population and that a SVR was associated with a decrease in fibrosis score; the authors speculated that excellent prognosis of sustained virological responders "is likely to hold true in the era of PEG-IFN and ribavirin". Our data confirm this important prognostic assumption. Moreover, extensive recent histological analyses have shown that most virological responders without cirrhosis had normalization of liver histology^[13]; that is, up to 82% had improved fibrosis scores^[14] and in addition to fibrosis stability/improvement in 88%, in 64% of patients (9 of 14) regression of cirrhosis was observed^[35]. Taken all together^[13,14,21,35], these data question the indication for a second liver biopsy in CHC patients with a SVR after antiviral combination therapy.

The long-term clinical outcome of patients with a SVR to PEG-IFN plus ribavirin is favorable. However, a risk of HCC development still remains, although it is very low, so we must clinically monitor SVR patients for a long time, even with undetectable HCV-RNA, normal ultrasonography, and normal aminotransferase and AFP levels after PEG-IFN plus ribavirin therapy.

COMMENTS

Background

The combination treatment of peginterferon (PEG-IFN) α plus ribavirin improved the sustained virological response (SVR) rate in chronic hepatitis C (CHC) patients. The long-term benefits of CHC combination therapy have been well established in several studies, but the results are limited by differing patient populations or treatment regimens and, importantly, a brief duration of follow-up.

Research frontiers

Occult hepatitis infection by hepatitis C virus (HCV) has been proposed and some cases of delayed relapses have been published.

Innovations and breakthroughs

The conclusions of long-term studies may be compromised by the small number of patients, and the fact that most studies used IFN monotherapy or IFN combined with ribavirin, with only 4 studies using PEG-IFN plus ribavirin combination therapy. This study analyzed patients with a SVR after antiviral combination therapy of PEG-IFN plus ribavirin with a mean follow-up greater than 5 years.

Applications

The study demonstrated that the long-term outcome of CHC patients who were sustained virological responders was good. It is important that evidence of a virological relapse must be assessed for a long time, as well as screening for hepatocellular carcinoma.

Terminology

A SVR is comparable with "clinical cure" in CHC patients. However, a minimal

percentage of patients present an activation of HCV replication, and are considered as "relapsers".

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

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S- Editor Sun H L- Editor Cant MR E- Editor Lin YP

EUS-guided drainage is more successful in pancreatic pseudocysts compared with abscesses

Riadh Sadik, Evangelos Kalaitzakis, Anders Thune, Jan Hansen, Claes Jönson

Riadh Sadik, Evangelos Kalaitzakis, Jan Hansen, Institute of Medicine, Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, SE 413 45, Sweden

Anders Thune, Claes Jönson, Institute of Medicine, Department of Surgery, Sahlgrenska Academy, University of Gothenburg, Gothenburg, SE 413 45, Sweden

Author contributions: All authors have been involved in the design and conduct of this work, data analysis and writing of the manuscript.

Supported by The Health and Medical Care Executive Board of the Västra Götaland Region, Sweden

Correspondence to: Riadh Sadik, MD, PhD, Institute of Medicine, Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, SE 413 45, Sweden. riadh.sadik@vgregion.se

Telephone: +46-31-3421000 Fax: +46-302-43889

Received: August 28, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: January 28, 2011

patient (4%) after 6 mo; the patient was successfully retreated.

CONCLUSION: EUS-guided drainage of pseudocysts is associated with a higher success rate and a lower complication rate compared with abscess drainage.

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Key words: Pancreas; Endoscopic ultrasound; Drainage; Pseudocyst; Abscess

Peer reviewer: Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

Sadik R, Kalaitzakis E, Thune A, Hansen J, Jönson C. EUS-guided drainage is more successful in pancreatic pseudocysts compared with abscesses. *World J Gastroenterol* 2011; 17(4): 499-505 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/499.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.499>

Abstract

AIM: To compare the results for endoscopic ultrasound (EUS)-guided drainage of clear fluid pancreatic pseudocysts with the results for abscess drainage.

METHODS: All patients referred for endoscopic drainage of a fluid collection were prospectively included. The outcome was recorded.

RESULTS: Altogether 26 pseudocysts or abscesses were treated in 25 (6 female) patients. One endoscopist performed the procedures. Non-infected pseudocysts were present in 15 patients and 10 patients had infected fluid collections. The cyst size ranged between 28 cm × 13 cm and 5 cm × 5 cm. The EUS drainage was successful in 94% of the pseudocysts and in 80% of the abscesses ($P = 0.04$). The complication rate in pseudocysts was 6% and in abscesses was 30% ($P = 0.02$). Recurrence of a pseudocyst occurred in one

INTRODUCTION

Endoscopic ultrasound (EUS)-guided drainage of pancreatic pseudocysts and abscesses has become a first line therapy in many centers^[1-4]. This is due to the ability of the EUS instrument to assess the wall thickness, identify major vessels and find the closest access to the fluid cavity^[5-7]. Moreover, this procedure will create an internal fistula and avoid the inconvenience of an external drainage and the risk for cutaneous fistula. A recent study has shown that percutaneous drainage failed in 36% of patients with normal pancreatic ductal anatomy. In patients with ductal abnormalities such as stricture, ductal cut-off and dilated duct the failure rate was 62%-100%. In those patients with an abnormal duct, cutaneous fistula developed in more than 50%^[8].

Surgery may be avoided in cases when successful

EUS-guided drainage is performed, with no recurrence of the cyst. However, acute surgery may be needed in some cases of complications such as perforation or major bleeding^[9,10].

In spite of these assumed advantages of EUS-guided drainage there are only a few prospective studies assessing the full scale of the advantages and shortcomings of this procedure. Moreover, prospective studies assessing this procedure in comparison to radiological intervention and surgery have not been performed. Furthermore, the results of non-infected pseudocyst drainage have not been prospectively compared with the drainage of abscesses. Kahaleh *et al*^[9] compared EUS-guided drainage and conventional transmural drainage in a prospective study. Long-term success assessed at 6 mo was 84% and 91%, respectively ($P = \text{NS}$). The complication rate was 18% and 19%, respectively ($P = \text{NS}$). The complications in this study by Kahaleh consisted of bleeding in three patients, infection in eight, stent migration into the cyst in three and pneumoperitoneum in five.

The aim of our prospective study was to compare the results for drainage of clear fluid pseudocysts with the results for abscess drainage. This prospective quality analysis may guide us in improving the performance of this procedure.

MATERIALS AND METHODS

All patients referred for EUS-guided drainage of a fluid collection were prospectively included in this study. The data were collected in the period between February 2006 and June 2010. Our center is the tertiary center for pancreatic surgery in the region of west Sweden, with access to EUS as well as interventional radiology.

The fluid collections were pseudocysts with clear fluid in 16 cases. The other fluid collections were 9 pseudocysts with infected fluid and one postoperative abscess. The abscess developed after an operation for a perforated duodenal ulcer. The indications for drainage were abdominal pain in 18 patients, infection in 10, food obstruction in one patient and jaundice in one patient. In some cases there was more than one symptom. All patients underwent a computed tomography (CT) scan before the procedure to assess accessibility of the pseudocyst from the stomach or duodenum and to assess an eventual communication between the cyst and the pancreatic duct. Hemoglobin, leucocytes, thrombocytes, CRP and INR were controlled before the procedure. The patients received verbal and written information about the procedure. The procedure was performed either under conscious sedation with pethidine and midazolam or, when possible, under intubation anaesthesia. Intravenous cefotaxime 1 g was administered before the procedure in patients without ongoing antibiotic treatment. After the procedure, therapeutic antibiotics were only given to patients with an infection. The procedures were performed by one endoscopist (RS) with extensive endoscopy experience.

A linear echoendoscope (Pentax EG3830UT and GF-

UCT 140, Olympus) was used to find the closest axes to the pseudocyst. Special attention was given to avoid vessels and to find areas of the wall that were not thicker than 1 cm.

Different procedures were used to enter and stent the cavity: (1) The Giovannini Needle Wire (Wilson-Cook Medical GI Endoscopy) stenting system was used to place a stent in one step^[10]. This was mainly used in clear fluid pseudocysts to place one stent; (2) A 19-gauge needle (Wilson-Cook Medical GI Endoscopy) was also used to access the pseudocyst and to place a 0.035-inch guide wire into the cyst before the opening was dilated using a balloon sized between 12 and 18.5 mm. The guide wire was then used to stent the cyst. This procedure was used to place more than one stent in infected cavities; and (3) A cystotome (Wilson-Cook Medical GI Endoscopy) was also used to enter the cyst and establish an opening. This procedure was also used to place more than one stent in infected cavities.

Both cut and coagulation current were used. Coagulation current and endocut (ERBE®) current were more often used in the procedures which took place during the later part of the study period in order to avoid bleeding.

Technical success of the EUS intervention was defined as the ability to improve clinical outcome of the pseudocyst or abscess without the need for surgical interventions. A complication was defined as an adverse event that led to a longer hospital stay and/or to emergency surgery. The ethics committee of the University of Göteborg had approved the study.

Statistical analysis

The primary endpoint was between-group differences in success rate and complication rate. McNemar's exact test was utilized to assess the difference between the results for pseudocysts and abscesses.

RESULTS

One patient with alcohol-related pancreatitis did not attend at the endoscopy department and was therefore excluded. All of the other 25 out of 26 referred patients were included in the study. Altogether 26 pseudocysts or abscesses were treated in 25 patients.

The mean follow up time of all patients was 20 mo, range 2-45 mo.

The EUS drainage was successful in 94% of the pseudocysts and in 80% of the abscesses ($P = 0.04$).

Table 1 shows the results for the patients with pseudocysts; Table 2 shows the results for abscesses; and Table 3 shows some technical aspects, etiologies and locations of the cysts.

Complications and recurrence

The complication rate after treatment of pseudocysts was 6% and after treatment of abscesses was 30% ($P = 0.02$). Overall, 3 patients (11.5% of the cases) needed surgery due to a procedure-related complication.

One of the patients developed a major bleed from

Table 1 Results for patients with pseudocysts

Patient age and gender	Etiology	Cyst size (cm)	No. of stents	Complication	Follow-up (mo)	Final result
(52)M	Idiopathic	13 × 10	1 (10F)	No	45	Total regression
(66)M	Malignancy	6 × 7	1 (10F)	No	12	Total regression
(61)M	Alcohol	15 × 13	3 (7F)	No	41	Total regression
(76)M	Alcohol	5 × 5	1 (10F)	No	34	Total regression
(47)M	Alcohol	7 × 5	3 (7F)	No	33	Total regression
(61)M	Alcohol	10 × 10	2 (10F)	No	32	Total regression
(43)M	Alcohol	12 × 10	1 (10F) + aspiration	No	23	Total regression
(36)M	Idiopathic	28 × 13	1 (10F)	No	22	Total regression
(24)M	Trauma	5 × 4	0	No	14	Spontaneous regression
(60)M	Idiopathic	12 × 11	1 (10F)	No	8	Total regression
(60)M	Idiopathic	13 × 9	1 (10F)	Pneumo-peritoneum	8	Regression, operation
(6)M	Medication related	10 × 8	0, aspiration	No	8	Regression to 3 cm × 4 cm after 7 mo, no symptoms
(22)M	Idiopathic	15 × 12	0, aspiration	No	6	Total regression
(43)M	Idiopathic	20 × 10	1 (10F) + aspiration	No	6	Regression, no symptoms
(29)F	Gallstone	8 × 5	1 (10F)	No	3	Regression, EUS control planned
(48)F	Idiopathic	11 × 14	1 (10F)	No	2	Regression, EUS control planned

EUS: Endoscopic ultrasound.

Table 2 Results for patients with abscesses

Patient age and gender	Etiology	Cyst size (cm)	No. of stents	Complication	Follow-up (mo)	Final result
(76)F	Gallstone	7 × 7	3 (7F)	No	37	Total regression
(32)M	Alcohol	5 × 4	Aspiration	No	36	Total regression
(57)M	Alcohol	5 × 5	1 (10F)	Bleeding	32	Operation
(68)F	Gallstone	18 × 13	5 (7F) + 1 naso-cystic	No	30	Total regression
(51)M	Hyperlipidemia	8 × 5	2 (7F)	Pneumoperitoneum	25	Regression, pneumo-peritoneum during a second procedure, operation
(53)F	Alcohol	7 × 5	Aspiration	No	18	Total regression
(64)M	Gallstone	23 × 10	1 (10F), 3 (7F), 1 naso-cystic	No	15	Total regression
(63)F	Postoperative abscess	6 × 5	1 (7F), 1 naso-cystic	Pneumomediastinum	10	Conservative treatment, total regression
(39)M	Systemic lupus erythematosus	12 × 7	Aspiration	No	3	Regression, Death due to renal insufficiency
(67)M	Idiopathic	20 × 6	1 (10F), 1 (7F)	No	5	Total regression

Table 3 Some technical aspects of the drainage, etiology and location of the cysts

Drainage technique	No. of procedures	Anesthesia	Etiology	Type of pancreatitis	Location
Giovannini Needle Wire ¹ in 16 cases	One in 19 patients	Sedation ² in 19 patients	Alcohol (11)	Chronic pancreatitis in 14 patients	Cauda in 12 patients
19-gauge needle ¹ in 11 cases	Two in 6 patients	Intubation anesthesia in 7 patients	Idiopathic (6)	Acute pancreatitis in 12 patients	Corpus in 11 patients
Cystotome ¹ in 1 case	Eight in one patient		Gallstone (3) 1 hyperlipidemia 1 trauma 1 postoperative 1 SLE 1 medication 1 malignant		Caput in 3 patients

¹Cook Endoscopy, Winston-Salem, NC; ²Pethidine + midazolam. SLE: Systemic lupus erythematosus.

the gastropiploic artery that was not possible to control conservatively. In this case the Giovannini Needle Wire

with cut current was used to enter the cyst according to the manufacturer's recommendations. The patient was



Figure 1 Pus drained from an infected pseudocyst.

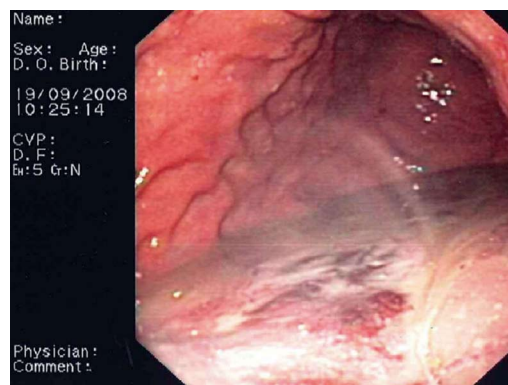


Figure 3 Pus accumulating in the stomach.

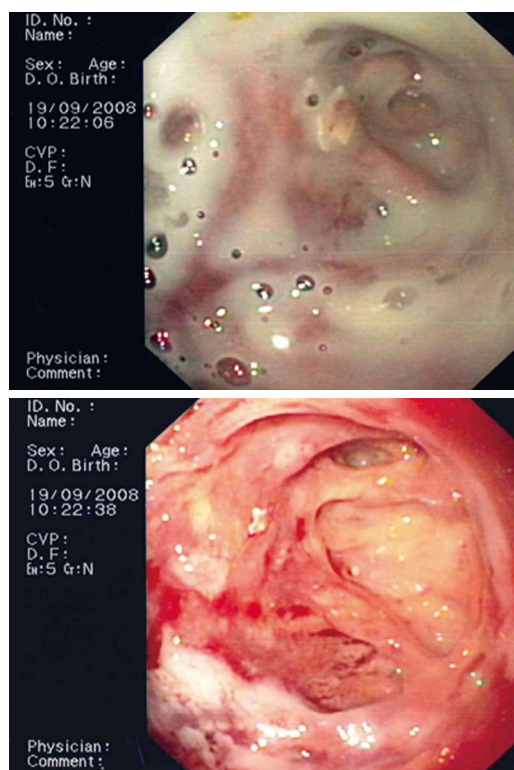


Figure 2 An infected pseudocyst before and after flushing.

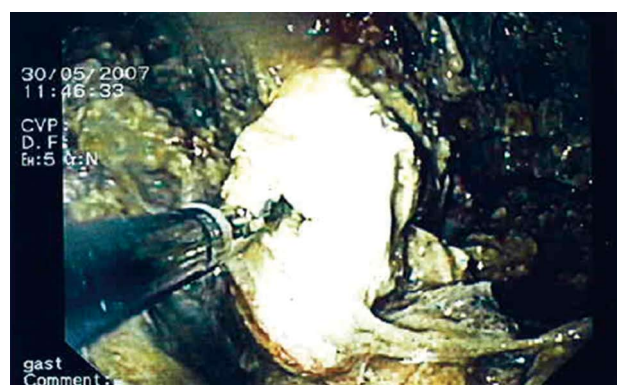


Figure 4 Necrosectomy in a pseudocyst.

operated upon immediately and received a cystoenteric anastomosis with a good final outcome.

In two patients perforation occurred in abscesses stented from the gastric fundus. The dilatation technique was used in one of them and a combination of diathermia and dilatation in the other. The first patient underwent surgical resection of the cyst with a good result. The second patient with a postoperative abscess was treated conservatively with total regression, and could be discharged in good health without further interventions.

A perforation after a technically successful stenting of the cyst occurred in one patient with a non-infected pseudocyst. This patient developed abdominal pain and a leak of both fluid and air was present on a CT-scan. The patient underwent surgery after two days with a resection of the cyst with a good outcome. The surgeon observed

that the stent was placed in a proper position; however, the pseudocyst was not attached to the gastric wall.

Two stents migrated during the procedure into the cyst and were retrieved endoscopically.

No procedure-related mortality was observed. One patient died of a non-procedure-related terminal renal insufficiency 3 mo after the aspiration of a pseudocyst.

Recurrence of a pseudocyst occurred in one patient (4%) after 6 mo (Pseudocyst No. 3 in Table 1). The patient underwent a repeat successful EUS drainage with a new follow up time of 29 mo (Pseudocyst no. 6 in Table 1).

Complicated cases

Nine patients had multiple pseudocysts. Two cases were complicated by gastric varices in the stomach. Despite this, it was possible to stent these patients. In the youngest patient (6 years) the pseudocyst was close to the heart; therefore, the cyst was aspirated and not stented. In two patients the distance between the cyst wall and the duodenum and stomach was 3 cm and 2 cm, respectively. Aspiration was therefore performed without stenting. In one patient the cyst was only accessible *via* the esophagus and was therefore not stented to avoid fistula formation to the esophagus.

Other procedures and aspects

Flushing of the abscess (Figures 1-3) and necrosectomy (Figure 4) were performed in 10 patients. Patients with

large amounts of necrotic material received a naso-cystic drain after a mechanical necrosectomy with a foreign body forceps. The naso-cystic drain was left in place for between a couple of days and three weeks. During this period the drain was flushed with 250-500 mL saline once or twice daily.

In six patients the stents were placed in the gastric fundus; in 12 patients in the gastric corpus; in one patient in the duodenum; and in one patient in the antrum.

The stents were withdrawn after 3 mo in four patients, in three other patients the stents were withdrawn after 4, 5 and 13 mo. In the other patients (13 patients) the stents have not been removed yet. Fluoroscopy was not utilized in 15 (58%) patients during the procedure.

Three patients underwent percutaneous drainage procedures before they were referred for the EUS-guided drainage. One patient received a percutaneous drainage after the EUS-guided aspiration without stenting because of the large distance (3 cm) between the duodenal wall and the cyst.

CT scan showed a communication between the cyst and pancreatic duct in three patients. These three patients underwent an endoscopic retrograde cholangiopancreatography (ERCP) to stent the pancreatic duct. The procedures were, however, not successful and the patients subsequently underwent an EUS-guided drainage.

In one patient a pseudocyst was clinically suspected and the appearance on the CT scan as well as the EUS findings supported the benign diagnosis. Accordingly, a drainage procedure was performed during the EUS session. The patient was stented to the duodenum and had a total regression of the cyst with symptom improvement. However, the patient developed jaundice after 5 mo and was then found to have a pancreatic cystadenocarcinoma.

DISCUSSION

This is the first prospective study to show that the success rate of EUS-guided drainage of pseudocysts is high compared with that of the drainage of abscesses. Moreover, the complication rate was lower for pseudocysts compared with abscesses.

The present study provides novel knowledge about the success rate of EUS-guided drainage of clear fluid pseudocysts and abscesses. The data indicate that the drainage of clear fluid pseudocysts is a more straightforward procedure whereas the success rate for abscesses is lower. This could be partially explained by the fact that patients with abscesses are sicker and more vulnerable and are therefore more prone to develop complications. Another contributing factor is that an abscess with necrosis is usually less clearly demarcated compared with a clear fluid pseudocyst. This may make the procedure more difficult. Moreover, the wall of an abscess with excessive inflammation may rupture more easily compared with the wall of a less inflamed pseudocyst. The data support a more cautious approach to abscesses compared with pseudocysts. Abscesses should be drained only when conservative therapy has failed and enough time has passed for the formation

of a well demarcated abscess wall.

The results presented in this work on pseudocyst drainage are comparable to previously reported data. A retrospective study by Cahen *et al.*^[11] showed a success rate of 97% and a complication rate of 34%. Kahaleh *et al.*^[9] published prospective data comparing EUS-guided drainage and conventional endoscopic drainage showing a long-term success rate of 84% and complication rate of 19% for EUS-guided drainage. There was no difference between EUS-guided drainage and conventional drainage. However, a recent prospective study^[12] has also compared the results of EUS-guided drainage and conventional endoscopic drainage, showing a clear advantage for EUS-guided drainage with a success rate of 100% and only 33% for conventional endoscopic drainage. The complication rate in the EUS group was 4% and 20% in the conventional group. The differences in success rate and complication rates between different series are probably due to different patient populations and heterogeneous interventions^[13].

EUS-guided drainage has been advocated as the first line treatment for pseudocysts^[12]. Moreover, there is an enthusiasm about the possibilities of EUS-guided procedures. However, our knowledge from large prospective studies is still limited. There is probably a publication bias for data coming from large and experienced centers with good results. Therefore, there is a need for large prospective multicenter studies to assess the full scale of advantages and complications of EUS-guided drainage.

Our results are comparable to previously published data. However, we do have to further improve the success rate and reduce the complication rate. Due to limited resources intubation anesthesia was possible only in a minority of our patients. This type of anesthesia should be adopted for the majority of patients whenever possible in order to provide stable conditions during the drainage procedure. However, the importance of anesthesia has not yet been addressed in a study. Available techniques also need further improvement and there are many issues that need to be addressed in multicenter studies. Whether to use a dilatation technique or diathermia to enter the cyst is such an issue. The type of current to use and the optimal site in the stomach in which to place the stents are other unanswered questions.

In our study we observed perforations in two abscesses stented from the gastric fundus. This area is rather thin and should be handled carefully during the procedure. The gastric cardia should be avoided because it is close to the diaphragm. In one patient we did not stent the cyst because it was only accessible from the esophagus. A stent to the esophagus may result in a permanent fistula^[8].

We experienced one perforation in a patient with a pseudocyst that was not attached to the gastric wall. This complication has been discussed for children but has not been published in adults so far. During the EUS procedure it can be difficult to know if the cyst is firmly attached to the gastric wall. However, a movement of the cyst under the gastric wall indicates that the cyst is not attached to the wall.

One of our patients developed a major bleed after the use of cutting current to enter a cyst from the stomach. After this complication more coagulation current and endocut (ERBE®) current was used to enter the cyst, rather than pure cut current, according to the manufacturer's recommendations. Moreover, the entrance to the cyst was performed slowly to avoid fast and large movements of the needle wire.

No procedure-related mortality was observed in this study. Mortality rate varies in different series between 1/29 in a patient who underwent conventional endoscopic drainage^[12] to 1% in retrospective material^[11]. We found a recurrence rate of 4%, which is comparable with 5% reported by Cahen *et al.*^[11].

Primary surgery may be an alternative treatment for pseudocysts and abscesses. There are no prospective studies comparing surgery with EUS-guided drainage. One study has addressed the results and complications of necrosectomy by open surgery or minimally invasive surgery. The study showed no difference between the two approaches and a mortality rate of 28% in these severely ill patients^[14]. Recent results from a large study by Seifert *et al.*^[15] indicate that initial endoscopic necrosectomy in the early phase of pancreatic necrosis is more risky and less successful and should be considered only if all other options have failed. Another study showed that delayed surgery correlated positively with reduced mortality^[16].

The length of time a stent should be left in place is still a matter for debate. In general, endosonographers tend to expand the time until the stent is withdrawn to avoid recurrence of the cyst. It has been recommended to leave the stent in place in selected patients^[13]. This is another issue that needs further studies to assess the optimal time for leaving the stent in place.

One patient was found to have a pancreatic adenocarcinoma and not a pseudocyst; this emphasizes the need for a close follow-up of the patients.

We have routine access to fluoroscopy during the drainage procedure. However, when the Giovannini Needle Wire was utilized there was no need for fluoroscopy in many cases. This is in agreement with a recent report showing that EUS-guided drainage was possible without access to fluoroscopy since not all endoscopy centers have fluoroscopy in the EUS room^[17].

In conclusion, this prospective study shows for the first time that the results of EUS-guided drainage are more favorable in pseudocysts compared with abscesses. This knowledge should guide the choice and timing of therapy in patients with fluid collection. Large and preferably multicenter studies are needed to further expand our knowledge regarding different aspects of EUS-guided drainage, including the optimal technical procedure.

COMMENTS

Background

Inflammation in the pancreas may cause the development of fluid collection in the abdomen. This fluid collection may become infected. These fluid collections may be treated by surgery or endoscopic ultrasound. Endoscopic ultrasound is an endoscope equipped with a camera and ultrasound probe. This instrument

gives a detailed visualization of the organs around the gut. These organs then become accessible for sampling and treatment. Using endoscopic ultrasound guidance it is possible to create a path between the fluid collection and the stomach to drain the fluid.

Research frontiers

Endoscopic ultrasound guidance is increasingly used to treat fluid collections instead of surgery. However, no study has compared the results of this treatment between infected and non-infected fluid collections.

Innovations and breakthroughs

This study shows that the results of endoscopic ultrasound treatment for non-infected fluid collections is very good and significantly better than the results for infected fluid collections.

Applications

Treatment using endoscopic ultrasound guidance should be applied in symptomatic patients with non-infected fluid collection. In patients with infected fluid collection the treatment should be applied when other non-surgical treatments have failed and not early in the course of the disease.

Peer review

Very interesting paper dealing with an alternative approach to one of the most frequent complications of chronic pancreatitis. Statistically sound (McNemar test was a fantastic choice), this study is very convincing.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH

Evaluation of Cladribine treatment in refractory celiac disease type II

Greetje J Tack, Wieke HM Verbeek, Abdul Al-Toma, Dirk J Kuik, Marco WJ Schreurs, Otto Visser, Chris JJ Mulder

Greetje J Tack, Wieke HM Verbeek, Department of Gastroenterology and Hepatology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Abdul Al-Toma, Department of Gastroenterology and Hepatology, St. Antonius Hospital, 3435 CM Nieuwegein, The Netherlands

Dirk J Kuik, Department of Epidemiology and Biostatistics, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Marco WJ Schreurs, Department of Pathology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Otto Visser, Department of Haematology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Chris JJ Mulder, Department of Gastroenterology and Hepatology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Author contributions: Tack GJ, Al-Toma A and Mulder CJJ designed the research; Tack GJ and Verbeek WHM performed the research; Kuik DJ contributed statistical analytic tools; Tack GJ and Verbeek WHM analyzed the data and wrote the paper; Mulder CJJ, Visser O and Schreurs MWJ drafted the article and revised it critically for important intellectual content.

Supported by An Unrestricted Grant from Astra Zeneca

Correspondence to: Greetje J Tack, MD, Department of Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands. g.tack@vumc.nl

Telephone: +31-20-4440613 Fax: +31-20-4440554

Received: June 8, 2010 Revised: September 27, 2010

Accepted: October 3, 2010

Published online: January 28, 2011

RESULTS: Overall, 32 patients were included with a median follow-up of 31 mo. Eighteen patients responded well to 2-CdA. Patients responsive to 2-CdA had a statistically significant increased survival compared to those who were unresponsive. The overall 3- and 5-year survival was 83% in the responder and 63% and 22% in the non-responder group, respectively. The overall 2-year clinical, histological and immunological response rates were 81%, 47% and 41%, respectively. Progression into EATL was reported in 16%, all of these patients died.

CONCLUSION: Treatment of RCD II with 2-CdA holds promise, showing excellent clinical and histological response rates, and probably less frequent transition into EATL.

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Key words: Cladribine; Refractory celiac disease; Clinical course; Enteropathy associated T-cell lymphoma; Survival

Peer reviewer: Hong-Xiang Liu, PhD, Department of Pathology, Division of Molecular Histopathology, University of Cambridge, Box 231, Level 3, Lab Block, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom

Tack GJ, Verbeek WHM, Al-Toma A, Kuik DJ, Schreurs MWJ, Visser O, Mulder CJJ. Evaluation of Cladribine treatment in refractory celiac disease type II. *World J Gastroenterol* 2011; 17(4): 506-513 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/506.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.506>

Abstract

AIM: To evaluate cladribine [2-chlorodeoxyadenosine (2-CdA)] therapy in refractory celiac disease (RCD) II.

METHODS: An open-label cohort-study of RCD II patients treated with 2-CdA was performed between 2000 and 2010. Survival rate, enteropathy associated T-cell lymphoma (EATL) occurrence, clinical course, and histological and immunological response rates were evaluated.

INTRODUCTION

Celiac disease (CD) is characterized by a permanent state of intolerance to dietary gluten leading to an inflammatory T-cell response in the small intestine with mu-

cosal injury in genetically susceptible individuals^[1,2]. Although reduction of this intestinal inflammatory activity is usually seen upon strict adherence to a gluten free diet (GFD), a small percentage (2%-5%) of the adult-onset CD patients, especially those diagnosed above the age of 50 years, display a lack of response to such a diet. They are regarded as suffering from refractory celiac disease (RCD) when clinical and histological symptoms persist or recur after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 mo, unless earlier intervention is necessary^[3,4]. Immunologically, this syndrome can be subdivided into RCD I and RCD II, with immunophenotypically normal and aberrant intraepithelial T lymphocytes (IELs) in the small intestinal mucosa, respectively. Clonal expansion of these aberrant IELs, lacking the T-cell surface markers T cell receptor (TCR), CD3, CD4 and CD8^[5], but expressing cytoplasmic CD3, is supposed to be responsible for the occurrence of enteropathy associated T-cell lymphoma (EATL)^[4-6]. This type of lymphoma occurs in 60%-80% of RCD II patients within 5 years and has a poor prognosis with a 2-year survival of only 15%^[7,8]. The latter is mainly due to incomplete response to currently available therapies, high rates of life-threatening complications such as perforation of the gut, and poor nutritional conditions^[7,8]. Therefore, it is of utmost importance to evaluate new treatment strategies for RCD II in order to improve clinical course and prevent or delay progression to overt EATL.

Since the late 90's, researchers have become increasingly interested in therapeutic alternatives for treating RCD II, however as far as has been published there is no standardised approach yet^[9,10]. RCD II is, at least in part, resistant to most evaluated therapies so far^[10-12]. Until 2005 in our tertiary referral centre for RCD II, patients were initially treated with conventional immunosuppressive drugs, mainly azathioprine and prednisone, and if clinically and histologically unresponsive cladribine [2-chlorodeoxyadenosine (2-CdA)] was prescribed. Since then, a modified treatment strategy has been initiated with cladribine being drug of first choice.

2-CdA is a synthetic purine nucleoside homologue being equally toxic to proliferating as to non-dividing lymphoid cells. Because of this unique feature it is supposed to be especially active against low-grade malignancies, including hairy cell leukaemia. Cladribine is metabolised into its pharmacologically active cladribine triphosphate, which induces apoptosis, necrosis and inhibition of DNA/RNA synthesis (Figure 1). Clinically, 2-CdA is of proven value in the treatment of a number of haematological malignancies and selected autoimmune disorders, including multiple sclerosis^[13-15]. Our pilot studies showed that 2-CdA therapy^[16] is feasible and well tolerated in these patients. Survival at that time seemed promising, but follow-up was short.

This analysis evaluates cladribine therapy in a large prospectively studied open-label cohort of RCD II patients, during a mean follow-up time of 3 years.

MATERIALS AND METHODS

This cohort study includes reports of extended follow-up of 14 out of the 17 RCD II patients included in the open-label prospective phase I study performed by Al-Toma *et al*^[16] with 18 new patients added. Three patients out of the previous study were not included in this study as they were followed over time outside our hospital. Between January 2000 and April 2010, 2-CdA was prescribed to 32 RCD II patients at the VU University Medical Centre in The Netherlands.

Inclusion criteria

Patients diagnosed as having RCD II and treated with one or two courses of 2-CdA at the VU University Medical Centre were included. Cladribine was intravenously given in a dose of 0.1 mg/kg per day for 5 consecutive days. The diagnosis of RCD II was based on persisting or recurring clinical symptoms and small intestinal villous atrophy after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 mo. Furthermore, the clinically validated cut-off value of more than 20% aberrant intraepithelial lymphocytes (IELs) detected by flow cytometric (FACS) analysis was used to distinguish RCD type I and type II^[5]. Although a clonal TCR gamma rearrangement determined by PCR is still a widely accepted method to define RCD II, Verbeek *et al*^[5] showed that the percentage of aberrant IELs detected by FACS analysis is a more accurate way to define RCD II. The presence of EATL was excluded by using selected investigations^[17-20] and its diagnosis was confirmed according to the World Health Organization Classification of Tumors of Haematopoietic and Lymphoid tissues^[21]. Furthermore, pre-treatment with immunomodulatory drugs within 6 mo or any experimental drug within 30 d of the study entry was not allowed.

Follow-up and criteria of response

Before and during follow-up after cladribine treatment a clinical assessment was carried out noting in particular signs and symptoms of malabsorption, body mass index (BMI), albumin, and haemoglobin (Hb). A nutritional screening was performed by a dietician who specialised in CD in almost all patients prior to treatment and nutritional support was given when indicated. Clinical remission was defined as improvement of the diarrhoea, abdominal discomfort and/or signs of malabsorption, combined with at least 2 out of the following parameters of intestinal integrity within the normal range or an improvement of ≥ 1 point: (1) Hb; (2) BMI; and (3) albumin. Multiple duodenal biopsies were taken by upper gastrointestinal endoscopy in order to detect histopathological abnormalities and to perform immunophenotyping of IELs by FACS analysis at different time points (planned at 3, 6 and 12 mo, and then every year during follow-up). Isolation of small intestinal T-lymphocytes and staining for immunophenotyping were performed as previously described^[5]. Complete histological remission has been defined as a normalisation of the architecture of the duodenum, clas-

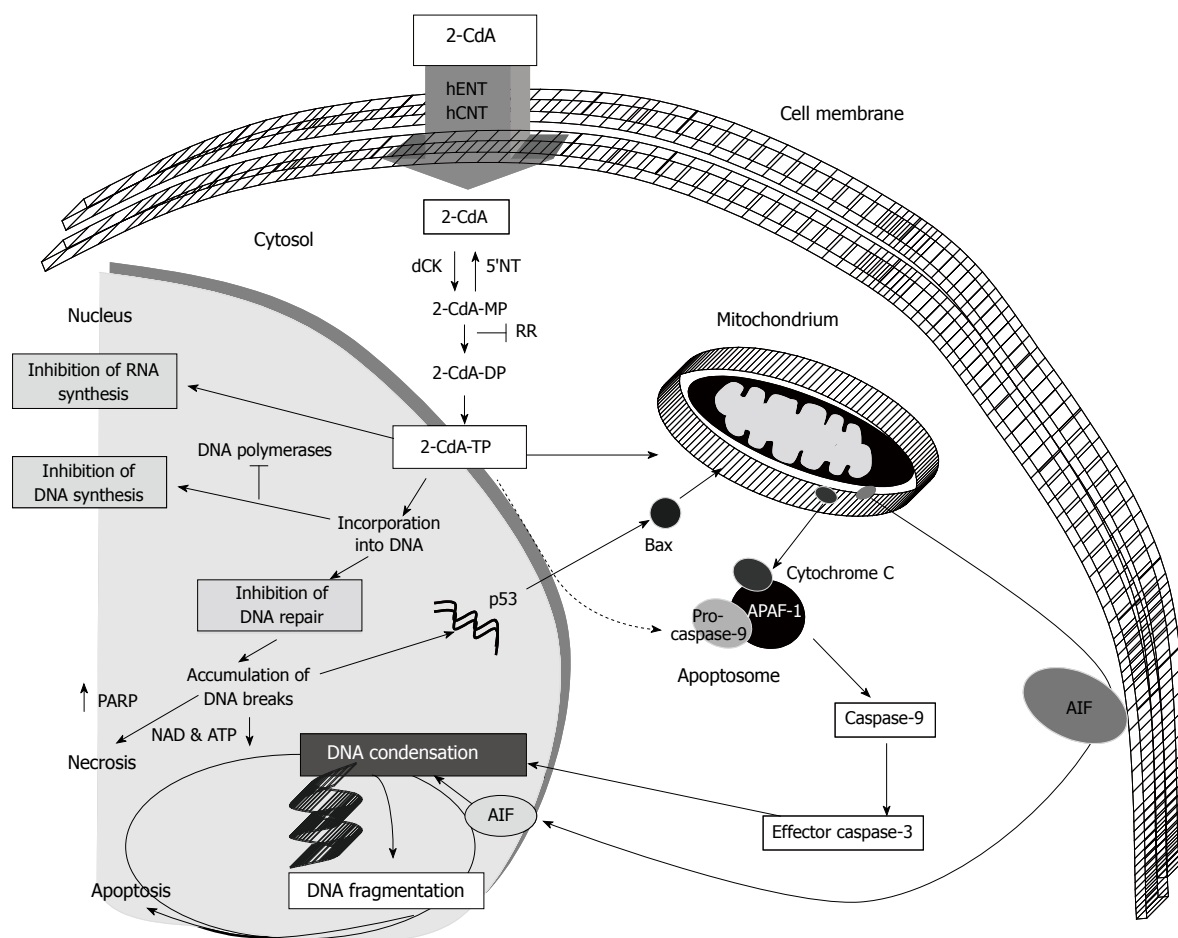


Figure 1 Schematic representation of intracellular pathways involved in 2-CdA cytotoxicity. (Adapted with permission from Borak, 2006). 2-CdA-MP: Cladribine monophosphate; 2-CdA-DP: Cladribine diphosphate; 2-CdA-TP: Cladribine triphosphate; dCK: Deoxycytidine kinase; 5'-NT: 5'-nucleotidase; hENT: Human equilibrative nucleoside transporter; hCNT: Human concentrative nucleoside transporter; AIF: Apoptosis inducing factor; APAF-1: Apoptotic protease activating factor; PARP: Poly (ADP-ribose) polymerase.

sified as a Marsh 0 or I lesion according to the Modified Marsh classification^[22]. A decline of 20% or more in the percentage of aberrant IELs was considered a significant immunological remission. In addition, survival rate and EATL occurrence were evaluated during follow-up.

Furthermore, following on from Verbeek *et al.*^[10] who hypothesised that pre-treatment with common immunosuppressive drugs, including azathioprine and prednisone might influence the response to 2-CdA treatment, this study compared RCD II patients pre-treated with immunosuppressive agents before 2-CdA was prescribed (group I) to those treated with upfront 2-CdA (group II).

Ethical approval and informed consent

Approval for this open label study protocol was obtained from the local ethics committee in 2000 and all patients gave their informed consent.

Statistical analysis

Quantitative data were expressed as medians and means. Kaplan-Meier survival curves were constructed using SPSS software (SPSS Inc., Chicago, Illinois, USA). In addition, the log rank test was used to assess the statistical

significance. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

In total, 32 RCD II patients who were treated with 2-CdA were included with a median follow-up time of 31 mo (range 4-120 mo). Patient characteristics (Table 1) show a median age of > 50 years, with a male predominance. Prior to the start of 2-CdA therapy, 10 patients failed to respond clinically and histologically to conventional immunosuppressive drugs (defined as group I), including high dose prednisone in 2 and azathioprine or 6-thioguanine added to prednisone in 8 patients. The remaining 22 patients (defined as group II) were initially treated with 2-CdA following diagnosis of RCD.

Disease status

In agreement with our previous study, 2-CdA was feasible and well tolerated without serious adverse events^[10]. Overall, 18 (56%) of the RCD II patients were responsive to one or two courses of 2-CdA based on the clinical and complete histological and/or immunological response

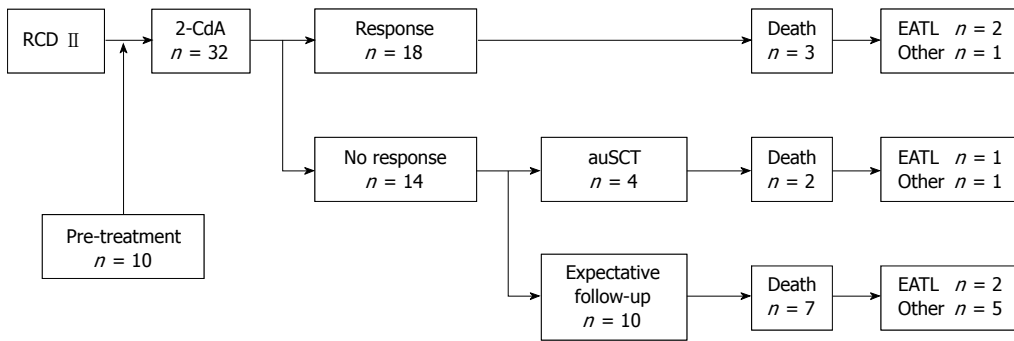


Figure 2 Flowchart of the response to cladribine treatment. RCD: Refractory celiac disease; 2-CdA: 2-chlorodeoxyadenosine; auSCT: autologous hematopoietic stem cell transplantation; EATL: Enteropathy associated T-cell lymphoma.

Table 1 Patient characteristics

Gender (M:F)	18:14
Age of CD diagnosis: median in years (range)	58.5 (38-74)
Age of RCD II diagnosis: median in years (range)	64 (42-78)
Age at the start of 2-CdA treatment: median in years (range)	64 (45-78)
Treatment prior to 2-CdA	
None	22
Immunosuppressive drugs	10
Follow-up time: median in months (range)	31 (4-120)
HLA-DQ status	
DQ2 heterozygous	17
DQ2 homozygous	12
DQ2 and DQ8	2
Unknown	1
TCR- γ gene rearrangement	
Monoclonal	18
Polyclonal	9
Unknown	5
Marsh classification before 2-CdA	
Marsh III A	13
Marsh III B	11
Marsh III C	8
Intestinal aberrant IELs before 2-CdA: median in % (range)	61 (21-96)
Body mass index before 2-CdA: median in kg/m ² (range)	21 (16-27)
Albumin level before 2-CdA: median (range, reference value 35-52 g/L)	36 (23-47)
Haemoglobin level before 2-CdA: median in mmol/L (range)	7.8 (6.0-9.8)

2-CdA: 2-chlorodeoxyadenosine; HLA: Human leukocyte antigen; TCR: T-cell receptor.

(Figure 2). Seven showed a clinical and histological response, 4 a clinical and immunological response, and 7 a clinical, histological and immunological remission. Out of the remaining 14 patients unresponsive to 2-CdA, 2 were admitted for a second course of 2-CdA in the last 6 mo and in anticipation of evaluation of response. Six non-responsive patients were evaluated for high dose chemotherapy followed by autologous hematopoietic stem cell transplantation (auSCT). In 2 of them stem cells could not be harvested, therefore 4 patients were actually transplanted^[23]. Another 6 patients had an expectative follow-up, 2 of them had an exacerbation after an initial response

Table 2 The 1- and 2-yr response rates and the median time to response in the pre-treated (I) and the upfront 2-CdA (II) group

	Treatment (n)	Response (%)		Time to a 50% response rate (mo)
		12 mo	24 mo	
Histological response	Group I	10	13	25
	Group II	22	20	58
	Overall	32	10	47
Immunological ¹ response	Group I	10	11	11
	Group II	22	52	58
	Overall	32	39	41
Clinical ² response	Group I	10	67	67
	Group II	22	95	95
	Overall	32	81	81
Overall response	Group I	10	22	38
	Group II	22	28	43
	Overall	32	25	41

¹P = 0.030 and ²P = 0.058 between group I and II.

to 2-CdA for almost 2 and 3.5 years.

Table 2 shows the clinical, histological and immunological 1- and 2-year response rates to 2-CdA treatment. In total, clinical remission was observed in 26 (81%), complete histological remission in 15 (47%) and immunological remission in 13 (41%) of the RCD II patients. The median levels of BMI, Hb and albumin increased from 20.9 kg/m², 7.8 mmol/L and 36 g/L at baseline to 23 kg/m², 7.9 mmol/L and 39 g/L at the end of follow-up, respectively. In addition to the 15 patients in whom complete histological remission, defined as Marsh 0 or I, was observed, 2 patients had a partial histological remission from Marsh 3B and 3C lesions at baseline to Marsh 2 and 3A at the end of follow-up respectively. The median percentage of intestinal aberrant IELs before 2-CdA treatment was 61% and declined to 56% after 2-CdA treatment. The time to a 50% response rate was 3 years. Approximately 33% of the patients lacked a clonal TCR-gamma gene rearrangement, although all patients had an aberrant IELs of more than 20%. A statistical significance between the percentage of aberrant IELs and the clonality status was not found.

Analysis of the overall response in patients pre-treated with immunosuppressive drugs prior to 2-CdA (group I)

and those with up-front 2-CdA treatment showed no statistical significance (log rank, $P = 0.856$). Immunological (log rank, $P = 0.030$) response, however, was significantly higher in group II. In addition, a trend towards a higher clinical response in group II (log rank, $P = 0.058$) was found. Yet, for the histological response rate a statistical significance was not observed (Table 2). Cox-regression analysis showed that the following parameters have no predictive value for response to 2-CdA: age at 2-CdA infusion ($P = 0.06$), sex ($P = 0.60$), TCR-gamma-clonality ($P = 0.604$), and percentage of aberrant IELs ($P = 0.646$), degree of small intestinal villous atrophy ($P = 0.610$), BMI ($P = 0.095$), albumin ($P = 0.936$) and Hb ($P = 0.953$) before treatment.

Survival

The overall median survival was almost 4.5 years. In total, 37% (12/32) of all included patients died, due to an EATL in 42% (5/12). All patients diagnosed with EATL (16%) died subsequently, having a median survival of only 4.4 mo (range 0-12 mo) after diagnosis.

The survival curve (Figure 3A) shows a statistically significant difference ($P = 0.037$) between responders and non-responders to 2-CdA treatment. The 3- and 5-years survival rate was 83% in the responding group and 63% and 22% in the non-responding group, respectively. One-sixth (3/18) of the responders died, 1 due to refractory disease status and 2 were diagnosed with EATL within 3 and 9 mo after the last infusion of 2-CdA. Approximately 65% (9/14) of the non-responders died, 3 as a consequence of developing an EATL within 8, 12 and 44 mo of 2-CdA treatment (Figure 2). The exceptionally delayed progression into EATL in the latter patient might be explained by treatment with high dose of chemotherapy followed by auSCT 8 mo after 2-CdA treatment. The remaining 6 unresponsive patients died as a result of refractory disease status; 1 treated with chemotherapy and auSCT died after 1 year of follow-up and 5 with expectative follow-up died despite lack of response to 2-CdA after a median follow-up of 18 mo (range: 4.5-60 mo). The patient in the latter group who died 5 years after 2-CdA treatment had an exacerbation after an initial remission to 2-CdA for 3.5 years and then refused further treatment.

In addition, there was no statistical significance ($P = 0.23$) between the overall survival in the pre-treatment group (I) and the upfront 2-CdA treatment group (II) as depicted in Figure 3B.

DISCUSSION

Approximately half of RCD II patients have an unfavourable clinical course as a consequence of non or partial response to current available therapies and subsequently the progression into EATL^[7,8]. For that reason clinicians have become increasingly interested in treating this condition alternatively and more effectively. However, there is no standardised approach reported so far. In this study a large cohort of RCD II patients treated

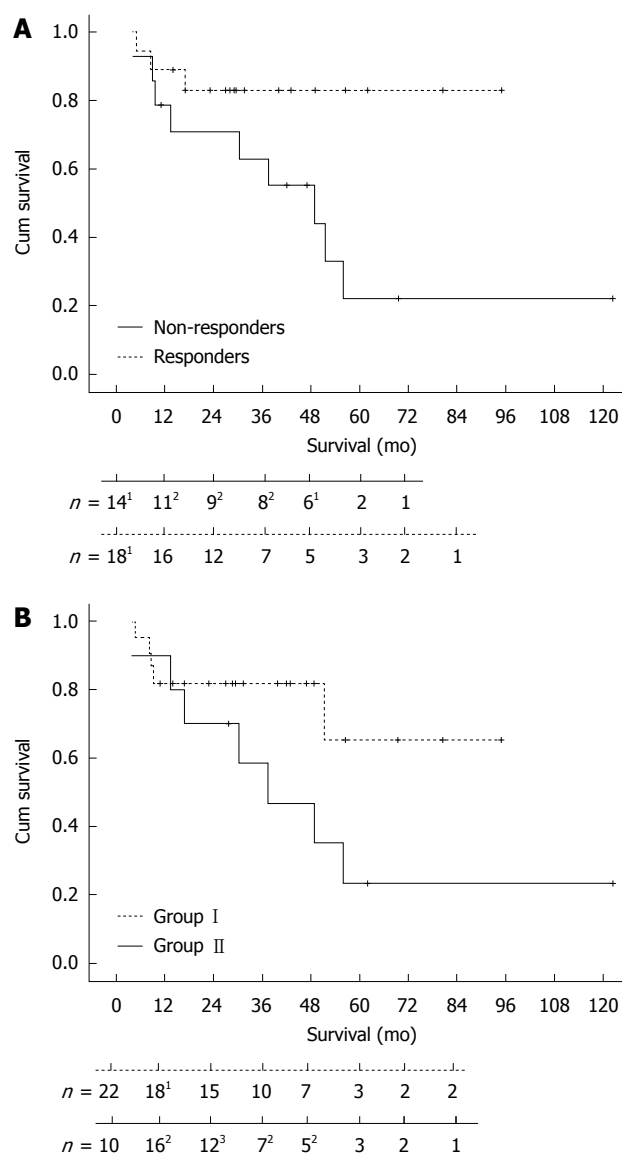


Figure 3 Kaplan-Meier survival curves. A: Survival rate of responders and non-responders to cladribine treatment (Log Rank: $P = 0.037$). ¹3 patients died in the 1st and 5th year; ²1 patient died in the 2nd, 3rd and 4th year of follow-up; B: Survival rate of the pre-treatment (I) and the upfront 2-chlorodeoxyadenosine (II) group (Log Rank: $P = 0.23$). ¹4 patients died in the 1st year; ²1 patient died in the 1st, 3rd and 4th year; ³2 patients died in the 2nd year of follow-up.

with 2-CdA has been analyzed.

In agreement with Al-Toma *et al.*^[16], this report describing almost twice as many patients with extended follow-up, showed that 2-CdA is indeed feasible and well tolerated without serious adverse events after short and long-term follow-up. Our data show that RCD II patients responsive to 2-CdA treatment have a statistically significant increased survival compared to those who are unresponsive. Although the median follow-up time was only 31 mo, after 1.5 year of follow-up none of the responders died compared to 4 non-responders, indicating a clear difference in survival rate. The overall 5-year survival rate of RCD II patients in this analysis is 46% so far, which is in line with other single-centre studies^[11,12], and is expected to improve after extended follow-up. Previous reports

included RCD II patients treated with diverse steroid-like drugs^[11,12], whereas in this study 10 RCD II patients unresponsive to such immunosuppressive drugs were included as well. However, unexpectedly the overall survival did not significantly differ between patients pre-treated with conventional immunosuppressive drugs (group I) compared to those with up-front 2-CdA treatment (group II). Nevertheless, a clearly increased median survival rate in group II was observed. The lack of statistical significance might be due to the small cohort included in group I. In addition, some of the RCD II patients treated with conventional immunosuppressive drugs died before 2-CdA treatment could be initiated as previously reported by our group^[24] and were therefore not included in the current analysis. Apart from favouring clinical course and increasing survival, preventing EATL represents the ultimate goal of treating RCD II. Although treatment with 2-CdA could not prevent progression into EATL, compared to previous reports showing EATL in 32%-52% of the RCD II patients within 5 years^[7,11], our results showed a much lower rate of lymphomagenesis (16%), yet approximately 50% had a 5-year follow-up.

Furthermore, our open-label and observational data showed that 2-CdA is an effective treatment in obtaining a clinical and immunohistological response in more than half of the RCD II patients. Previous studies reported a good clinical response after treatment, but histological response rates up to only 30% were found depending on the type of treatment^[11,12]. Consistent with these findings, a clinical response rate of 81% was found. The potential advantage of 2-CdA, however, seems to be a good histological response. In fact, histologic healing (Marsh 0 or I) was observed in almost half of the cases and a partial response of at least two Marsh scores in another 3 cases. Although not statistically significant, the 2-year histological response rate in up-front 2-CdA group (II) was twice that of the pre-treatment group (I), possibly indicating a beneficial effect of up-front 2-CdA treatment.

Furthermore, Malamut *et al.*^[11] recently described a 74% steroid-dependency in a large cohort of RCD II patients treated with corticosteroids. Since there is no need for corticosteroids if 2-CdA therapy is prescribed, steroid-dependency and its complications will not occur.

In this cohort, the proportion of RCD II patients lacking a clonal TCR-gamma gene rearrangement was relatively high (33%) compared with that reported in other studies^[4,11,12], although all patients had more than 20% aberrant IELs determined by FACS analysis. This discrepancy might be the result of some technical aspects of the DNA analysis, for instance a reduced DNA quality due to formalin fixation of the biopsies and a limited sensitivity of PCR in the case of a low percentage of aberrant IELs. Therefore, the tested polyclonal status in this cohort is most likely overestimated. However, we have previously shown^[5] that flow cytometry of aberrant IELs is superior to clonality analysis for risk stratification in RCD II.

Approximately 40% of the RCD II patients showed an immunological response after 2-CdA treatment, defined

as a decrease of more than 20% of the aberrant intestinal IELs determined by FACS analysis. However, the majority still showed more than 30% aberrant IELs during follow-up. Expansion of these aberrant T-lymphocytes, which reside in the intraepithelial as well as lamina propria layer of the small intestine^[25], is generally accepted as the culprit factor in the progression into EATL. Conversely, in our series the persisting high percentage of aberrant mucosal T-lymphocytes did not correlate with the relatively low EATL occurrence found during the time of follow-up so far. Unfortunately, reports from other centres on the immunological response determined by FACS analysis after treatment are lacking. Whereas the percentage of aberrant IELs is established to be important in the diagnostic work-up for distinguishing RCD type I and II^[5], apparently it is questionable whether this percentage is a predictive marker for progression into EATL during monitoring of the therapeutic response. Further research is mandatory to further elucidate EATL risk stratification. Immunophenotyping of the aberrant T-cells by FACS analysis of small intestinal biopsies and probably also genotyping seem to be appropriate methods to search for predictive markers. In addition, future studies on quantifying the mass of aberrant IELs using immuno-PET techniques, instead of the currently determined percentage of aberrant IELs, given the relatively equal depletion of normal and aberrant IELs upon 2-CdA treatment, have to be conducted.

In the current analysis approximately half of the RCD II patients were unresponsive to 2-CdA, for yet unknown reasons. A significant association with the degree of mucosal villous atrophy, the percentage of aberrant IELs, and levels of BMI, Hb and albumin before 2-CdA treatment, clonal TCR-gamma gene rearrangement and HLA-DQ status was not revealed. Although dose-finding studies with 2-CdA infusion in refractory celiac disease are not conducted, clinical dose-finding studies in lymphoproliferative diseases showed good response rates with an identical treatment schedule^[13,26]. In addition, compared to intravenous infusions and subcutaneous injections which provide identical plasma 2-CdA levels, oral administration has a much lower bioavailability (approximately 40%) due to degradation through acid in the stomach and intestinal bacteria^[14]. A higher dose and/or a prolonged treatment schedule might result in a higher response rate, yet the maximum tolerated dose established in lymphoproliferative diseases without serious adverse events was 0.1 mg/kg per day for 7 d^[13]. A recent clinical trial of oral cladribine for relapsing multiple sclerosis showed that short-course and high dose (3.5 mg/kg) therapy is effective, yet lymphocytopenia is frequently reported^[15]. Furthermore, the high sensitivity of hairy cell leukaemia (HCL) to treatment with 2-CdA showing low resistance levels is hypothesised to be the result of p53-dependent pathways required for killing resting cells and its inhibitory effect on the cholesterol metabolism which is highly active in HCL cells^[14]. Physiological conditions such as increased repair of DNA, increased anti-apoptotic effects and decreased activation of deoxycytidine kinase, an enzyme required for the cytotoxicity of 2-CdA, might be contributing factors

to resistance as well^[27]. Whether the same results regarding administration route, treatment schedule and resistance pattern of 2-CdA also correlate with such good response rates in RCD II, remains to be further elucidated. The registration of oral 2-CdA for multiple sclerosis in Europe, might be a further step forward towards the application of this drug in RCD as well as some other gastroenterological diseases refractory to currently available therapies, including Crohn's disease, ulcerative colitis and autoimmune hepatitis.

In conclusion, 2-CdA appears to be a promising treatment in RCD II. This analysis showed excellent clinical and histological response rates after 2-CdA treatment. Furthermore, 2-CdA therapy does not necessitate the additional use of corticosteroids and subsequently prevents steroid-dependency and its complications. Although EATL could not be fully prevented, its incidence was restricted to 16%. Multicentre, randomised clinical trials with 2-CdA and/or other new treatment options are mandatory to standardise the treatment strategy for RCD II, in order to further decrease morbidity and mortality in this patient group.

COMMENTS

Background

A small percentage (2%-5%) of patients with adult-onset celiac disease, especially those diagnosed above the age of 50, show a lack of response to a gluten-free diet. They are diagnosed as suffering from refractory celiac disease (RCD) when clinical and histological symptoms persist or recur after a former good response to a strict gluten-free diet and despite strict adherence to the diet for more than 12 mo, unless earlier intervention is necessary. RCD can be subdivided into type I with phenotypically normal and type II with aberrant intraepithelial T lymphocytes in the small intestinal mucosa. Patients with RCD I have a less dismal prognosis compared with those diagnosed as having RCD II: the 5-year survival rates are 96% and 58%, respectively. As the majority of RCD type II patients are unresponsive to common immunosuppressive therapy, it is of utmost importance to evaluate new treatment strategies. Apart from clinical and histological remission, the ultimate goal of treatment is to prevent progression into a lethal enteropathy associated T-cell lymphoma (EATL) which occurs in more than half the patients within 4-6 years.

Research frontiers

This is the first report evaluating a standardised treatment strategy for RCD type II with cladribine [2-chlorodeoxyadenosine (2-CdA)] therapy in a large cohort ($n = 32$) with a long-term follow-up (median 31 mo).

Innovations and breakthroughs

This report shows that cladribine was feasible and well tolerated without serious adverse events. More than half of the RCD II patients were responsive to cladribine therapy and had a significantly increased 3 and 5 year survival compared to those who were unresponsive. In line with previous reports, very high clinical response rates were observed. The potential advantage of cladribine, however, seems to be a good histological response. In fact, histologic healing (Marsh 0 or I) was observed in almost half of the cases and a partial response of at least two Marsh scores in another 3 cases, compared to reported histological response rates up to only 30%. Although EATL could not be fully prevented, its incidence was restricted to 17%. Furthermore, 2-CdA therapy does not necessitate the additional use of corticosteroids and subsequently prevents steroid-dependency and its complications.

Applications

The results suggest that cladribine therapy may represent a promising option for RCD II, however, multicentre randomised clinical trials with 2-CdA and/or other new treatment options are mandatory to standardise the treatment strategy for this group, in order to further decrease morbidity and mortality in this patient group.

Terminology

Aberrant IELs: Intraepithelial T lymphocytes are considered aberrant when expressing cytoplasmic CD3, but lacking surface expression of the T-cell markers CD3, CD4, CD8 and the T-cell receptor. 2-CdA: Cladribine, 2-chlorodeoxyadenosine, a synthetic purine nucleoside homologue. EATL: Enteropathy associated T-cell lymphoma. RCD: Refractory celiac disease. Immunologically, this disorder can be subdivided into two types: type I without and type II with aberrant IELs in the small intestinal mucosa. TCR: T-cell receptor.

Peer review

In this manuscript, the authors reported an open-label study on the treatment of type II refractory coeliac disease (RCD II) with cladribine (2-chlorodeoxyadenosine) in a cohort of 32 patients. After a mean follow up time of 3 years, cladribine, which was given to 22 patients as the first line treatment and 10 patients who failed to respond to conventional immunosuppressive drugs, was shown to have induced apparent clinical, histological and/or immunological response in over half of the patients. In addition, all the responders had a significantly increased 3 and 5 year survival compared to those who were unresponsive although progression of RCD-II to enteropathy associated T-cell lymphoma had not been prevented and overall 5 year survival (46%) was not different from that reported by other centres using alternative therapies. This open-label trial was uncontrolled. However, a favourable outcome was observed in most of patients treated with cladribine. The results suggest that cladribine therapy may represent a promising option for RCD II patients.

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S- Editor Wang YR L- Editor O'Neill M E- Editor Ma WH

Proximal and distal esophageal sensitivity is decreased in patients with Barrett's esophagus

Anne L Krarup, Søren S Olesen, Peter Funch-Jensen, Hans Gregersen, Asbjørn M Drewes

Anne L Krarup, Søren S Olesen, Hans Gregersen, Asbjørn M Drewes, Mech-Sense, Department of Gastroenterology, Aalborg Hospital, Aarhus University Hospital, DK-9000 Aalborg, Denmark

Peter Funch-Jensen, Department of Surgery L, Aarhus University Hospital, DK-8000 Aarhus, Denmark

Author contributions: Krarup AL, Funch-Jensen P, Gregersen H and Drewes AM designed the research; Krarup AL and Olesen SS performed the research; Krarup AL, Olesen SS, Gregersen H and Drewes AM analyzed the data; Krarup AL, Olesen SS, Funch-Jensen P, Gregersen H and Drewes AM wrote the paper.

Supported by "Hertha Christensens Fond" and "The Danish Agency for Science, Technology and Innovation"

Correspondence to: Asbjørn M Drewes, MD, PhD, DMSc, Professor, Mech-Sense, Department of Gastroenterology, Aalborg Hospital, Aarhus University Hospital, DK-9000 Aalborg, Denmark. drewes@smi.auc.dk

Telephone: +45-99326264 Fax: +45-99326507

Received: June 5, 2010 Revised: September 18, 2010

Accepted: September 25, 2010

Published online: January 28, 2011

Abstract

AIM: To investigate sensations to multimodal pain stimulation in the metaplastic and normal parts of the esophagus in patients with Barrett's esophagus (BE).

METHODS: Fifteen patients with BE and 15 age-matched healthy volunteers were subjected to mechanical, thermal and electrical pain stimuli of the esophagus. Both the metaplastic part and the normal part (4 and 14 cm, respectively, above the esophago-gastric junction) were examined. At sensory thresholds the stimulation intensity, referred pain areas, and evoked brain potentials were recorded.

RESULTS: Patients were hyposensitive to heat stimulation both in the metaplastic part [median stimulation time to reach the pain detection threshold: 15 (12-34) s vs 14 (6-23) s in controls; $F = 4.5$, $P = 0.04$] and the normal part of the esophagus [median 17 (6-32) s vs 13

(8-20) s in controls; $F = 6.2$, $P = 0.02$]. Furthermore, patients were hyposensitive in the metaplastic part of the esophagus to mechanical distension [median volume at moderate pain: 50 (20-50) mL vs 33 (13-50) mL in controls; $F = 5.7$, $P = 0.02$]. No indication of central nervous system abnormalities was present, as responses were comparable between groups to electrical pain stimuli in the metaplastic part [median current evoking moderate pain: 13 (6-26) mA vs 12 (9-24) mA in controls; $F = 0.1$, $P = 0.7$], and in the normal part of the esophagus [median current evoking moderate pain: 9 (6-16) mA, vs 11 (5-11) mA in controls; $F = 3.4$, $P = 0.07$]. Furthermore, no differences were seen for the referred pain areas (P -values all > 0.3) or latencies and amplitudes for the evoked brain potentials (P -values all > 0.1).

CONCLUSION: Patients with BE are hyposensitive both in the metaplastic and normal part of esophagus likely as a result of abnormalities affecting peripheral nerve pathways.

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Key words: Barrett's esophagus; Heat; Multimodal; Pain; Sensitivity

Peer reviewer: Jean Paul Galmiche, MD, Professor, Department of Gastroenterology and Hepatology, Hôpital Hôtel Dieu, 44093 Nantes cedex, France

Krarup AL, Olesen SS, Funch-Jensen P, Gregersen H, Drewes AM. Proximal and distal esophageal sensitivity is decreased in patients with Barrett's esophagus. *World J Gastroenterol* 2011; 17(4): 514-521 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/514.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.514>

INTRODUCTION

Barrett's esophagus (BE) is characterized by intestinalized

columnar epithelium in the esophageal mucosa, and patients have an elevated risk of developing esophageal adenocarcinoma^[1]. The etiology is still not fully elucidated, but is thought to involve excessive acid exposure^[2]. Abnormal acid exposure of the esophageal mucosa can be the result of increased reflux or slow esophageal clearing of acid. Several studies have confirmed that patients with BE have pathologic gastro-esophageal reflux and that the length of the Barrett's segment is correlated to reflux duration^[3,4]. However, the magnitude of pathological reflux in patients with BE and patients with erosive reflux disease without BE seems to be similar^[5]. Thus the progression to BE has yet to be clarified. The missing link could be slow clearance of acid and non acidic reflux from the esophagus due to a defect in the afferent (sensory) or efferent (motor) part of the reflex arch. This will reduce clearance due to less saliva production and less secondary contractions. When studying the motility related to BE, conflicting results have been found^[6-8]. More consistency is seen in studies of esophageal sensation, where most studies have reported a decreased sensitivity to esophageal acid in patients with BE^[8-11]. It is not yet known whether this change in esophageal sensation is a result of the characteristics of the metaplastic mucosa or whether it is present before development of the metaplasia. If the first assumption is true only the metaplastic part of the esophagus will be hyposensitive. However, if the hyposensitivity is a predisposing pathogenetic factor it will be expected to be present both in the metaplastic distal part and the normal proximal part of the esophagus, but this has never been investigated.

Esophageal sensitivity can be examined on several levels and with many stimulation modalities using the multimodal pain model previously developed in our group^[12]. The model has been used to study pain mechanisms in patients with erosive esophagitis, non-erosive esophagitis and non-cardiac chest pain, thereby proving its validity^[13-15]. We hypothesized that patients with BE are hyposensitive both in the metaplastic part and in the normal proximal part of the esophagus. Using the multimodal pain model in patients and healthy volunteers, the aims were as follows: (1) to compare the sensory response to thermal and mechanical pain stimuli in the metaplastic and normal part of the esophagus; and (2) to determine from the response to electrical stimuli, referred pain areas and evoked brain potentials, whether the origin of symptoms in BE is suggestive of peripheral or central nervous system (CNS) abnormalities.

MATERIALS AND METHODS

Ethics

The study was conducted according to the Declaration of Helsinki and approved by the local Ethical Committee (VN 2003/120mch). Oral and written informed consent was obtained from all participants.

Selection of patients

Fifteen patients with BE aged 18-70 years were selected

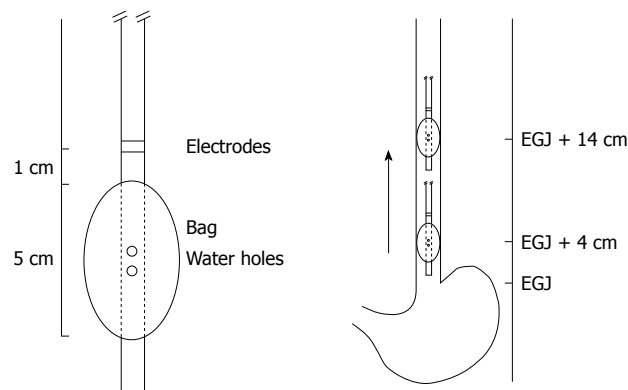


Figure 1 Probe design and probe placement in the esophagus during the study. After stimulation 4 cm above the esophago-gastric junction (EGJ) the probe was retracted 10 cm and the remaining protocol completed. Inlet and an outlet channels were used for circulation of water during mechanical and thermal stimulation. Electrodes were used for electrical stimulation (see text for details).

from our outpatient unit. All patients had typical endoscopic features of BE confirmed by histology within the last 2 years. Patients with a hiatus hernia rated by endoscopists as moderate or large were excluded as were patients who previously had esophageal surgery. In addition, we excluded patients with concomitant painful visceral diseases or other critical medical, surgical, or psychiatric illness. Patients were compared to a control group of 15 age- and sex-matched healthy volunteers.

Sensory assessment

Before the study all subjects were instructed in the use of a 0-10 visual analogue scale, where 1 = sensation threshold; 3 = vague perception of moderate sensation; 5 = pain threshold; and 7 = moderate pain. This scale has previously been described in detail, and has been shown to be reliable in discriminating sensations in the esophagus^[16]. The stimulation intensities at these defined sensory levels were measured for all stimulation modalities.

Probe design

The multimodal probe is illustrated in Figure 1. A custom-made esophageal probe measuring 60 cm in length with a diameter of 6.2 mm was fitted with a bag near the tip (Ditens A/S, Aalborg, Denmark). The bag was made of 25 μ m thick polyether urethanes had a maximum volume of 80 mL and was 5 cm long. An inlet and an outlet channel permitted recirculation of water in the bag during mechanical and thermal stimulation. A temperature wire with a tip sensor was placed in a separate channel for continuous temperature assessment during stimulation (TC Ltd., Uxbridge, England). Stainless steel electrodes for electrical stimuli were mounted on the probe 1 cm proximal to the bag. Detailed information on the probe and stimulation system has been described previously^[12].

Protocol

No analgesics or other medication potentially interfering with pain assessments were allowed in the 24 h prior to

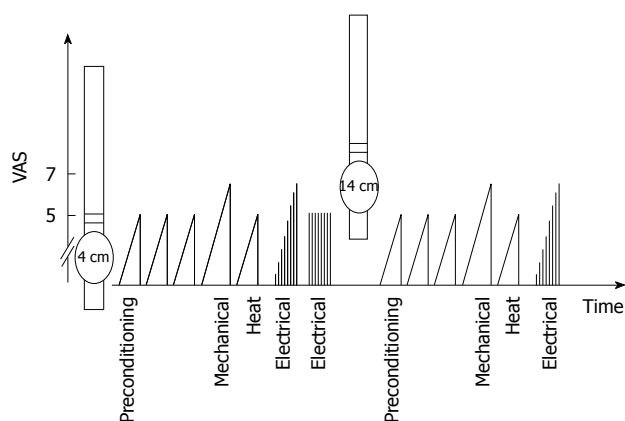


Figure 2 Schematic illustration of the stimulation protocol. The following stimulations were applied in the metaplastic part 4 cm above the esophago-gastric junction: three mechanical preconditioning stimuli to the pain threshold [visual analogue scale (VAS) = 5]; mechanical stimulation to moderate pain (VAS = 7); thermal stimulation to the pain threshold; electrical stimulation to moderate pain; 50 electrical stimulations at the pain threshold were done to record evoked brain potentials. The mechanical, thermal and electrical stimulations were repeated 14 cm above the sphincter.

experimental testing. Treatment with proton pump inhibitors was stopped 4 d before the study day and the subjects fasted 4 h prior to probe intubation. The probe was inserted through the mouth. The bag close to the tip of the probe was positioned in the stomach and filled with 25 mL water. Retraction of the probe until resistance was met identified the position of the esophago-gastric junction (EGJ) corresponding to the middle of the bag. Finally, the bag was emptied and retracted 4 cm to ensure placement above the EGJ and the probe was taped to the chin.

Figure 2 shows the stimulation protocol. Initially, three mechanical distensions were applied to precondition the tissue and to train the subjects in assessing sensations from the esophagus. These distensions were stopped at the pain threshold and have previously been shown to facilitate discrimination of the different sensory ratings^[17]. Hereafter, pain tests were initiated: mechanical distension until moderate pain, heat stimulation to the pain threshold, and electrical stimulation until moderate pain. The participants outlined the area of referred pain after each stimulation type. Recordings of pain-specific evoked brain potentials were performed twice following 50 electrical stimulations at the pain threshold. The probe was then retracted 10 cm corresponding to 14 cm above the EGJ, and the mechanical, thermal and electrical stimulations were repeated after a break of 10 min.

Mechanical stimulation

Mechanical distension was done by inflating the bag with water (37°C) at a rate of 15 mL/min using a syringe pump (PHD 22/2000, Harvard Apparatus, Holliston, Massachusetts, USA). The maximum volume was set at 50 mL for safety reasons. The bag cross-sectional area, pressure and volume were continuously registered and stored for later analysis. If the maximum of 50 mL failed to evoke

moderate pain, the remaining sensory levels were assigned a volume of 50 mL.

Heat stimulation

Heat stimulation was done by recirculating heated water (60°C) using the above described pump at a rate of 180 mL/min. The stimulation was terminated at the pain threshold as a safety precaution and has been described in detail previously^[18]. Time from stimulation onset until the patient reached the predefined sensory levels was measured.

Assessment of the CNS response

The sensory response to (1) electrical stimulation; (2) the size of the referred pain area; and (3) the pain specific evoked brain potentials served as proxies for abnormalities in CNS pain processing. When the CNS is sensitized clinically or experimentally these measurements change with a decrease in pain threshold, increase in referred pain area and shortened latency of the evoked brain potential^[18-20].

Electrical stimulation: Mucosal contact was secured by keeping the inter-electrode impedance below 3 kΩ during stimulations. A computer-controlled constant-current stimulator (University of Aalborg, Denmark) delivered the electrical stimulations to the esophagus^[12]. A single stimulus consisted of five square-wave constant-current pulses at 200 Hz with duration of 1 ms, and each stimulation in total had a duration of 25 ms. The current intensity was increased in 0.5 mA increments with random sham stimulations having the same or lower intensity until moderate pain was evoked. Calculations were based on current at the predefined sensory levels.

Referred pain area: After each stimulation the subjects outlined their referred pain area on transparent paper. The size of the referred pain area was later digitized (Trust, 1200 wireless tablet, Trust International BV, Dordrecht, The Netherlands).

Electroencephalographic recordings and evoked brain potentials

The electroencephalographic (EEG) was recorded with a 64 surface electrode cap (Quick-Cap, Neuroscan, El Paso, TX, USA) and an amplifier (Nuamp, Neuroscan, Compumedics, Hamburg, Germany). Four additional electrodes for eye movement detection were used. Electrode gel was applied to reduce the electrode impedance below 8.2 kΩ.

Fifty identical esophageal stimulations were applied at 0.2 Hz with a current intensity corresponding to the pain threshold. The subjects relaxed quietly with open eyes in light-dimmed room. The EEG signals were recorded in continuous mode with a sampling rate of 1000 Hz and the online notch filter switched off (SynAmp, Neuroscan, El Paso, TX, USA).

The evoked potentials from each session were analyzed offline (Neuroscan software v 4.3.1, Neuroscan, El Paso, TX, USA) and stimulations containing artefacts (from eye

Table 1 Stimulation intensities to evoke the predefined sensory levels on the visual analogue scale while stimulating with distension, heat and electricity 4 and 14 cm above the esophago-gastric junction, median (range)

VAS level	Patients				Controls			
	1	3	5	7	1	3	5	7
Heat (s)								
4 cm ¹	12 (7-18)	14 (11-20)	15 (12-34)	NA	11 (5-18)	13 (6-20)	14 (6-23)	NA
14 cm ¹	12 (5-14)	15 (6-24)	17 (6-32)	NA	9 (4-17)	11 (7-18)	13 (8-20)	NA
Mechanical (mL)								
4 cm ¹	11 (1-38)	25 (12-50)	36 (18-50)	50 (20-50)	8 (2-24)	21 (6-50)	28 (10-50)	33 (13-50)
14 cm	5 (3-17)	14 (9-26)	18 (10-34)	25 (11-46)	5 (1-27)	14 (4-40)	18 (8-44)	19 (10-46)
Electrical (mA)								
4 cm	7 (4-17)	9 (5-21)	12 (6-25)	13 (6-26)	7 (4-15)	8 (5-20)	10 (8-21)	12 (9-24)
14 cm	4 (2-6)	5 (3-10)	7 (5-13)	9 (6-16)	4 (2-9)	6 (4-12)	8 (4-13)	11 (5-15)

¹Overall statistically significant difference between patients and controls. Heat stimulation is expressed as stimulation time to reach the thresholds. On the visual analogue scale, 1 = the sensation threshold, 3 = vague perception of moderate sensation, 5 = the pain threshold, and 7 = moderate pain. VAS: Visual analogue scale; NA: Not applicable.

blinking or movements) were rejected. Amplitudes and latencies for the evoked potentials were obtained from the vertex electrode (Cz) with the electrodes above the ears (TP7 and TP8) as reference. Data was processed in the following steps: (1) zero-phase notch filtering (49-51 Hz) with a filter order of 24; (2) zero-phase band-pass filtering (1-70 Hz) with a filter order of 12; (3) epoching in the time window 50 ms prestimulus to 350 ms post-stimulus; (4) baseline correction; (5) linear detrending; (6) rejecting artefact sweeps manually, and (7) calculating average of accepted sweeps. Peak latencies to negative (N) and positive (P) deflections (N1, P1, N2, and P2) and peak-to-peak amplitudes were extracted for each subject.

Statistics

Normality was checked by QQ-plots and the assumption of variance homogeneity by the Levene median test. Descriptive statistics for data that were normally distributed are reported as mean (standard deviation), not normally distributed data as median (range). The Student *t*-test was used for measurements with single endpoints and the Mann-Whitney Rank Sum test for non-parametric data. Two-way analysis of variance was used for measurements where stimulus intensities were measured at several sensory thresholds. The Holm-Sidak method was chosen for *post hoc* comparisons^[21]. The chi-square test compared the dichotomous data. SigmaPlot 11.0 statistical software was used and *P* < 0.05 was considered statistically significant.

RESULTS

Thirty-three patients were invited to participate: 17 patients declined and 16 accepted. One of the 16 patients could not tolerate the probe and was excluded. The 15 remaining patients (12 male) had a mean age of 54 years (range, 25-68 years) and mean body mass index (BMI) of 30 kg/m² (range, 23-56 kg/m²). Seven patients had long segment BE and the remaining patients had short segment BE, e.g. less than 3 cm. Three patients did not complete the protocol 14 cm above the EGJ due to nausea evoked by the mechanical distension. Mechanical

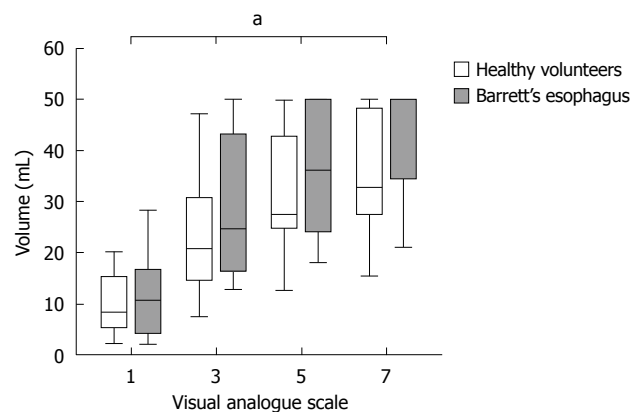


Figure 3 Mechanical stimulation evoking moderate pain in patients with Barrett's esophagus and healthy volunteers. Patients were hyposensitive to mechanical stimulation 4 cm above the esophago-gastric junction compared to healthy volunteers, *P* = 0.02. On the visual analogue scale, 1 = the sensation threshold, 3 = vague perception of moderate sensation, 5 = the pain threshold, and 7 = moderate pain. ^a*P* < 0.05.

and thermal data from one patient were discarded due to technical errors.

The patients were compared to 15 healthy volunteers (10 male) with a mean age of 47 years (range, 28-63 years) (*P* = 0.07), and mean BMI of 25 kg/m² (range, 19-30 kg/m²) (*P* = 0.1). Volunteers were recruited among hospital staff and had no gastrointestinal symptoms. All volunteers completed the protocol. No adverse events were observed.

BE patients were hyposensitive to distension and heat in the metaplastic segment

It was possible to evoke moderate pain in 13 of 15 healthy volunteers compared to only 5 of 14 patients when stimulating mechanically in the metaplastic part 4 cm above the EGJ (*P* = 0.01). Four of these hyposensitive patients, but none of the controls, responded with nausea or vomiting rather than pain (*P* = 0.03). To achieve moderate pain, patients with BE required higher mechanical stimulus intensity compared to healthy volunteers (*F* = 5.7, *P* = 0.02; Table 1, Figure 3). Furthermore, patients required heat

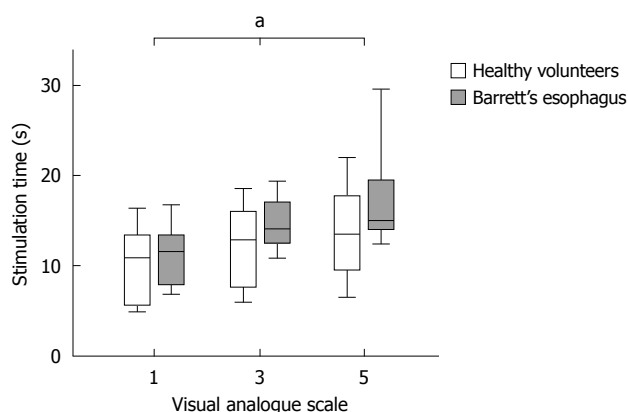


Figure 4 Response to heat stimulation in patients with Barrett's esophagus compared to healthy volunteers. Patients were hyposensitive to heat stimulation 4 cm above the esophago-gastric junction ($P = 0.04$). On the visual analogue scale, 1 = the sensation threshold, 3 = vague perception of moderate sensation, 5 = the pain threshold. $^aP < 0.05$.

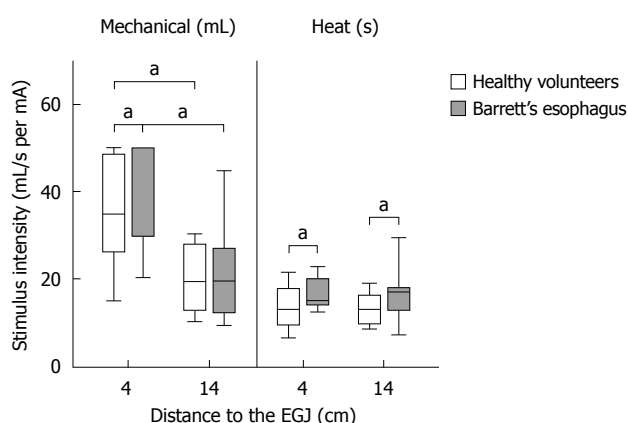


Figure 5 Response to mechanical and thermal stimulations at maximum pain in patients with Barrett's esophagus and healthy volunteers. Patients were hyposensitive to mechanical and thermal stimuli 4 cm above the esophago-gastric junction (EGJ). Heat hyposensitivity was also found in the normal part of the esophagus 14 cm above the EGJ. Both patients and controls were less sensitive to mechanical stimulation 4 cm above the EGJ, whereas the sensitivity for heat was the same on the two levels. Numbers on the X-axis indicate the level of stimulations 4 and 14 cm above the EGJ. $^aP < 0.05$.

stimulation for a longer time to reach the pain threshold ($F = 4.5$, $P = 0.04$; Table 1, Figure 4).

Patients were hyposensitive to heat in the normal mucosa

Patients with BE were also hyposensitive to heat in the normal part of esophagus 14 cm above the EGJ ($F = 6.2$, $P = 0.02$), but not to mechanical distension ($F = 0.2$, $P = 0.6$) (Table 1, Figure 5).

Lower esophagus is less sensitive to mechanical but not to heat stimulation

Both patients and controls were less sensitive to mechanical pain stimulation 4 cm above compared to 14 cm above the EGJ (patients with BE: $F = 26.9$, $P < 0.001$; controls: $F = 25.2$, $P < 0.001$) (Table 1, Figure 5). In contrast, no differences in sensitivity were found for thermal pain

Table 2 Traditional peaks in evoked brain potentials as response to esophageal electrical stimulations in patients and controls, mean \pm SD

	Patients	Controls
N1 latency (ms)	76 (20)	69 (14)
P1 latency (ms)	105 (19)	106 (15)
N2 latency (ms)	177 (15)	172 (25)
P2 latency (ms)	255 (35)	266 (39)
N1P1 amplitude (mV)	2.5 (2.5)	3.0 (1.7)
P1N2 amplitude (mV)	6.8 (5.1)	9.3 (5.5)
N2P2 amplitude (mV)	7.6 (5.9)	12.6 (7.6)

There were no overall differences between groups ($F = 2.7$, $P = 0.1$).

stimuli comparing stimulations 4 cm and 14 cm above the EGJ (patients with BE: $F = 0.3$, $P = 0.6$; controls: $F = 1.1$, $P = 0.3$) (Table 1, Figure 5).

BE patients had normal central pain processing

Electrical stimulation: No differences between patients and controls were found for the response to electrical pain stimuli in the metaplastic part 4 cm above the EGJ ($F = 0.1$, $P = 0.7$) (Table 1). Furthermore, no differences were found for the normal area 14 cm above the EGJ ($F = 3.4$, $P = 0.07$) (Table 1). Within groups, the area 4 cm above the EGJ was less sensitive to current compared to the area 14 cm above (patients with BE: $F = 32.5$, $P < 0.001$; controls: $F = 21.7$, $P < 0.001$) (Table 1).

Referred pain areas: Referred pain areas to the following stimulations did not differ between patient and control groups in the metaplastic part: mechanical [median 8.9 cm^2 (0.8-66.6) *vs* controls 14.9 cm^2 (2.3-38.6), $P = 0.5$], thermal [mean 23.7 cm^2 (20.7) *vs* controls 16.0 cm^2 (14.8), $P = 0.3$], and electrical [median 2.3 cm^2 (0.1-33.4) *vs* controls 3.2 cm^2 (0.2-40.7), $P = 0.7$]. Also in the upper part of the esophagus the results from both patients and controls were comparable: referred pain area in response to mechanical [median 14.5 cm^2 (2.1-72.9) *vs* controls 10.0 cm^2 (1.0-53.5), $P = 0.4$], thermal [mean 23.2 cm^2 (22.8) *vs* controls 19.5 cm^2 (23.2), $P = 0.5$], and electrical stimulation [median 20.6 cm^2 (2.9-70.0) *vs* controls 15.1 cm^2 (1.2-92.2), $P = 1.0$].

Recordings of evoked brain potential: No differences were found between patients and controls for the evoked brain potentials ($F = 2.7$, $P = 0.1$) (Table 2).

DISCUSSION

In this study the main findings were that patients with BE were hyposensitive to heat stimulation both in the metaplastic and normal parts of the esophagus. Furthermore, they were hyposensitive to noxious mechanical distension in the metaplastic part of the esophagus and many reported nausea rather than pain in response to noxious stimulations. The hyposensitivity is most likely a result of abnormalities affecting peripheral nerves, since there were no differences between groups for the response to

the stimuli reflecting central pain processing.

In clinical assessment, characterization of symptoms may be confounded by psychological, cognitive, and social aspects of the illnesses. To encompass these problems, human experimental pain models have been developed. In this study, we used one of these models: the multimodal pain model that has proven reliable as an esophageal research tool^[18,22]. An abnormal response to one or more of the stimulation modalities indicate pathology in different receptors or visceral nerve pathways^[23,24]. Validity has been proven in studies of pain mechanisms in erosive esophagitis, non-erosive esophagitis and non-cardiac chest pain proving its validity^[13-15]. Patients with both short and long segment BE were included. When stimulating the "metaplastic area", the balloon reached from 1.5-6.5 cm above the EGJ, therefore patients with short segment BE were stimulated partly in an area with normal mucosa and partly in the metaplastic area. However, as the patients were hyposensitive both low and high in the esophagus, bias was not a problem in the present study. The controls were slightly but non significantly leaner than the patients, but this is not thought to have influenced the results.

Thermal sensation

To our knowledge, studies examining thermal sensation in BE have not previously been conducted. In the current study, patients were hyposensitive both in the metaplastic part of the esophagus as well as in the normal control area 14 cm above the EGJ. The hyposensitivity in BE patients is interesting as these patients often have pathological reflux and could be expected to have sensory thresholds comparable to patients with gastro-esophageal reflux disease. However, both patients with erosive and non-erosive reflux disease have previously been shown to be hypersensitive to heat and have signs of CNS abnormalities underlining the differences between them and patients with BE^[13,15]. The hyposensitivity to heat in the normal part of the esophagus in BE patients suggests that heat hyposensitivity may be generalized in the esophagus and therefore independent of the metaplastic mucosa. Hence, it can be hypothesized that dysfunction of the afferent pathways may be one of several pathogenetic factors in BE. This fits well with previous studies that have demonstrated hyposensitivity to acid in patients with BE, and several acid sensitive receptors could potentially be involved^[5,9,10,25]. One of them - the transient receptor potential vanilloid 1 (TRPV1) - is of special interest as it is activated by heat and acid^[26]. Although speculative, the hyposensitivity to both heat and acid found in patients with BE could therefore be due to a defect or downregulation of TRPV1 receptors in the esophagus. A study involving assessment of heat and acid sensitivity as well as TRPV1 quantification in biopsies could elucidate this further.

Mechanical sensation

Trimble and coworkers found that patients with BE were mechanically hyposensitive, but their data may have been

biased by the large age difference between the patients and the control group^[27,28]. Compared with an age-matched control group we also found patients with BE to be hyposensitive to mechanical stimulation in the metaplastic area 4 cm above the EGJ. Some patients developed nausea rather than pain in response to noxious esophageal distension. It should be kept in mind that nausea rather than pain for some BE patients may be their only "warning response" to noxious distension, and further studies are needed to establish if nausea is a predictor of serious disease.

We found no differences for the response to mechanical distension between patients and controls in the area with normal mucosa 14 cm above the EGJ. There were four dropouts and a statistical type 2 error could be responsible. Hence, it is not possible to conclude from this study whether mechanical hyposensitivity is present in the normal part of the esophagus.

No indication of central sensitisation

Changes in the sensory response to electrical stimulation, referred pain areas, and evoked brain potentials has been shown to reflect changes in the CNS, such as seen experimentally after esophageal acid perfusion^[18-20]. Electrical stimulation bypasses the receptors stimulating the nerve fibers directly, and the sensitivity increases after sensitization. Referred pain areas increase, possibly as a result of stimulation of sensitized neurons receiving converging neural information from the esophageal nerves and the somatic nerves in the chest. Pain specific EEG recordings evoked brain potentials are believed to change due to faster spinal conduction and altered brain processing. Such changes have been reported in erosive reflux disease, non-erosive reflux disease and non-cardiac chest pain, where central sensitization is thought to play a role in symptom generation^[24,29]. We found comparable results in patients with BE and controls to electrical stimulation, referred pain areas, and waveforms of evoked brain potentials. Consequently, the cause of sensory changes in patients with BE is probably not found in the CNS, but rather at receptor level in the peripheral nervous tissue.

The distal oesophagus is less sensitive

We measured differences between the sensory responses evoked 4 and 14 cm above the EGJ and found that both the patients and the controls were more sensitive to mechanical and electrical stimuli 14 cm above the EGJ. Patel *et al.*^[30] previously found the same in healthy volunteers for mechanical stimulation and an explanation could be a greater number of nociceptors and mechanoreceptors proximal in the esophagus as seen in animal studies^[31,32]. However, other nociceptors seem to be responsible for heat sensation as no differences in heat sensitivity was observed at the two esophageal levels.

In conclusion, patients with BE are hyposensitive both in the metaplastic and normal part of the esophagus likely as a result of abnormalities affecting peripheral nerves. Hence, hyposensitivity is likely not to be a consequence of sensory changes due to the metaplastic epithelium, but

may be a pathogenetic factor in the development of the disease.

ACKNOWLEDGMENTS

The authors acknowledge the extensive work done by research nurses Karen-Marie marstal, Heidi Stenstrup, Isabelle Larsen, and Birgit Koch-Henriksen

COMMENTS

Background

Barrett's esophagus (BE) is a complication of long-term severe acid reflux to the esophagus and patients are at higher risk of developing esophageal cancer. In several studies it has been demonstrated that patients with BE are less sensitive to acid than healthy controls. It is not known if this decreased sensitivity precedes the development of the condition or if it is a result of the mucosa changes of the disease. This study investigated the sensitivity in the esophagus of patients with BE in comparison to healthy controls. The esophagus was examined both in the part where the disease was present and in the normal part. Furthermore, the pain processing of the spinal cord and brain was examined.

Innovations and breakthroughs

In the patients, a decreased sensitivity to heat both in the normal part and the diseased part of esophagus was demonstrated. Distension was also felt later in the diseased part of esophagus. The spinal cord and brain was not responsible for the decreased sensitivity as they had a normal reaction to esophageal pain. The decreased sensitivity in the normal part of the esophagus could indicate that sensory changes are present before development of BE.

Applications

Results of this study has led to a better understanding of the disease and may, in the future, help to identify subjects with a higher risk of developing this disease. When identified, a more aggressive treatment of their acid reflux can be chosen and perhaps thereby prevent development of BE.

Terminology

BE is a metaplastic mucosal change in the distal esophagus resulting from long term acid exposure.

Peer review

This is an excellent work, carefully done and very well interpreted. The data are extremely original and represent a major breakthrough in the understanding of hyposensitivity in BE.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Zheng XM

T2* magnetic resonance imaging of the liver in thalassemic patients in Iran

Farhad Zamani, Sara Razmjou, Shahram Akhlaghpour, Seyyedeh-Masoomeh Eslami, Azita Azarkeivan, Afsaneh Amiri

Farhad Zamani, Sara Razmjou, Afsaneh Amiri, Gastroenterology and Liver Disease Research Center, Firoozgar Hospital, Tehran University of Medical Sciences, Tehran 0098-3137875661, Iran

Shahram Akhlaghpour, Department of Computed Tomography, Noor Medical Imaging Center, Tehran 0098-3137875661, Iran

Seyyedeh-Masoomeh Eslami, Clinic of Thalassemia, Imam Reza Hospital, Amol 0098-3137875661, Iran

Azita Azarkeivan, Research center of Iranian Blood Transfusion Organization, Tehran 0098-3137875661, Iran

Author contributions: Zamani F designed the study; Zamani F, Akhlaghpour S, Eslami SM and Azarkeyvan A performed the research; Razmjou S, Akhlaghpour S and Amiri A analyzed the data and wrote the paper.

Supported by The Gastrointestinal and Liver Disease Research Center of Tehran University of Medical Sciences

Correspondence to: Dr. Farhad Zamani, Associate Professor, Gastroenterology and Liver Disease Research Center, Firoozgar Hospital, Valadi Street, Valiasr Square, Tehran 0098-3137875661, Iran. zamani.farhad@gmail.com

Telephone: +98-21-1547311 Fax: +98-21-88942622

Received: August 18, 2010 Revised: September 27, 2010

Accepted: October 3, 2010

Published online: January 28, 2011

Abstract

AIM: To investigate the accuracy of T2*-weighted magnetic resonance imaging (MRI T2*) in the evaluation of iron overload in beta-thalassemia major patients.

METHODS: In this cross-sectional study, 210 patients with beta-thalassemia major having regular blood transfusions were consecutively enrolled. Serum ferritin levels were measured, and all patients underwent MRI T2* of the liver. Liver biopsy was performed in 53 patients at an interval of no longer than 3 mo after the MRIT2* in each patient. The amount of iron was assessed in both MRI T2* and liver biopsy specimens of each patient.

RESULTS: Patients' ages ranged from 8 to 54 years with a mean of 24.59 ± 8.5 years. Mean serum ferritin level was 1906 ± 1644 ng/mL. Liver biopsy showed a moderate negative correlation with liver MRI T2* ($r = -0.573$, $P = 0.000$) and a low positive correlation with ferritin level ($r = 0.350$, $P = 0.001$). Serum ferritin levels showed a moderate negative correlation with liver MRI T2* values ($r = -0.586$, $P = 0.000$).

CONCLUSION: Our study suggests that MRI T2* is a non-invasive, safe and reliable method for detecting iron load in patients with iron overload.

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Key words: T2*-weighted magnetic resonance imaging; Liver; Iron overload; Major thalassemia; Ferritin

Peer reviewer: Dr. Paul Sharp, BSc (Hons), PhD, Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London, SE1 9NH, United Kingdom

Zamani F, Razmjou S, Akhlaghpour S, Eslami SM, Azarkeivan A, Amiri A. T2* magnetic resonance imaging of the liver in thalassemic patients in Iran. *World J Gastroenterol* 2011; 17(4): 522-525 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/522.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.522>

INTRODUCTION

Conventional treatment of beta-thalassemia major requires regular blood transfusions to maintain pre-transfusion hemoglobin level above 90 g/L^[1]. A major drawback of this treatment is transfusion siderosis, which, in association with the increased intestinal iron absorption, apoptosis of the erythroid precursors and peripheral hemolysis,

leads to inexorable iron accumulation in various organs such as the heart, liver and endocrine organs^[2]. The assessment of body iron is still dependent upon indirect measurements, such as levels of serum ferritin, as well as direct measurements of the liver iron content^[3]. Serum ferritin has been widely used as a surrogate marker but it represents only 1% of the total iron pool, and as an acute phase protein, it is not specific because the levels can be raised in inflammation (e.g. hepatitis) and liver damage^[4]. Liver iron concentration measured by needle biopsy is the gold standard for evaluation of siderosis. However, it is an invasive technique which is not easily repeated and its accuracy is greatly affected by hepatic inflammation-fibrosis and uneven iron distribution^[4]. More recently, biomagnetic susceptometry and magnetic resonance imaging (MRI) have been validated for measuring iron overload, and these techniques have great merit in being noninvasive^[5]. Biomagnetic susceptometry is a non-invasive, well calibrated and validated method as a quantitative measurement technique, but it has limited clinical value because of its high cost and technical demands^[6]. MRI has been considered a potential method for assessing tissue iron overload, as iron accumulation in various organs causes a significant reduction in signal intensity stemming from a decrease in the T2 relaxation time^[7,8].

The objective of the present study was to report our experience of the MRI technique in assessing hepatic iron overload in thalassemic patients.

MATERIALS AND METHODS

Between January 2008 and April 2009, 210 patients with beta-thalassemia major (114 females, 96 males) referred to the thalassemia clinic of Firuzgar Hospital were consecutively enrolled in this cross-sectional study. Ages ranged from 8 to 54 years with a mean of 24.59 ± 8.5 years.

Patients were treated conventionally with regular blood transfusion, in order to maintain the pre-transfusion hemoglobin concentration above 90 g/L. Regarding chelation therapy, all patients were receiving deferoxamine at a dose of 40 mg/kg, 5-7 times per week, by 8-hourly subcutaneous infusion.

The study was approved by the Institutional Review Board Ethics Committee of Iran University of Medical Sciences and written informed consent was obtained from all patients for the procedures studied.

Serum ferritin

A 5 mL blood sample was obtained from each patient for routine laboratory tests and measurement of ferritin level. Serum ferritin concentrations were assayed in all patients before the MRI scan, using an enzyme-linked radioimmunoassay method (Monobind Kit, USA).

Liver biopsy

Liver biopsy was performed with a 16-gauge Tru-Cut needle (TSK Laboratory, Japan) in 53 patients who gave written informed consent to undergo the biopsy for this

study. Each specimen was at least 2 cm in length. The specimens were kept in 10% formaldehyde solution, and were sent to the Department of Pathology of Firuzgar Hospital. The specimens were stained with hematoxylin & eosin and viewed by an expert pathologist. The amount of stainable iron was graded 0-4 according to the Scheuer *et al.*^[9] method.

It is notable that the interval between liver biopsy and MRI of the liver and heart was less than 3 mo in all patients.

MRI technique

MRI scans were performed using a 1.5 Tesla Magnetom Siemens Symphony scanner (Siemens Medical Solution, Erlangen, Germany). Each scan lasted about 10-15 min and included the measurement of hepatic and myocardial T2* quantities. A standard quadrature radiofrequency body coil was used in all measurements for both excitation and signal detection. Respiratory triggering was used to monitor the patients' breathing. Cardiac electrocardiographic gating was used. Spatial presaturation slabs were used to suppress motion-related artifacts.

The MRI T2* of the liver was determined using a single 10 mm slice through the center of the liver scanned at 12 different echo times (TE 1.3-23 ms). Each image was acquired during an 11-13 s breathhold using a gradient-echo sequence (repetition time 200 ms, flip angle 20°, base resolution matrix 128 pixels, field of view 39.7 cm × 19.7 cm, sampling bandwidth 125 kHz).

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences, version 15 for Windows™ (SPSS® Inc., Chicago, IL). Continuous variables are presented as mean \pm SD and count (percent) for categorical variables. The relationship between continuous variables was evaluated by the Pearson correlation coefficient for normally distributed data and Spearman's Rank correlation coefficient for non-normally distributed data. All tests of significance were two-tailed and considered to be significant at $P < 0.05$.

RESULTS

All patients underwent MRI and 53 patients had a liver biopsy. The mean serum ferritin level of all patients was 1906 ± 1644 ng/mL. Serum ferritin levels showed a moderate negative correlation with liver T2* MRI values ($r = -0.586$, $P = 0.000$) (Figure 1). Of the 53 patients who had a liver biopsy, 5 patients had grade I liver siderosis, 19 had grade II, 17 had grade III and 12 had grade IV liver siderosis. The degree of siderosis assessed by liver biopsy showed a moderate negative correlation with liver T2* MRI ($r = -0.573$, $P = 0.000$) and a low positive correlation with ferritin level ($r = 0.350$, $P = 0.001$).

DISCUSSION

It is evident that different non-invasive methodologies

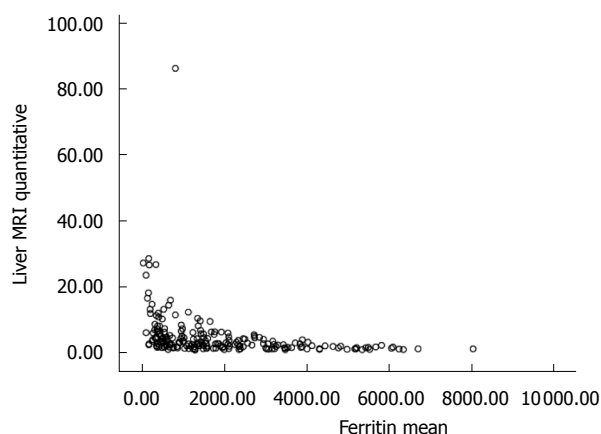


Figure 1 Serum ferritin levels showed a moderate negative correlation with liver T2* magnetic resonance imaging values. MRI: Magnetic resonance imaging.

have been implemented for the detection of organ-specific iron burden in patients with thalassemia major. Among these, MR relaxometry has the potential to become the method of choice for non-invasive, safe and accurate assessment of organ-specific iron load^[10]. Until recently serum ferritin levels and liver biopsy have been the most commonly used methods for estimating body iron stores in the thalassemic population. However, ferritin levels are not fully acceptable because there have been significant variations due to inflammation, infections and chronic disorders^[3].

Liver iron load vs serum ferritin

We found a moderate correlation ($r = -0.59$, $P < 0.001$) between serum ferritin levels and hepatic T2* levels. These findings are compatible with other reported studies with highest correlation^[3,11,12]. Attempts to correlate serum ferritin levels and hepatic iron concentrations have failed to demonstrate a linear relationship between the two parameters^[11].

Histological grade of siderosis vs liver T2*

Liver biopsy has been regarded as the most precise method to measure body iron content if direct measurement of the iron concentration was applied. However, this is an invasive procedure which is not available in most clinical settings.

Our results revealed no reasonable correlation between histological grade of siderosis (HGS) and serum ferritin. However, a moderate correlation with liver T2* ($r = 0.57$, $P < 0.001$) indicated that HGS could still be considered as a method of evaluating thalassemic patients. Liver iron concentration showed significant correlation with hepatic T2* ($r > 0.9$). These results indicated that MRI T2* measurement is of more value than HGS in thalassemic patients.

The most important limitation of our study was the lack of a consideration of intervening factors that may affect the serum ferritin levels such as C-reactive protein,

white blood cell count and liver function tests.

In conclusion, although hepatic iron content and serum ferritin levels have been considered as the gold standards in evaluating body iron load for several years, iron accumulation in different organs proceeds independently. This emphasizes the importance of direct iron load measurement in each involved organ and direct evaluation of the efficacy of different therapeutic measures.

According to our study, the serum ferritin level is not a reliable method for estimating the level of iron overload in thalassemic patients. MRI T2* is a more accurate and non-invasive method which we recommend for measurement of iron load in these patients.

COMMENTS

Background

Iron overload is a common and serious problem in thalassemic major patients. As iron accumulation is toxic in the body's tissues, accurate estimation of iron stores is of great importance in these patients to prevent iron overload by an appropriate iron chelating therapy.

Research frontiers

Liver biopsy is the gold standard for evaluating iron stores but it is an invasive method which is not easily repeatable in patients. Introduction of other more applicable methods seems to be necessary.

Innovations and breakthroughs

The authors found that MRI T2* might be an accurate method for estimation of whole body iron.

Applications

According to the findings, the authors suggest that clinicians consider MRI as an accurate method to evaluate the iron overload in patients with thalassemia major.

Peer review

This is an interesting report.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Ma WH

Silence of HIN-1 expression through methylation of its gene promoter in gastric cancer

Yan Gong, Ming-Zhou Guo, Zhi-Jia Ye, Xiu-Li Zhang, Yong-Liang Zhao, Yun-Sheng Yang

Yan Gong, Ming-Zhou Guo, Xiu-Li Zhang, Yun-Sheng Yang, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing 100853, China

Zhi-Jia Ye, Department of Genetics, Yale Medical School TAC, S320 1 Gilbert Street, New Haven, CT 06520, United States

Yong-Liang Zhao, Department of General Surgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

Author contributions: Gong Y and Yang YS performed the majority of experiments; Guo MZ and Ye ZJ provided vital reagents and analytical tools and edited the manuscript; Zhao YL and Zhang XL collected all the specimens for this work; Gong Y and Guo MZ designed the study and wrote the manuscript.

Supported by National Basic Research Program (973 Program No. 2010CB912802) and the Postdoctoral Fund of China, No. 20080441314

Correspondence to: Yun-Sheng Yang, MD, PhD, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing 100853, China. sunny888@medmail.com.cn

Telephone: +86-10-68182255 Fax: +86-10-68212267

Received: September 2, 2010 Revised: November 2, 2010

Accepted: November 9, 2010

Published online: January 28, 2011

of gastric cancer cells with or without 5-aza-2'-deoxycytidine treatment.

RESULTS: HIN-1 was not expressed in 4 of 5 gastric cancer cell lines. The demethylation reagent 5-aza-2'-deoxycytidine was able to induce or upregulate *HIN-1* expression in gastric cancer cell lines, which is associated with reduction of tumor cell viability. Furthermore, methylation of the *HIN-1* gene promoter was shown in 57.8% (26/45) of the primary gastric cancer and 42.1% (17/38) of adjacent tissue samples, but was not shown in normal gastric mucosa (0/10). From the clinicopathological data of the patients, methylation of the *HIN-1* gene promoter was found to be associated with tumor differentiation ($P = 0.000$).

CONCLUSION: High methylation of *HIN-1* gene promoter results in silence of HIN-1 expression in gastric cancer. 5-aza-2'-deoxycytidine reverses *HIN-1* methylation and reduces viability of gastric cancer cells.

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Key words: High in normal-1; Gene methylation; 5-aza-2'-deoxycytidine; Tumor differentiation; Gastric cancer

Peer reviewer: Huanbiao Mo, PhD, Associate Professor, Department of Nutrition and Food Sciences, Texas Woman's University, PO Box 425888, Denton, TX 76204, United States

Gong Y, Guo MZ, Ye ZJ, Zhang XL, Zhao YL, Yang YS. Silence of HIN-1 expression through methylation of its gene promoter in gastric cancer. *World J Gastroenterol* 2011; 17(4): 526-533 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/526.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.526>

Abstract

AIM: To clarify the role of high in normal-1 (*HIN-1*) gene promoter methylation during gastric cancer development.

METHODS: Gastric cancer cell lines and tissue specimens were analyzed for expression of HIN-1 mRNA and protein using the semi-quantitative reverse transcription polymerase chain reaction and immunohistochemistry. The methylation of the *HIN-1* gene promoter was detected in gastric carcinoma cells and tissues using methylation-specific polymerase chain reaction. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell viability assay and flow cytometry were used to assess the changes in behaviors

INTRODUCTION

Gastric cancer is the second most common cause of can-

cer death worldwide after lung cancer^[1,2]. Gastric carcinogenesis, like all other cancers, is a multistep process, involving numerous genetic and epigenetic alterations, such as abnormalities in growth factors/receptors, angiogenic factors, cell cycle regulators, and DNA mismatch repair genes. These abnormalities also define biological characteristics of gastric cancer cells, which can serve as therapeutic targets for gastric cancer^[3,4]. Although genetic abnormalities including gene mutation and deletion are prominent in causing oncogene activation and tumor suppressor gene inactivation, epigenetic silence of tumor suppressor genes via aberrant promoter hypermethylation have also been shown to be frequent events in gastric carcinoma^[5,6]. DNA high methylation of tumor suppressor genes frequently occurs in the early stage of human carcinogenesis, and investigating the methylation of these gene promoters may contribute to the diagnosis, prognosis and target therapy in gastric carcinoma^[7,8].

High in normal-1 (*HIN-1*) gene was originally isolated through a serial analysis of gene expression from normal and ductal carcinoma *in situ* luminal mammary epithelial cells. The latter is believed to be the precursor of invasive ductal carcinoma^[9]. *HIN-1* is highly expressed in normal luminal mammary epithelial cells but lost in the majority of breast cancers. Restoration of *HIN-1* expression suppressed growth of breast cancer cells^[10]. *HIN-1* can also regulate cell-cycle reentry, suppresses tumor cell migration and invasion, and induces apoptosis in breast cancer cell lines^[10]. Although *HIN-1* processes the putative tumor suppressor function, no somatically genetic changes of *HIN-1* gene were found in breast cancer^[9]. Previous studies demonstrated frequent methylation of *HIN-1* gene promoter in breast cancer, prostate cancer, malignant mesotheliomas, non-small cell lung cancer, lymphoma, retinoblastoma, Wilms' tumor, and rhabdomyosarcoma^[11-14].

However, expression of this putative tumor suppressor gene in gastric cancer has not been fully studied. Therefore, in this study, we first confirmed the methylation of *HIN-1* gene promoter in human gastric cancer cell lines and determined the role of 5-aza-2'-deoxycytidine [5-aza-dc, a drug that inhibits the DNA methyltransferase (DNMT)-mediated hypermethylation of promoter region CpG islands] in regulation of *HIN-1* expression in gastric cancer cells. We also detected the methylation of *HIN-1* gene promoter in tissue specimens and found the association between *HIN-1* gene promoter methylation and clinicopathologic characteristics of gastric cancer.

MATERIALS AND METHODS

Cell lines and culture

Gastric carcinoma cell lines KATOIII, AGS, PHM82, NUGC3, and BCG823 were obtained from American Type Culture Collection (Manassas, VA) and cultured in either RPMI 1640 medium or RPMI 1640/Ham's F-12 medium (all from Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO₂ and 95% air at 37°C. These cells were pas-

saged at a ratio of 1:3 with trypsin once they reached confluence (approximately 10⁶ cells) into 75 cm² culture flasks (Sarstedt, Newton, NC). For treatment with 5-aza-2'-deoxycytidine, these cell lines were split and cultured at a low density (30% confluence) overnight and then treated with 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) at a concentration of 1 μmol/L for up to 96 h. The growth medium was refreshed every 24 h, and at the end of the treatment, DNA and RNA from these cells were isolated as described below.

Human tissue samples

In the current study, 45 surgically resected and pathologically confirmed gastric tumors and 38 adjacent non-tumor tissues were obtained from the PLA General Hospital, Beijing, China between January 2009 and January 2010 and stored in liquid nitrogen until use. Ten cases of normal gastric mucosa were also obtained from the gastric endoscopic biopsies of tumor-free patients. This study was approved by our hospital's Institutional Review Board.

DNA extraction and methylation-specific polymerase chain reaction

Genomic DNA from these cell lines and tissue specimens were extracted using a proteinase-K method described previously^[15]. The extracted DNA was then dissolved in Tris-EDTA (TE) buffer and stored at -20°C. To assess the methylation levels of the *HIN-1* gene promoter, genomic DNA from gastric cancer cell lines and tissue specimens were first subjected to bisulfite treatment and then methylation-specific polymerase chain reaction (MSP) as described previously^[16]. The MSP primers for *HIN-1* were designed and synthesized according to genomic sequences skirting the presumed transcription start sites for *HIN-1*. The *HIN-1* MSP primers spanned a region of 92 base pairs for unmethylation (location is from +128 to +41) and 88 base pairs for methylation (location is from +131 to +40). The primer sequences were: *HIN-1*-UN 5'-GAAGTTTGTGGTTTGTGTTGGGTAGTT-3', *HIN-1*-UN-AS 5'-CACACAAAACCCCAAAAAACAACA-3', *HIN-1*-ME-S 5'-GTTTCGTGGTTTGTTCGGGTAGTC-3' and *HIN-1*-ME-AS 5'-GCAAAACCCCAAAAAACGACG-3'. Each MSP reaction incorporated approximately 100 ng of bisulfite-treated DNA, 25 picomoles of each primer, 100 pmoles dNTPs, 2.5 μL 10 × PCR buffer, and 1 unit of JumpStart Red *Taq* Polymerase (Sigma) in a final reaction volume of 25 μL. The PCR amplification conditions were an initial 95°C for 5 min and then 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min and then stored at 4°C. The MSP products were separated on 2% agarose gel electrophoresis and visualized under the ultraviolet (UV) light.

RNA isolation and semi-quantitative reverse transcription PCR

Total cellular RNA from the cell lines was isolated using the TRIzol reagent (Invitrogen) according to the manu-

facturer's instructions. RNA quality and quantity were assessed using agarose gel electrophoresis (1%) and spectrophotometric analysis of 260/280 ratios. The RNA was stored at -70°C prior to use. The first strand cDNA was synthesized with oligo-(dT) primer using a reverse transcriptase kit from Invitrogen.

Two micrograms RNA was subjected to the first strand cDNA synthesis, and 1 µL cDNA from RT reaction was subjected to PCR amplification of gene expression in a total 25 µL reaction volume. The PCR amplification was carried out using primer sets derived from the published *HIN-1* gene sequences: *HIN-1* primers were 5'-TCTGCGTGGCCCTGTCCTG-3' (sense) and 5'-GCTCAGCCAAACACTGTCAG-3' (antisense)^[14]. This primer set, designed to cross the intronic sequences, can prevent from amplification of genomic DNA for control of genomic DNA contamination during RNA isolation. A total of 32 cycles of PCR amplification were performed based on our pre-experiment for semi-quantitative measurement of *HIN-1* gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified for 25 cycles as an internal control of equal loading and cDNA quality and quantity. The sequence of GAPDH primers: 5'-GACCACAGTCATGCCATCAC-3' (sense) and 5'-GTCCACCACCCTGTTGCTGTA-3' (antisense). The PCR products were then electrophoresed in 1.5% agarose gels containing ethidium bromide and reviewed under the UV light.

Protein extraction and Western blotting

The cells were grown and treated with or without 5-aza-2'-deoxycytidine for 6 d and total cellular protein was then extracted from these cells in 200 µL ice-cold mild lysis buffer containing 10 µL nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 µg/mL aprotinin. The cell mixture was centrifuged at 20000 r/min for 15 min and supernatants were then collected. The concentration of protein was quantified by the BCA protein assay from Pierce (Rockford, IL, USA) and an equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PDVF membranes (Millipore, Billerica, USA). Western blotting analyses were then carried out using an anti-HIN-1 (Novus Biologicals, Littleton, USA) or an anti-β-actin antibody (Boster, Wuhan, China). The blots were developed with chemiluminescence substrate solution from Pierce and exposed to X-ray film.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

Gastric cancer cells were grown in 96-well plates and treated with or without 5-aza-2'-deoxycytidine for up to 6 d, and then cell proliferation was determined using CCK-8 solution (Beyotime, China) according to the manufacturer's instructions. The optical density was measured at 492 nm using an ELISA plate reader (TECAN, Switzerland). The

experiments were performed in triplicate and repeated three times.

Detection of apoptosis

Gastric cancer cells were treated with or without 5-aza-2'-deoxycytidine for up to 6 d. Both attached and floating cells were harvested and fixed with 70% ethanol for at least 48 h. After resuspension in 50 µg/mL, the cells were treated with 100 µg/mL RNase for 30 min and stained with propidium iodide and then analyzed by flow cytometry (FACScalibur; Becton Dickinson, Franklin Lakes, NJ).

Immunohistochemistry

Sections 5 µm thick were formalin-fixed and paraffin-embedded in xylene and rehydrated through an ethanol series. Antigen retrieval was carried out at this stage in a microwave oven. Sections were then blocked with 3% hydrogen peroxidase followed by incubation with a 50% protein blocking agent. Fetal bovine serum (10%), with or without HIN-1 antibody (1:60), was applied to each slide, and the slides were incubated for 30 min, and counter-stained with hematoxylin. Tissues without the specific antibody were used as negative controls. Anti-HIN-1 (Novus Biologicals, Littleton, USA) and PV-6000-G Kit (Beijing Zhongshan Jinqiao Biotechnology, Beijing, China) were used for the immunohistochemical (IHC) staining. HIN-1 expression was regarded as positive when 10% or more cancer cells exhibited HIN-1 expression.

Statistical analysis

The statistical analyses of the experimental data were carried out using SPSS 13.0 software for Windows (Chicago, IL). *P* values for dichotomous variables were two-tailed and based on the Pearson χ^2 test or the Pearson χ^2 test with continuity correction. Continuous variables were analyzed with Student's *t* test. A value of *P* < 0.05 was considered statistically significant.

RESULTS

Silence of HIN-1 expression through methylation of HIN-1 gene promoter and 5-aza-2'-deoxycytidine induction of HIN-1 gene expression in gastric cancer cell lines

To find out whether the silence of *HIN-1* gene expression is caused by methylation of the *HIN-1* gene promoter, we first detected the methylation status of *HIN-1* in 5 gastric cancer cell lines. The MSP analysis showed that *HIN-1* gene promoter was highly methylated in AGS, PHM82, and BCG-823 cells, but not methylated or partially methylated in NUGC 3 and KATOIII cell lines (Figure 1A). We detected HIN-1 expression in five gastric cancer cell lines and found that HIN-1 mRNA was not expressed in AGS, PHM82, and BCG 823 cells, but expressed in NUGC 3 and weakly expressed in KATOIII cells. HIN-1 expression was induced or upregulated in these cell lines after we treated them with 5-aza-2'-deoxycytidine (Figure 1B).

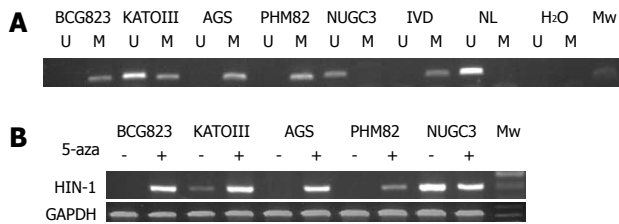


Figure 1 Silencing of *high in normal-1* gene expression due to methylation of *high in normal-1* gene promoter in gastric carcinoma cell lines. A: Methylation-specific polymerase chain reaction analysis of *high in normal-1* (*HIN-1*) gene promoter methylation in five gastric carcinoma cell lines. U: Unmethylated alleles; M: Methylated alleles. *In vitro* methylated DNA (IVD) and DNA from normal human peripheral lymphocytes were used as methylated and unmethylated controls; B: Gastric cancer cell lines were treated with or without 5-aza-CdR (-AZ) for up to 96 h. *HIN-1* mRNA levels were measured by semi-quantitative reverse transcription polymerase chain reaction analysis, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. The 1-kb marker indicated an appropriate size for the amplified products. *HIN-1* expression varied among cell lines. The presence of methylation of *HIN-1* corresponds directly to the loss of expression of the genes in each cell line.

Suppression of gastric cancer cell viability with 5-aza-2'-deoxycytidine treatment

We determined the ability of 5-aza-2'-deoxycytidine to regulate gastric cancer cell viability using BCG-823 cells treated with 5-aza-2'-deoxycytidine. The results showed that treatment with 1 $\mu\text{mol/L}$ of 5-aza-2'-deoxycytidine for up to 6 d significantly upregulated expression of *HIN-1* but reduced the number of the viable cells (Figure 2A) and induced them to undergo apoptosis compared with the untreated tumor cells ($20.46\% \pm 1.24\%$ vs $11.28\% \pm 1.01\%$, $P = 0.001$, Figure 2B). These data were associated with *HIN-1* expression induced by 5-aza-2'-deoxycytidine (Figure 1B and Figure 2A).

Aberrant hypermethylation of *HIN-1* gene promoter in primary gastric carcinomas

To translate this *in vitro* finding into *ex vivo* tissue specimens, MSP analysis of *HIN-1* gene promoter methylation was conducted in 45 patients with human gastric carcinoma (32 male and 13 female). The patients' average age was 55 ± 13 years and other clinicopathological data are listed in Table 1. MSP analysis showed that methylation of the *HIN-1* gene promoter was frequently detected in gastric cancer (57.78%, 26/45) and adjacent non-tumor tissues (42.1%, 17/38), but not in normal gastric mucosa. Statistically, there was no difference in methylation of the *HIN-1* gene promoter between gastric cancer and adjacent non-tumor tissues. However, there were statistically significant differences between gastric cancer and normal gastric mucosa, and between adjacent non-tumor tissues and normal mucosa (Figure 3A and B, $P = 0.002$ and $P = 0.005$, respectively). To correlate *HIN-1* gene promoter methylation with *HIN-1* expression, 29 gastric cancer tissues (GCs) were subjected to immunohistochemistry analysis. Representative immunostaining is shown in Figure 4A and B. GC cases with low *HIN-1* immunostaining had more frequent DNA methylation than GCs with high immunos-

Table 1 Association of *high in normal-1* methylation with clinicopathologic characteristics in gastric cancer

Variable	Patients	<i>HIN-1</i> methylation	P value
Sex			0.161
Male	32	17	
Female	13	9	
Age (yr)			0.401
≤ 50	14	9	
> 50	31	17	
Tumor size (cm)			0.283
< 5	25	16	
≥ 5	19	10	
Tumor differentiation			0.000 ^a
Moderate/poor	21	17	
Well	23	8	
Stage			0.683
I - II	13	7	
III - IV	29	17	
Nodal status			0.903
-	9	5	
+	35	20	

^aPearson's χ^2 test using SPSS 13.0 software for Windows. The methylation frequency of well-differentiated tumor vs moderately/poorly tumor. TNM was staged according to the guidelines of the International Union against Cancer. *HIN-1*: High in normal-1.

taining (53.33% vs 14.29%, $P = 0.027$, Figure 4C). These data demonstrate that DNA methylation contributes to the decreased expression of *HIN-1* in GCs.

Association of *HIN-1* gene promoter methylation with clinicopathological data in gastric cancer patients

Methylation status of *HIN-1* gene promoter was associated with tumor differentiation. The methylation frequency in well-differentiated and moderately/poorly-differentiated tumors was 34.78% (8/23) and 80.95% (17/21), respectively, indicating that *HIN-1* was more frequently methylated in poorly-differentiated gastric cancer than that in well-differentiated gastric cancer ($P = 0.000$, Table 1). However, there was no correlation between *HIN-1* methylation and other parameters (such as age, tumor size, and lymph node metastasis) (Table 1).

DISCUSSION

In the current study, we determined *HIN-1* gene expression and the methylation status of the *HIN-1* gene promoter in gastric cancer cells. We found that the expression of *HIN-1* mRNA was lost in gastric cancer cells. MSP analysis revealed high methylation of the *HIN-1* gene promoter in these tumor cells. 5-aza-2'-deoxycytidine treatment induced *HIN-1* expression, but reduced viability of gastric cancer cells. Furthermore, *ex vivo* data demonstrated that the *HIN-1* gene promoter is frequently methylated in gastric cancer and the adjacent non-tumor tissues, but not in normal gastric mucosae. *HIN-1* gene promoter methylation was associated with differentiation of gastric cancer. This study demonstrated frequent methylation of the *HIN-1* gene promoter in gastric cancer. Therefore, the

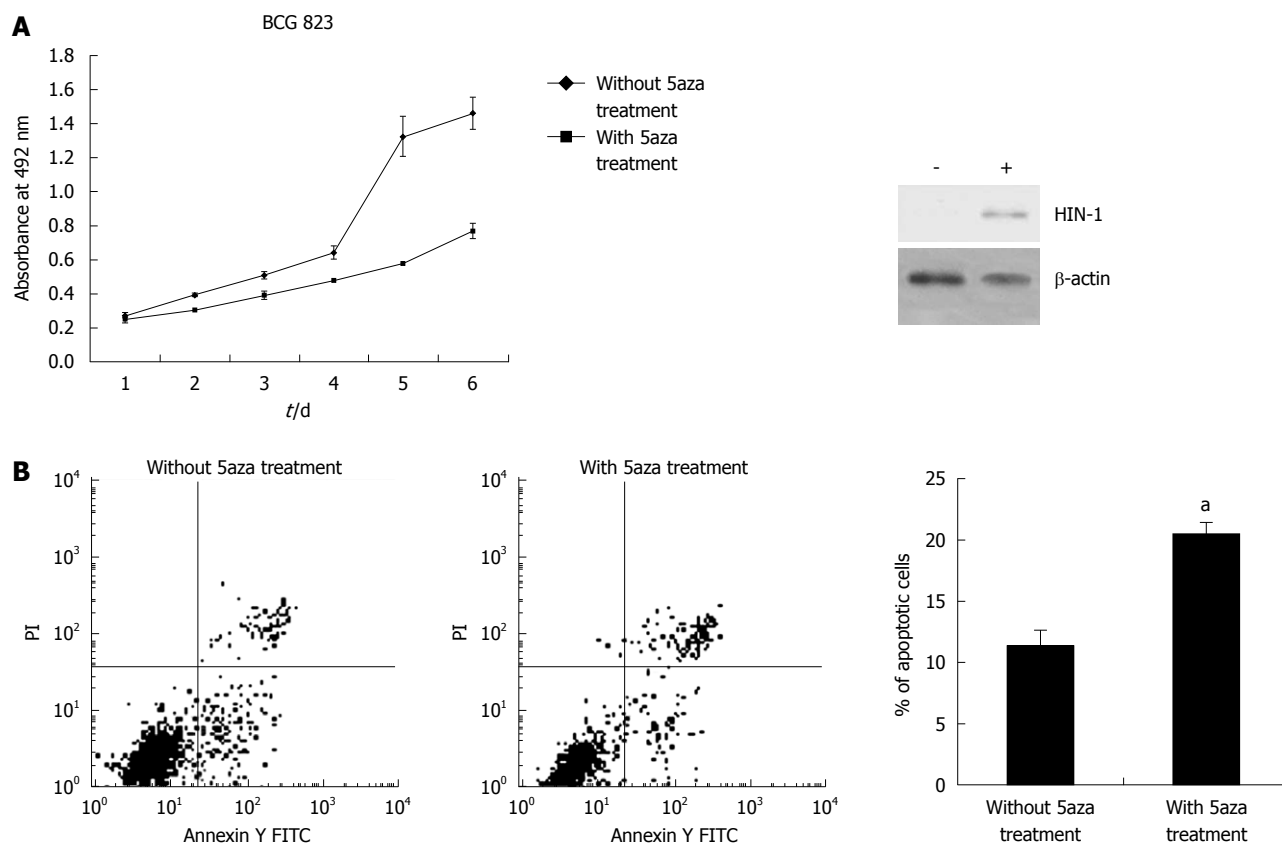


Figure 2 5-aza-2'-deoxycytidine inhibition of gastric cancer cell BCG823 viability through induction of high in normal-1 expression. A: Gastric cancer BCG823 cells were treated with or without 5-Aza-CdR (AZ) for up to 6 d and then subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium analysis of cell viability before and after 5-Aza-CdR treatment. The high in normal-1 (HIN-1) protein levels were measured by immunoblotting. -: Without 5aza treatment; +: With 5aza treatment; B: BCG-823 cells were subjected to FACs for apoptosis analysis. 5-aza-2'-deoxycytidine treatment inhibits BCG-823 cell proliferation (A) and induces them to undergo apoptosis (B) vs the controls (^a $P < 0.05$).

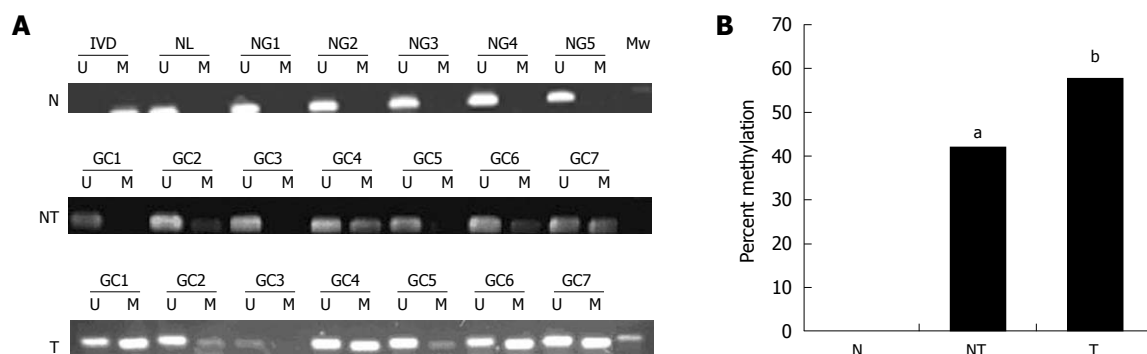


Figure 3 Methylation-specific polymerase chain reaction analysis of high in normal-1 gene promoter methylation in gastric cancer, adjacent non-tumor tissues, and normal gastric mucosa. A: Representative data of MS-PCR analysis of high in normal-1 (*HIN-1*) genes in tumor tissues (T), paired adjacent non-tumor tissues (NT) and normal gastric mucosa (N). U: Unmethylated alleles; M: Methylated alleles. *In vitro* methylated DNA and DNA from normal human peripheral lymphocytes were used as methylated and unmethylated controls; B: Comparison of *HIN-1* gene methylation among gastric cancer (T), adjacent non-tumor tissue (NT) and normal gastric mucosa (N). ^aStudent's *t* test by SPSS 13.0 software, NT vs N, $P = 0.005$; ^bT vs N, $P = 0.002$.

HIN-1 gene promoter methylation may be further evaluated as a biomarker for early detection of gastric cancer.

Inactivation of tumor suppressor genes contributes to cancer development. Such inactivation may be caused by genetic or epigenetic alterations, including gene mutation, deletion, promoter methylation, abnormal splicing, deregulation of imprinting and haploinsufficiency^[4]. Among these abnormalities, loss of heterozygosity (LOH) was

shown to cause inactivation of most candidate tumor suppressor genes in the critical regions of chromosomes 3p, 5q, 8p and 9p^[17-20]. However, changes in methylation status of these genes also frequently occur. The *HIN-1* gene is located at 5q35 and plays a role in epithelial cell differentiation. *HIN-1* can also regulate cell-cycle reentry, suppresses tumor cell migration and invasion, and induces apoptosis in breast cancer cell lines^[10]. The *HIN-1* gene is

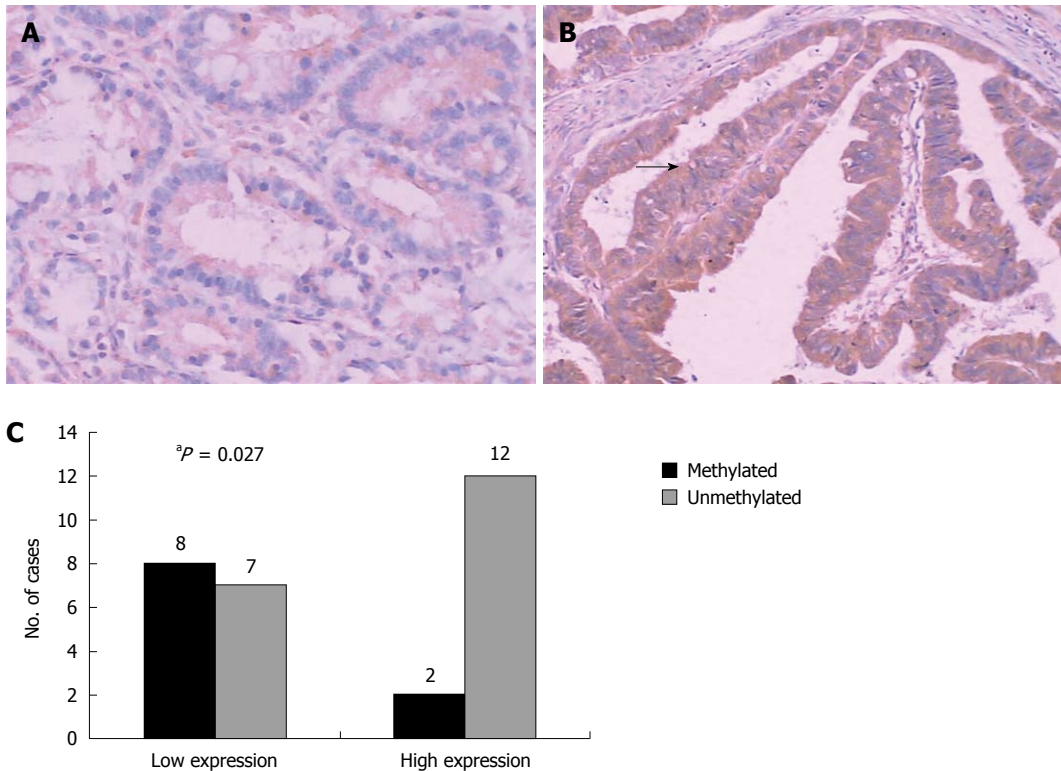


Figure 4 Immunohistochemical analysis of high in normal-1 protein expression in gastric cancer tissue samples. A: Tumor cells with methylated alleles of high in normal-1 (*HIN-1*) gene promoter exhibited negative staining; B: Cancer cells without *HIN-1* gene promoter methylation exhibited positive staining. *HIN-1* expression in gastric cancer (membrane staining, arrow); C: The association of *HIN-1* methylation with *HIN-1* expression level was analyzed in 29 gastric cancers. High expression: +++++ staining intensity with 10% or more cancer cells positively stained, otherwise it is considered as low expression. The staining intensity and percentage of staining were compared with a non-cancerous area of the same section. ^aPearson χ^2 test or Pearson χ^2 test with continuity correction by SPSS 13.0 software. A, B: IHC, $\times 200$.

frequently methylated in different cancers, but not by mutation^[11]. For example, *HIN-1* gene promoter hypermethylation was found in the majority (70%) of breast cancer and pre-invasive lesions. Hypermethylation of the *HIN-1* promoter region also occurs in cancer and the adjacent tissues of the lung, prostate, pancreas, and esophagus, but not in normal tissues^[21]. Methylation of the *HIN-1* gene promoter was associated with esophageal squamous carcinoma progression^[14]. Our current data demonstrated aberrant methylation of *HIN-1* gene promoter regions and subsequent loss of *HIN-1* expression in gastric cancer cell lines and tumor tissue specimens. These results are consistent with previous studies on other cancers^[9,12]. *HIN-1* methylation existed in 57.78% (26/45) of gastric cancer and 42.1% (17/38) of adjacent non-tumor tissues, which indicated that it is a common feature of gastric cancer and may be the early stage accident in gastric carcinogenesis.

The pathogenesis of intestinal-type gastric cancer is usually initiated or caused by *Helicobacter pylori* (*H. pylori*) infection^[22]. However, the underlying mechanism remains to be defined, and a better understanding of pathogenesis of gastric cancer could help develop molecular diagnostic and patient-tailored therapeutic targets^[23]. In the previous studies, we reported that field defect, an area of abnormal tissue that precedes and is predisposed to the development of cancer, could be predicted by detection of gene promoter methylation^[24]. Such abnormal fields are of interest because they give insight into the early

stages of carcinogenesis and may provide biomarkers of cancer risk^[25,26]. Aberrant promoter hypermethylation has been shown to be a common event in human cancer mainly due to the loss of function of tumor suppressor. This neoplasia-related event is thought to occur early in carcinogenesis, and hence, promoter hypermethylation is being widely studied as a biomarker for the diagnosis and detection of early lesions. In this context, *HIN-1* was frequently methylated in gastric carcinoma adjacent tissues but not in normal gastric mucosa. It suggests that *HIN-1* methylation may represent the field defect of gastric carcinoma. *HIN-1* gene promoter methylation may be an early event in gastric cancer. However, further studies are required to determine whether *H. pylori* infection is responsible for this.

Our current data showed a statistical difference between methylation of the *HIN-1* gene promoter and gastric cancer differentiation, *HIN-1* was more frequently methylated in poorly-differentiated gastric carcinomas than in well-differentiated ones, which may suggest the role of *HIN-1* in regulation of cell differentiation.

We also found that methylation of *HIN-1* gene promoter only occurred in gastric cancer but not in normal gastric mucosa. 5-aza-2'-deoxycytidine induced expression of *HIN-1*, which is associated with reduced viability of gastric cells, indicating that *HIN-1* plays an important role in suppressing gastric carcinogenesis. However, we cannot rule out whether other tumor suppressor genes

are also induced and restored by 5-aza-2'-deoxycytidine, which plays a role in regulation of tumor cell viability. The latter warrants further studies because some other studies showed that epigenetic modification of pro-apoptotic genes is one of the mechanisms by which the tumor cells are resistant to chemotherapy^[27,28]. Therefore, treatment with a demethylating agent like 5-aza-2'-deoxycytidine prior to chemotherapy may help improve the therapeutic efficacy for gastric cancer.

In summary, silence of HIN-1 expression is achieved through the gene methylation in gastric cancer. Methylation of *HIN-1* is correlated with tumor differentiation. Future studies will evaluate whether *HIN-1* gene promoter methylation can be used as a biomarker for the early detection of gastric cancer.

COMMENTS

Background

Gastric cancer is the second most common cause of cancer death worldwide. However, the cause of gastric cancer development remains to be determined. Lost expression of tumor suppressor genes, such as high in normal-1 (*HIN-1*), may contribute to the development of gastric cancer. This study determined the cause of *HIN-1* gene inactivation: epigenetic silence through methylation of the gene promoter.

Research frontiers

Silence of *HIN-1* gene through hypermethylation of the gene promoter is a common event in different cancers including breast, prostate, and non-small cell lung cancers and malignant mesotheliomas, lymphoma, retinoblastoma, Wilms' tumor, and rhabdomyosarcoma. This study investigated the role of *HIN-1* in gastric cancer and showed for the first time that the hypermethylation of *HIN-1* gene promoter was the mechanism for *HIN-1* gene silence in gastric cancer.

Innovations and breakthroughs

The authors confirmed the methylation of *HIN-1* gene promoter in human gastric cancer cell lines and determined the role of 5-aza-2'-deoxycytidine in regulation of *HIN-1* expression in gastric cancer cells.

Applications

The *HIN-1* gene promoter methylation may be further evaluated as a biomarker for early detection of gastric cancer.

Terminology

HIN-1 gene was originally isolated through a serial analysis of gene expression from normal and ductal carcinoma *in situ* luminal mammary epithelial cells. *HIN-1* gene promoter is frequently methylated in gastric cancer and the adjacent non-tumor tissues, but not in normal gastric mucosa.

Peer review

This manuscript demonstrated promising data illustrating the methylation status of *HIN-1* gene promoter and its potential role in suppression of gastric carcinoma development.

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S- Editor Sun H L- Editor Ma JY E- Editor Zheng XM

Anorectal malignant melanomas: Retrospective experience with surgical management

Xu Che, Dong-Bing Zhao, Yong-Kai Wu, Cheng-Feng Wang, Jian-Qiang Cai, Yong-Fu Shao, Ping Zhao

Xu Che, Dong-Bing Zhao, Yong-Kai Wu, Cheng-Feng Wang, Jian-Qiang Cai, Yong-Fu Shao, Ping Zhao, Department of Abdominal Surgical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Author contributions: Che X, Zhao DB, Wang CF, Shao YF, Cai JQ and Zhao P contributed equally to this work; Zhao DB, Shao YF, Wang CF and Cai JQ performed the research; Che X, Wu YK and Zhao DB analyzed the data; Che X and Zhao DB wrote the paper.

Correspondence to: Dong-Bing Zhao, MD, Department of Abdominal Surgical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China. dbzhao2003@sina.com

Telephone: +86-10-87788521 Fax: +86-10-67730386

Received: March 14, 2010 Revised: July 20, 2010

Accepted: July 27, 2010

Published online: January 28, 2011

Abstract

AIM: To present the experience and outcomes of the surgical treatment for the patients with anorectal melanoma from the Cancer Hospital, Chinese Academy of Medical Sciences.

METHODS: Medical records of the diagnosis, surgery, and follow-up of 56 patients with anorectal melanoma who underwent surgery between 1975 and 2008 were retrospectively reviewed. The factors predictive for the survival rate of these patients were identified using multivariate analysis.

RESULTS: The 5-year survival rate of the 56 patients with anorectal melanoma was 20%, 36 patients underwent abdominoperineal resection (APR) and 20 patients underwent wide local excision (WLE). The rates of local recurrence of the APR and WLE groups were 16.13% (5/36) and 68.75% (13/20), ($P = 0.001$), and the median survival time was 22 mo and 21 mo, respectively ($P = 0.481$). Univariate survival analysis

demonstrated that the number of tumor and the depth of invasion had significant effects on the survival ($P < 0.05$). Multivariate analysis showed that the number of tumor [$P = 0.017$, 95% confidence interval (CI) = 1.273-11.075] and the depth of invasion ($P = 0.015$, 95% CI = 1.249-7.591) were independent prognostic factors influencing the survival rate.

CONCLUSION: Complete or R0 resection is the first choice of treatment for anorectal melanoma, prognosis is poor regardless of surgical approach, and early diagnosis is the key to improved survival rate for patients with anorectal melanoma.

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Key words: Anorectal melanomas; Prognostic factors; Surgical management; Survival rate

Peer reviewers: Philip H Gordon, Professor, Department of Surgery, McGill University, 3755 Cote Ste. Catherine Road, Suite G-314, Montreal, Quebec, H3T 1E2, Canada; Jai Dev Wig, MS, FRCS, Former Professor and Head, Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Che X, Zhao DB, Wu YK, Wang CF, Cai JQ, Shao YF, Zhao P. Anorectal malignant melanomas: Retrospective experience with surgical management. *World J Gastroenterol* 2011; 17(4): 534-539 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/534.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.534>

INTRODUCTION

Anorectal malignant melanoma (ARMM) is an infrequent fatal tumor. ARMM accounts for $< 1\%$ of anorectal malignant tumors and approximately 1%-2% of all melanomas^[1]. Although there has been progress in the treatment of melanomas, the prognosis of ARMM is still very

poor, and the 5-year disease-specific survival (DSS) is 10% or less. In most cases, when patients are diagnosed with ARMM, local invasion or distal metastasis already exists^[2], and because ARMM is not sensitive to radiotherapy and chemotherapy, selection of treatment methods is limited. At present, surgical treatment remains the main therapeutic method for ARMM, with abdominoperineal resection (APR) and wide local excision (WLE) as the most common approaches used. Nevertheless, there are controversies regarding which technique is superior in terms of long-term survival and overall quality of life. Because of the low incidence and poor biological behavior of ARMM, there has been no consensus about the therapeutic method for ARMM^[3]. The current study involved a retrospective analysis of 56 patients with ARMM who underwent surgery in our hospital between 1975 and 2008. We compared the survival status of the patients after APR and WLE to determine the relationship between the surgical approaches and prognosis of ARMM. Cases with distal metastasis at the time of diagnosis were not included in this study.

MATERIALS AND METHODS

Clinical data

Among the 56 patients, 22 were men and 34 were women; the ratio of males to females was 1:1.55. The mean age of the patients was 55 years (range, 36-81 years). The main symptoms were hematochezia in 32 cases, anal tumors in 11 cases, change in bowel habits in 6 cases, and bulge or pain in the anus in 7 cases. The distance from the tumor to the anal verge was < 5 cm in all the 56 patients, and < 3 cm in 47 (83.92%) patients. Misdiagnoses occurred in 32 (57.14%) cases. Thirteen cases were misdiagnosed as hemorrhoids, 8 cases as adenomas or polypus, 4 cases as cancer, and 1 case each as carcinoid, fibroma, malignant fibroma, eczema, and dysentery.

Pathologic examination

Routine pathologic examinations were carried out in all 56 cases and the diagnoses were made by two pathologists. There were 49 cases of pigmented melanomas, 7 cases of amelanotic melanomas, 50 cases of single lesions, 6 cases of multiple lesions. Ten cases had lesions invading into the mucosa, 6 cases into the submucosa, 19 cases into the muscle layer and 7 cases into the fibrous membrane, and the depth of lesion was not recorded in 14 cases. Among 36 patients undergoing APR, lymph node metastases occurred in 21 cases, while no lymph node metastases were noted in 14 cases, and the status of metastasis was not identified in 1 case. Immunohistochemical examinations were carried out for differential diagnosis in 28 cases.

Surgical methods

Thirty-six cases underwent APR, 19 cases underwent WLE, 1 case underwent WLE+ lymph node dissection, Assisted radiotherapy was performed in 4 cases, and assisted biotherapy and chemotherapy were carried out in

19 cases. The main biological agent was interferon, and the main chemotherapeutic agents were dacarbazine and vincristine.

Follow-up and statistical analysis

Postoperative follow-ups were made until December 2009 through outpatient visits, telephone interviews, and questionnaires, with a rate of 91% (51/56). The survival time was calculated from the day of surgery. SPSS13.0 software was used for statistical analyses. χ^2 test was used to ascertain the relationship between the clinical pathologic parameters and local recurrence. The Kaplan-Meier method and long-rank univariate analysis were used for calculating the survival rates and multivariate analysis was conducted for the COX model.

RESULTS

Comparison of different surgical methods between local recurrence and distal metastasis

All 56 cases underwent tumorectomy. The local recurrence rate was 32.14% (18/56) and the rate of metastasis was 58.92% (33/56). Thirty-six cases underwent APR, with a local recurrence rate of 16.13% (5/36). Twenty cases underwent WLE, with a local recurrence rate of 68.75% (13/20). The results of the χ^2 test showed that there was a correlation between the surgical method and local recurrence ($P = 0.001$).

Comparison of different surgical methods in survival time

The 5-year overall survival rate for all the patients was 20%. The 5-year overall survival rate was 24.6% in the APR group and 9.9% in the WLE group. The median survival time was 22 mo in the APR group and 21 mo in the WLE group ($P = 0.645$, Figure 1A).

Overall prognosis and its influencing factors

In this study, the mean follow-up period was 4-144 mo, the median survival time was 21 mo, and the 5-year overall survival rate was 20%. Based on univariate analysis, the factors correlated with prognosis were the number of tumors and the depth of invasion (Table 1). When these factors were subjected to a Cox model with stepwise regression, the results showed that the number of tumors [$P = 0.017$, OR = 3.755, 95% confidence interval (CI) = 1.273-11.075 (Figure 1B)] and the depth of invasion [$P = 0.015$, OR = 3.079, 95% CI = 1.249-7.591 (Figure 1C)] were the most important influencing factors for prognosis.

DISCUSSION

ARMM is an infrequent fatal tumor and accounts for 1%-2% of all melanomas. The rectum and the anal canal are the most common organs for ARMM onset, except the skin and eyes. In this study, the clinical characteristics of ARMM included: an age of onset of 55 years, hematochezia as the most common primary symptom, similar

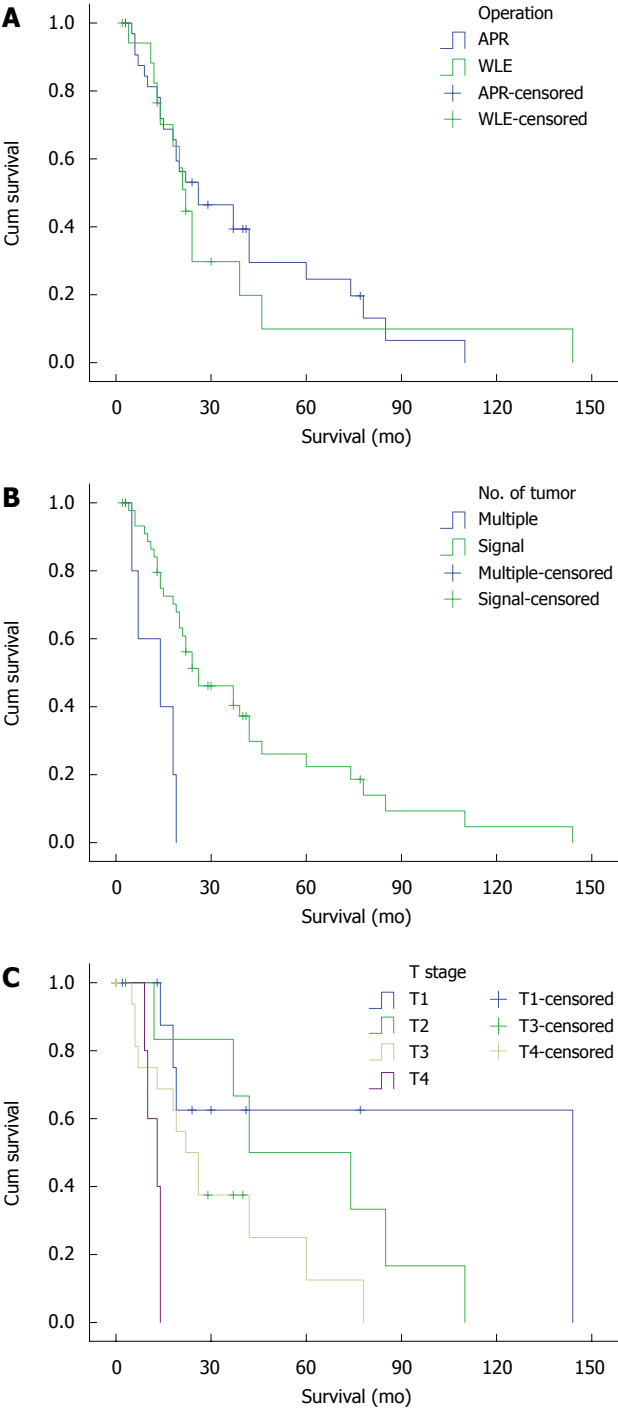


Figure 1 Overall survival by different surgical methods and prognosis factors. A: Survival curves for different surgical methods ($P = 0.645$); B: Survival curves for number of tumors ($P = 0.001$); C: Survival curves for depth of invasion ($P = 0.002$). APR: Abdominoperineal resection; WLE: Wide local excision.

incidences of the tumor in the rectum and anal canal, more females than males affected, and a ratio of males to females of 1:1.6, which is consistent with a previous report^[4]. Although the gastrointestinal tract contains melanocytes, ARMM is most likely located around the anocutaneous line, including the anocutaneous line and the anal canal. In our study, the distance from the tumor to the anal verge was < 5 cm in all 47 cases; < 3 cm in 43 (91.5%) cas-

Table 1 Clinical pathologic factors and prognosis-related factors of 56 cases of anorectal melanoma

Factors	Cases	5-yr survival (%)	P value
Gender			0.759
Male	22	23.4	
Female	34	18.1	
Age (yr)			0.524
≤ 45	14	16.7	
> 45	42	21.1	
Tumor size (cm)			0.815
≤ 2	17	19.9	
> 2	29	22.2	
Lesion			0.001
Single	50	22.4	
Multiple	6	0.0	
Pigment			0.245
Yes	43	17.4	
No	4	33.33	
Depth of invasion			0.002
T1	10	62.5	
T2	6	50.0	
T3	19	12.5	
T4	7	0.0	
Lymph metastasis			0.275
Yes	21	20.6	
No	15	30.0	
Surgical method			0.645
Abdominoperineal resection	36	24.6	
Local excision	20	9.9	
Postoperative chemotherapy			0.256
Yes	19	17.8	
No	15	0.0	

es; and < 2 cm in 16 cases. Zhang *et al*^[4] summarized 216 cases of ARMM, carried out a descriptive analysis of the characteristics of tumor growth, and found that ARMM was more likely benign. For example, the maximum diameter of the tumor was relatively small; 43.6% were polypoid type in gross morphology, and only 23.6% of the tumors were invading the surrounding tissues and fixed. Although most tumors have hard surfaces and ulcers on the surface, there are tumors with soft and smooth surfaces. Melanin has been reported under light microscope in 70%-80% of tumors; however, the percentage was 91% in the current study.

Misdiagnosis is a significant characteristic of ARMM. Because ARMM is rare and its clinical features lack specificity, the rate of misdiagnosis is high. In this study the rate of misdiagnosis was 57.14% (32/56), which is similar to Zhang's report^[5]. The early symptoms of ARMM resemble some anorectal benign diseases, such as thrombosed hemorrhoids, mixed hemorrhoids, and rectal adenomas. In the advanced stage, ARMM is similar to rectal cancer. Amelanotic ARMM is more likely to be misdiagnosed. The causes of misdiagnosis are as follows: (1) physicians do not have sufficient knowledge about this disease; (2) the clinical features lack specificity; and (3) the pathologic diagnosis is difficult; in particular, immunohistochemical methods are needed for a correct diagnosis of amelanotic melanomas. The data of this study suggest that the prog-

nosis for ARMM is better when the tumor is limited in the mucosa and submucosa, and in such cases the tumor can be treated by WLE, therefore, lowering the misdiagnosis rate is very important for early treatment. There are several methods that can help improve the diagnosis of ARMM. (1) Rectal examination and endoscopy: ARMM is most likely located in the anocutaneous line and the anal canal. In this study, the distance from the tumor to the anal verge was < 5 cm in all cases, therefore, the rectal examination and the endoscopy are very important. Most patients with such melanomas complained of bleeding, pain, or an anal mass. Digital examination provides information concerning size, fixation and ulceration of the tumor, and proctosigmoidoscopy may be suggestive of anorectal melanoma^[6]. When the pathologic examination is performed in patients with suspected ARMM, the whole tumor should be resected to prevent iatrogenic dissemination; (2) Light microscopy: Light microscopy can localize pigment granules in the cytoplasm of tumor cells in most cases. If the existence of pigment granules is not clear, other methods, such as Fontana-Masson-stained sections for melanin, dopamine staining, or enzyme reactions, can be used to confirm the diagnosis; and (3) Immunohistochemical staining: Because the aforementioned methods can not diagnose amelanotic ARMM, the immunohistochemical method combining HMB45, S-100 protein, and the vimentin test can be used^[7].

The malignancy of ARMM is high. The 5-year disease-specific survival (DSS) is < 10% and the mean survival time is 12-18 mo^[8,9]. ARMM is not sensitive to radiotherapy or chemotherapy; surgical excision remains the most important therapy. APR and WLE are the most common surgical methods used; however, since the tumor is located in the anorectal area, there have been differences in the viewpoint regarding which surgical method is most suitable for long-term survival and overall life quality^[10]. At present, the results of most studies have indicated no difference between the two surgical methods with respect to the survival rate. Yeh *et al*^[11] performed a retrospective analysis of 46 patients with ARMM who were treated surgically; among them, 19 patients underwent APR and 26 patients underwent WLE. The rates of local recurrence were 21% and 26%, respectively, and there were no statistical differences. The DSS was 34% and 35%, respectively. The neural invasion around the tumor was the only independent prognosis factor ($P = 0.01$). It was considered that the range of surgical excision was not correlated with the prognosis. Because WLE has significant advantages, such as minor surgical trauma, quick recovery, less effect on the function of the intestinal tract, and preservation of anal function, it was suggested that WLE should be the first choice for treatment of ARMM. Another point of view considered that APR has a certain advantage in the control of local recurrences, thus it should be the first choice for patients with early-stage disease.

Because of the infrequency of ARMM, it is difficult to carry out a prospective, randomized, controlled study, and there have been only some retrospective data of small

samples studied. Thibault *et al*^[12] summarized 24 references involving 428 cases of AMM patients who underwent APR and WLE. There was no significant difference between the two groups with respect to the 5-year DSS. A series of 26 patients from the MD Anderson Cancer Center reported fewer local recurrences after APR (29%) than after wide local excision (58%), but these authors noted that the majority of recurrences occurred in patients who also had regional or systemic metastases and that local recurrence did not affect survival^[13]. In our series, the 5-year overall survival rate was 24.6% in the APR group and 9.9% in the WLE group ($P = 0.645$). The rate of local recurrence was lower in the APR group than in the WLE group (16.13% and 68.75%, respectively, $P = 0.001$). It is suggested that APR can decrease local recurrence, but no significant difference between the two groups with respect to the 5-year overall survival rate.

The definition of relevant pathological prognostic parameters which might be able to guide the clinical decision is also lacking in anorectal melanomas. Tumor thickness less than 2 mm has been advocated as a good prognostic factor by Roumen in his study^[14]. Weyandt *et al*^[15] reported that, in early-stage disease with a tumor thickness below 1 mm, a local sphincter saving excision with a 1-cm safety margin would be appropriate. In the cases of a tumor thickness between 1 and 4 mm, a local sphincter saving excision with a safety margin of 2 cm seems to be adequate. In this study, multivariate analysis showed that a single lesion and the depth of tumor invasion were the most important factor influencing prognosis. Therefore, early diagnosis is the key to improved survival rate for patients with anorectal melanoma^[16].

In this study, the surgical method affected the rate of local recurrence, but it did not affect the prognosis of ARMM, confirming that the quick development of a tumor over the body obscures the effect of surgical method on the control of a local tumor. Hence, as with other malignant tumors, the metastatic capability of ARMM was determined at the moment of tumorigenesis, and it is independent from other factors, such as the size of tumor and metastasis to the lymph nodes^[17]. ARMM is a systemic disease and its prognosis is not affected by the surgical method, so that the goal of surgical therapy is to improve the quality of life of those patients maximally, by now, most scholars hold the same view^[18].

Because ARMM is highly invasive and the blood supply to the area of the anocutaneous line is abundant, lymph node and distal metastases may occur. Therefore, biotherapy and chemotherapy are necessary as postoperative adjunctive therapies. The rate of complete remission is 11% when metastatic ARMM is treated by chemotherapy and biotherapy^[19]. Cytotoxic chemotherapeutics (cisplatin, catharanthine, or dacarbazine), combined with immunomodulators (interleukin-2 and interleukin- α) can improve the survival status of some patients. Ballo *et al*^[20] reported postoperative radiotherapy in a retrospective analysis of 23 patients with AM who were managed with sphincter-sparing procedures and adjuvant radiotherapy.

Although the overall survival was not improved, the actuarial 5-year local and nodal control was 74% and 87%, respectively. This degree of locoregional control is superior to the standard WLE alone, in which local control is typically poor (a crude estimate of 35%). This study did not investigate the effects of adjuvant chemotherapy and postoperative radiotherapy on the prognosis, which is also a controversial topic. Further studies are needed to demonstrate the benefits of adjuvant chemotherapy and postoperative radiotherapy.

It can be concluded that the surgical method does not affect the prognosis of ARMM. If the tumor can be resected totally, WLE should be the first choice of treatment. APR can be performed as a rescue therapy when WLE is impossible, the margins of the local excision are positive, or in the event of recurrence^[21].

Endorectal ultrasonography is increasingly employed in the preoperative staging of rectal cancers. Accuracy in evaluating tumor depth ranges from 81% to 94%, and accuracy in detecting lymph node metastases ranges from 58% to 80%^[22], and preoperative magnetic resonance imaging (MRI) is often of great importance when planning rectal cancer surgery^[23]. Use of endoanal ultrasonography and MRI for preoperative staging of patients with anorectal melanoma has rarely been reported. In this study, no patient underwent preoperative ultrasonographic and MRI evaluation, but some patients are being followed postoperatively in an attempt to detect early recurrence. We are currently evaluating the accuracy of endoanal ultrasonography and MRI in the preoperative assessment of melanoma as well as other anal canal malignancies.

This study was hampered by several limitations. First, it is retrospective, based mainly on data from medical documentation. Second, data are incomplete. In some patients, medical documentation was absent, and follow-up data were missing. Finally, patients received heterogeneous treatment and no prospective protocol was followed. Therefore, planning of surgery after thorough clinical and radiological investigations, including MRI of the pelvis and endoluminal ultrasound for tumor depth, may aid in defining the appropriate surgical approach for anorectal melanoma.

COMMENTS

Background

Anorectal malignant melanoma (ARMM) is an infrequent fatal tumor. ARMM represents < 1% of anorectal malignant tumors and approximately 1%-2% of all melanomas. Although there has been progress in the treatment of melanomas, the prognosis of ARMM is still very poor, and the 5-year disease-specific survival (DSS) is 10% or less. At present, surgical therapy remains the main treatment method for ARMM, with abdominoperineal resection (APR) and wide local excision (WLE) as the most common methods used. Nevertheless, there are considerable controversies regarding which technique is superior in terms of long-term survival and overall quality of life. Because of the low incidence and poor biological behavior of ARMM, there has been no consensus reached about the treatment method for ARMM.

Research frontiers

The authors compared the survival status of the patients after APR and WLE to determine the relationship between the surgical approaches and prognosis of ARMM, and found that complete or R0 resection is the first choice of treatment

for anorectal melanoma, prognosis is poor regardless of surgical approach, and early diagnosis is the key to improved survival rate for patients with anorectal melanoma.

Innovations and breakthroughs

It can be concluded that the surgical method does not affect the prognosis of ARMM. If the tumor can be resected totally, WLE should be the first choice of treatment. APR can be performed as a rescue therapy when WLE is impossible, the margins of the local excision are positive, or in the event of recurrence.

Applications

Planning of surgery after thorough clinical and radiological investigations, including MRI of the pelvis and endoluminal ultrasound for tumor depth, may aid in defining the appropriate surgical approach for anorectal melanoma.

Terminology

ARMM is an infrequent fatal tumor. At present, surgical therapy remains the main treatment method for ARMM, with APR and WLE as the most common methods used. If the tumor can be resected totally, WLE should be the first choice of treatment. APR can be performed as a rescue therapy when WLE is impossible, the margins of the local excision are positive, or in the event of recurrence.

Peer review

The authors should address how the decision was made as to which of the treatments was recommended to the patients. This may very well have influenced the results in local recurrence.

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S- Editor Wang YR L- Editor Ma JY E- Editor Ma WH

Isolated pancreatic granulocytic sarcoma: A case report and review of the literature

Xin-Ping Li, Wen-Fang Liu, Shu-Rong Ji, She-Hua Wu, Jian-Jun Sun, Yue-Zu Fan

Xin-Ping Li, Wen-Fang Liu, Shu-Rong Ji, She-Hua Wu, Jian-Jun Sun, Yue-Zu Fan, Department of Surgery, Tongji Hospital of Tongji University, Shanghai 200065, China
Author contributions: Li XP and Liu WF contributed equally to this work; Li XP and Liu WF collected the data and wrote the article; Li XP, Ji SR and Wu SH analyzed the data; Liu WF, Sun JJ and Fan YZ designed the study.

Correspondence to: Dr. Wen-Fang Liu, Department of Surgery, Tongji Hospital of Tongji University, Shanghai 200065, China. lwf112@163.com

Telephone: +86-21-66111082 Fax: +86-21-66111082

Received: August 19, 2010 Revised: November 25, 2010

Accepted: December 1, 2010

Published online: January 28, 2011

Li XP, Liu WF, Ji SR, Wu SH, Sun JJ, Fan YZ. Isolated pancreatic granulocytic sarcoma: A case report and review of the literature. *World J Gastroenterol* 2011; 17(4): 540-542 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/540.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.540>

INTRODUCTION

Granulocyte sarcoma (GS), also known as chloroma, is a localized malignant tumor composed of myeloid cells, and is difficult to establish its diagnosis. GS may precede or occur concurrently with acute or chronic myeloid leukemia or less often with polycythemia vera and primary myelofibrosis. It was reported that GS occurs in a variety of tissues but presents as an abdominal mass, and infiltration of the pancreas is particularly rare^[1]. In this paper, we report an unusual case of pancreatic GS in a patient without acute myeloid leukemia.

CASE REPORT

The patient was a 48-year-old woman with no significant past medical history. She was referred because of acute abdomen with no history of abdominal trauma. She complained of persistent severe epigastric pain accompanying a high fever for three days, but not of vomiting, diarrhea or shortness of breath. On physical examination, the patient appeared to be in acute distress with pale complexion. Her abdomen was slightly distended with tenderness. No mass was palpable and bowel movements were decreased. Laboratory test showed 79 g/L hemoglobin, 6400/mm³ white blood cells, 60 × 10³/mm³ platelets, 128 U/L aspartate aminotransferase (AST), 270 U/L alanine aminotransferase (ALT), 1365 U/L lactate dehydrogenase (LDH), and 17.4 U/L carbohydrate antigen 19-9 (CA19-9). Computed tomography scan revealed a 4.5 cm × 4.0 cm fuzzy mass at the pancreatic tail with splenomegaly and splenic infarction (Figure 1). Bone marrow infiltration was assessed and no evidence of acute myeloid leukemia (AML) was

Abstract

Granulocytic sarcoma (GS) is an extramedullary tumor mass consisting of immature myeloid cells. Isolated pancreatic granulocyte sarcoma is extremely rare. We report a very unusual pancreatic granulocytic sarcoma in a patient without acute myeloid leukemia. The patient presented with acute epigastric pain because of splenic infarction due to a mass consisting of myeloblasts in the pancreatic tail. The patients underwent splenectomy and distal pancreatectomy. Pathology and immunohistochemistry suggested a GS. Despite local surgery, an isolated tumor recurred 2 mo after operation and the patient died 3 mo after removal of the tumor. Only 7 reported cases of pancreatic GS were identified in the literature and the mass was located in the pancreatic head. This is the first report of GS in the pancreatic tail with splenic infarction.

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Key words: Granulocytic sarcoma; Pancreatic mass

Peer reviewer: Claudio Sorio, MD, PhD, Department of Pathology, Assistant Professor, University of Verona School of Medicine, General Pathology Section, Strada Le Grazie, 8, 37134 Verona, Italy

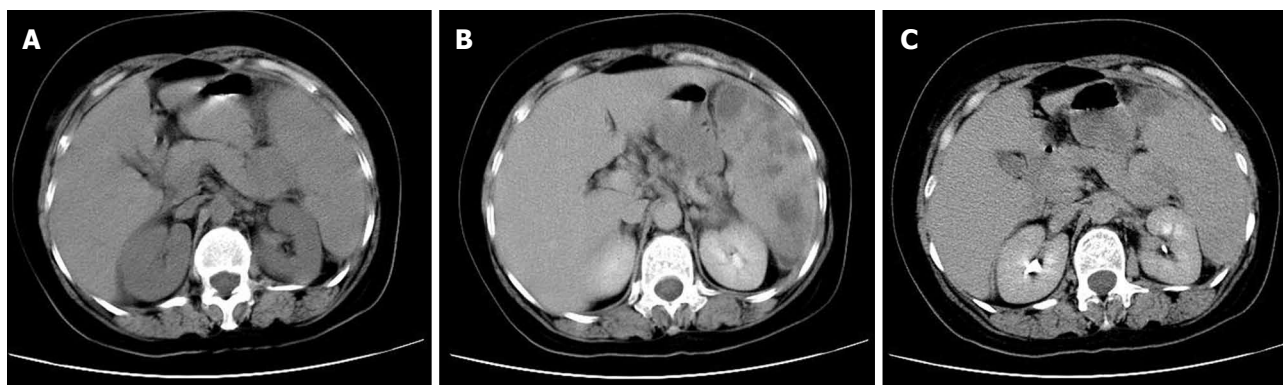


Figure 1 Computed tomography scan showing a low density mass at the pancreatic tail (A), splenomegaly and splenic infarction (B), and a mass at the pancreatic tail (C) in a patient with isolated pancreatic granulocytic sarcoma.

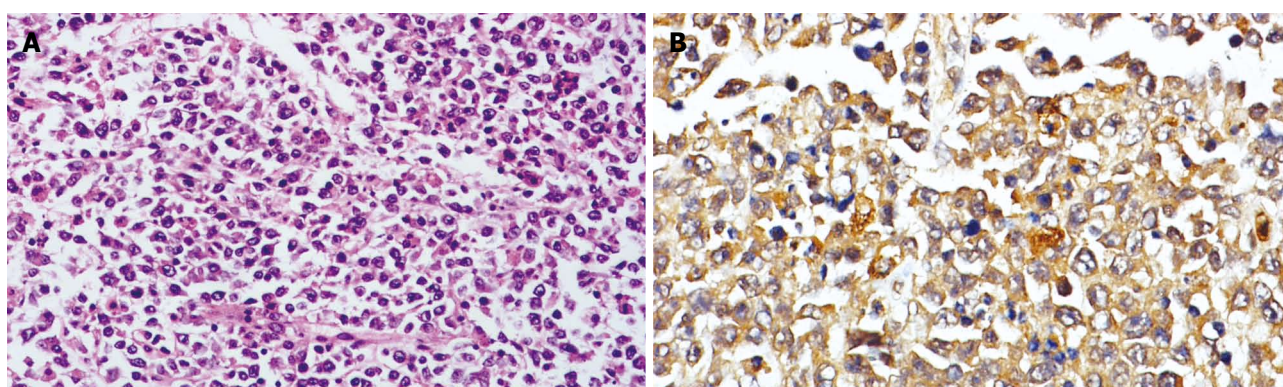


Figure 2 Hematoxylin and eosin staining showing infiltration of immature neoplastic myeloid cells in pancreatic tissue (A, magnification at $\times 100$) and positive reaction of myeloid cells to myeloperoxidase antibodies (B, magnification at $\times 200$) in a patient with isolated pancreatic granulocytic sarcoma.

found. Barium meal examination was negative. Exploratory laparotomy was performed because of persistent severe abdominal pain probably due to pancreatic mass and splenic infarction. An invasive tumor was detected in the pancreatic tail with lymphadenectasis around the hilus of spleen. Consequently, the patient underwent splenectomy and distal pancreatectomy. Histological examination revealed diffusely infiltrating mono-morphous immature blast-like cells (Figure 2A), which were round to oval in shape with mild-moderate basophilic cytoplasm but without granules. Paraffin-embedded sections were examined with immunohistochemical staining. The tumor cells reacted to myeloperoxidase (MPO) antibodies (Figure 2B) but not to CD20 monoclonal antibodies. Therefore, a diagnosis of GS was made and intensive AML-type chemotherapy was recommended. Unfortunately, the patient refused further chemotherapy and was discharged from hospital. Follow-up showed an isolated tumor recurred 2 mo after operation and the patient died 3 mo after operation.

DISCUSSION

GS, also known as chloroma for their green appearance, occurs in association with different hematological diseases, especially FAB M1 and M2 AML^[2,3]. Translocation t (8; 21) is the most common cytogenetic abnormality found in

leukemia patients with GS, which is associated with a relatively good prognosis when treated with chemotherapy^[4]. GS is often confused with non-Hodgkin lymphoma of the lymphoblastic type, Burkitt lymphoma, large-cell lymphoma and small round cell tumor^[5]. It is more difficult to isolate pancreatic GS than to diagnose GS during the course of AML. Immunohistochemical methods are essential to obtain the correct diagnosis. GS cells often react to MPO, CD43, CD68 antibodies, but not to lymphoid antigens such as CD20 and CD30 monoclonal antibodies^[6,7].

Isolated pancreatic GS is most commonly located in periosteum, soft tissue, bone, lymph nodes and skin^[8,9]. However, it is extremely uncommon. Only 7 cases of isolated pancreatic GS, located in the pancreatic head, are available in the literature^[10,11] (Table 1). Consequently, the symptoms of these patients were jaundice and epigastric pain. This is the first report of isolated pancreatic GS located in the pancreatic tail accompanying splenic infarction. The isolated pancreatic GS in the present case invaded the splenic vessels, leading to splenomegaly and splenic infarction and finally severe epigastric pain.

Although isolated pancreatic GS can be treated with radiotherapy or surgical resection, it would recur if not treated with intensive AML-type chemotherapy^[12]. Different strategies are available for improving the disease-free interval of patients with isolated pancreatic GS^[10,11]. All the reported

Table 1 Clinical and laboratory features of patients with isolated pancreatic granulocytic sarcoma

Author	Sex/age	Symptoms	Mass sites	Bone marrow	Therapy	Response
King <i>et al</i> ^[13]	F/36	Jaundice Epigastric pain	Head of pancreas	Normal	Chemotherapy Local XRT	CR
Moreau <i>et al</i> ^[14]	F/37	Jaundice Epigastric pain Respiratory infections	Head of pancreas	AML with 60% blasts	Not available	Unknown
Marcos <i>et al</i> ^[15]	M/32	Right upper guardant epigastric pain	Head of pancreas	Normal	Whipple surgery Chemotherapy	CR
Ravandi-Kashani <i>et al</i> ^[6]	M/31	Jaundice Epigastric pain	Head of pancreas	AML with 6% blasts	Chemotherapy	CR
Ravandi-Kashani <i>et al</i> ^[6]	F/61	Epigastric pain	Head of pancreas	AML with 78% blasts	Chemotherapy	CR Died of relapse 10 mo later
Servin-Abad <i>et al</i> ^[10]	M/64	Jaundice Mild epigastric pain	Head of pancreas	Normal	Chemotherapy	CR Died of Stroke
Rong <i>et al</i> ^[11]	M/40	Jaundice Weight loss	Head of pancreas	Normal	Whipple surgery Chemotherapy	CR
Our case	F/48	Severe left upper guardant epigastric pain, pancytopenia, high fever	Tail of pancreas	Normal	Splenectomy Distal pancreatectomy	Died of relapse 3 mo later

CR: Complete remission; AML: Myeloid leukemia.

cases of isolated pancreatic GS responded well to chemotherapy. However, our patient refused advice of further chemotherapy and died of relapse 3 mo after operation.

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S- Editor Tian L L- Editor Wang XL E- Editor Ma WH

Pernicious anemia: What are the actual diagnosis criteria?

Daniel Cattan

Daniel Cattan, Department of Internal Medicine and Hepato-Gastroenterology, Centre Hospitalier, 94195 Villeneuve Saint George, France

Author contributions: Cattan D contributed to all of this letter.
 Correspondence to: Daniel Cattan, Professor, Department of Internal Medicine and Hepato-Gastroenterology, Centre Hospitalier, 94195 Villeneuve Saint George, France. danielcattan@yahoo.fr

Telephone: +33-1-60470829 Fax: +33-1-43310249

Received: October 21, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: January 28, 2011

Abstract

A gastric intrinsic factor output under 200 U/h after pentagastrin stimulation ($V > 2000$ U/h) is specific for pernicious anemia. The other findings are either variable or non specific. Serum intrinsic factor antibodies, considered as specific in general practice, are present only in half of the patients with pernicious anemia. In their absence, since the disappearance of the Schilling tests, the gastric tubage currently used for the study of gastric acid secretion, is obligatory for the simultaneous study of intrinsic factor output. This study is important to eliminate another disease much more frequent than pernicious anemia, the protein bound to cobalamin malabsorption was observed in achlorhydric simple atrophic gastritis in the presence of intrinsic factor secretion.

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Key words: Pernicious anemia; Intrinsic factor; Achlorhydria; Schilling test; *Helicobacter pylori*

Peer reviewer: Yu-Yuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

Cattan D. Pernicious anemia: What are the actual diagnosis criteria? *World J Gastroenterol* 2011; 17(4): 543-544 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i4/543.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i4.543>

TO THE EDITOR

The recent article entitled "new insights in pernicious anemia (PA) from a gastroenterological point of view" published in issue 41 of the *World Journal of Gastroenterology* 2009^[1], does not clearly describe the actual diagnosis criteria for PA. The recent disappearance of Schilling tests and the difficulties in finding a laboratory able to appreciate the intrinsic factor (IF) output have raised the question about the secure diagnosis of this disease.

A gastric IF output under 200 U/h post pentagastrin stimulation ($N > 2000$ U/h) is specific for PA. The other findings are either variable or non specific. Variable findings include elevated serum gastrin, serum IF antibodies (considered specific in current general practice), normal antral mucosa, normal or elevated serum level of folate and reduced level of erythrocyte folate. Non specific findings include fundic atrophic gastritis, achlorhydria, and hypergastrinemia^[2]. Hyperplasia of enterochromaffin-like cells exists in atrophic gastritis with hypergastrinemia, achlorhydria with conservation of good IF secretion^[3]. Parietal cells antibodies (PCA) are observed in a high proportion of normal middle aged women.

In fact, PA diagnosis is easily feasible in half of the patients in the presence of IF serum antibodies and hypergastrinemia. The absence of any these findings does not eliminate the diagnosis. Replacement of hypergastrinemia by a fundic atrophic gastritis is perhaps admissible. However, this gastritis alone and hypergastrinemia alone are not sufficient. PCA have no place^[4].

In old patients with cobalamin deficiency, the demand is evidently less once intestinal diseases (gluten enteropathy being not forgotten) are eliminated.

In scientific studies, particularly in those on the relation between *Helicobacter pylori* and PA, however, the demand has to be greater than in recent articles^[5,6] to make sure that the patient does not have a simple atrophic gastritis with achlorhydria and conservation of good IF secretion, a disease much more frequent than PA and responsible for a non dissociation of alimentary cobalamin from protein nutriment. In this disease (food bound to cobalamin malabsorption^[7]), the most common disorder of cobalamin absorption^[4], cobalamin deficiency is moderate (the role of chlorhydric acid

in the dissociation of cobalamin from alimentary proteins varies with the nature of these proteins. Moreover IF secretion is important for the reabsorption of cobalamins from bilio-pancreatic and intestinal secretions), the anemia is discrete and sometimes only macrocytosis is observed. Schilling tests show a good absorption of crystalline cobalamin and a malabsorption of various proteins bound to cobalamins^[8]. Treatment can be oral cobalamin^[4]. Longitudinal studies^[4,9] showed that the frequency of the evolution of this gastritis toward PA is very low. This disease is another disease rather than PA. The presence of cobalamin deficiency in these simple achlorhydric gastritis can explain the demand of the earliest authors^[2,9,10] for the diagnosis tests of PA in patients without IF serum antibodies. These diagnosis tests include either gastric tubage with study of the IF output by 15 min fractions in the hours before and after stimulation or Schilling test done in good conditions, that is using the two-stage test (with then without oral administration of IF eventually repeated for the elimination of cobalamin malabsorption due to the cobalamin deficiency's effect itself on the intestinal mucosa)^[10].

Since Schilling tests are no longer available, the diagnostic criteria have changed^[4,11]. In the absence of serum IF antibodies in a patient with a low serum cobalamin level, the gastric tubage for study of IF output is obligatory for scientific purposes.

Deficiency in IF secretion is the "gold standard" for the diagnosis of PA, which should be used to evaluate the value of associations between serological markers, including eventually new PCA.

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S- Editor Tian L L- Editor Wang XL E- Editor Lin YP



ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Alexander Becker, MD, Department of Surgery, Haemek Medical Center, Afula 18000, Israel

Lisardo Boscá, Dr., Professor, Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain

Jan Bures, MD, PhD, Professor, University Department of Gastroenterology, Charles University, Faculty of Medicine, University Teaching Hospital, Sokolska 581, 50005 Hradec Kralove, Czech Republic

José Liberato Ferreira Caboclo, Dr., Professor, Rua Antônio de Godoy, 4120, São José do Rio Preto, Brazil

Eugene P Ceppa, MD, Department of Surgery, DUMC 3443, Durham, NC 27710, United States

Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Henning Gerke, MD, Associate Clinical Professor, Medical Director, Diagnostic and Therapeutic Unit, Digestive Disease Center, Director, Endoscopic Ultrasound, Division of Gastroenterology-Hepatology, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52246, United States

Mark D Gorrell, PhD, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Hai-Yong Han, PhD, MS, BS, Division of Clinical Translational Research, The Translational Genomics Research Institute (TGen), 445 N. Fifth Street, Phoenix, AZ 85004, United States

Kevin Cheng-Wen Hsiao, M.D., Assistant Professor, Colon and Rectal Surgery, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-Kung Rd, Nei-Hu district, Taipei 114, Taiwan, China

Satoru Kakizaki, MD, PhD, Assistant Professor, Department of Medicine and Molecular Science, Gunma University, Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan

Seon Hahn Kim, MD, Department of Surgery, Korea University Anam Hospital, #126-1, 5-ga, Anam-dong, Sungbook-gu, Seoul, 136-705, South Korea

Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Kyu Taek Lee, M.D., Ph.D., Professor, Department of Medicine Samsung Medical Center, Sungkyunkwan, University School of Medicine, #50, Irwon-dong, Gangnam-gu, Seoul, 135-710, South Korea

Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euroclinic, 19 Mavromateon Street, Athens 10 34, Greece

Kazuichi Okazaki, Professor, Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka, 570-8506, Japan

Giuseppe Orlando, MD, PhD, Department of Health Sciences, Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston Salem, NC 27101, United States

Mark Pines, PhD, Institute of Animal Sciences, Volcani Center, PO Box 6, Bet Dagan 50250, Israel

Tokihiko Sawada, MD, PhD, Associate Professor, Second Department of Surgery, Dokkyo Medical University, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi, 321-0293, Japan

Beat Schnüriger, MD, University of Southern California, Keck School of Medicine, Department of Surgery, Division of Acute Care Surgery, (Trauma, Emergency Surgery and Surgical Critical Care), 1200 North State Street, Inpatient Tower (C), 5th Floor, Room C5L100, Los Angeles, CA 90033-4525, United States

Andreas G Schreyer, Dr., Department of Radiology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Virendra Singh, Dr., MD, DM, Additional Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, 160012, India

Vincenzo Stanghellini, MD, Professor of Medicine, Department of Internal Medicine and Gastroenterology, Policlinico S.Orsola-Malpighi, University of Bologna, VIA MASSARENTI 9, Bologna-40138, Italy

Bruno Stieger, Professor, Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

Shoichiro Sumi, MD, PhD, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan

Masahiro Tajika, MD, PhD, Department of Endoscopy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan

Yuk Him Tam, Dr., Department of Surgery, Prince of Wales Hospital, Shatin, Hong Kong, China

Rakesh Kumar Tandon, Professor, Pushpawati Singhan Research Institute for Liver, Renal and Digestive Diseases, Sheikh Sarai- Phase II, New Delhi 110017, India

Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu, 51014, Estonia



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

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International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

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Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

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9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
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May 19-22, 2011
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in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
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Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
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September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
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September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
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September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
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Hotel, Place Rogier 3, 1210 Brussels,
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Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
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Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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World Journal of Gastroenterology

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ISSN 2219-2840 (online)

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Books

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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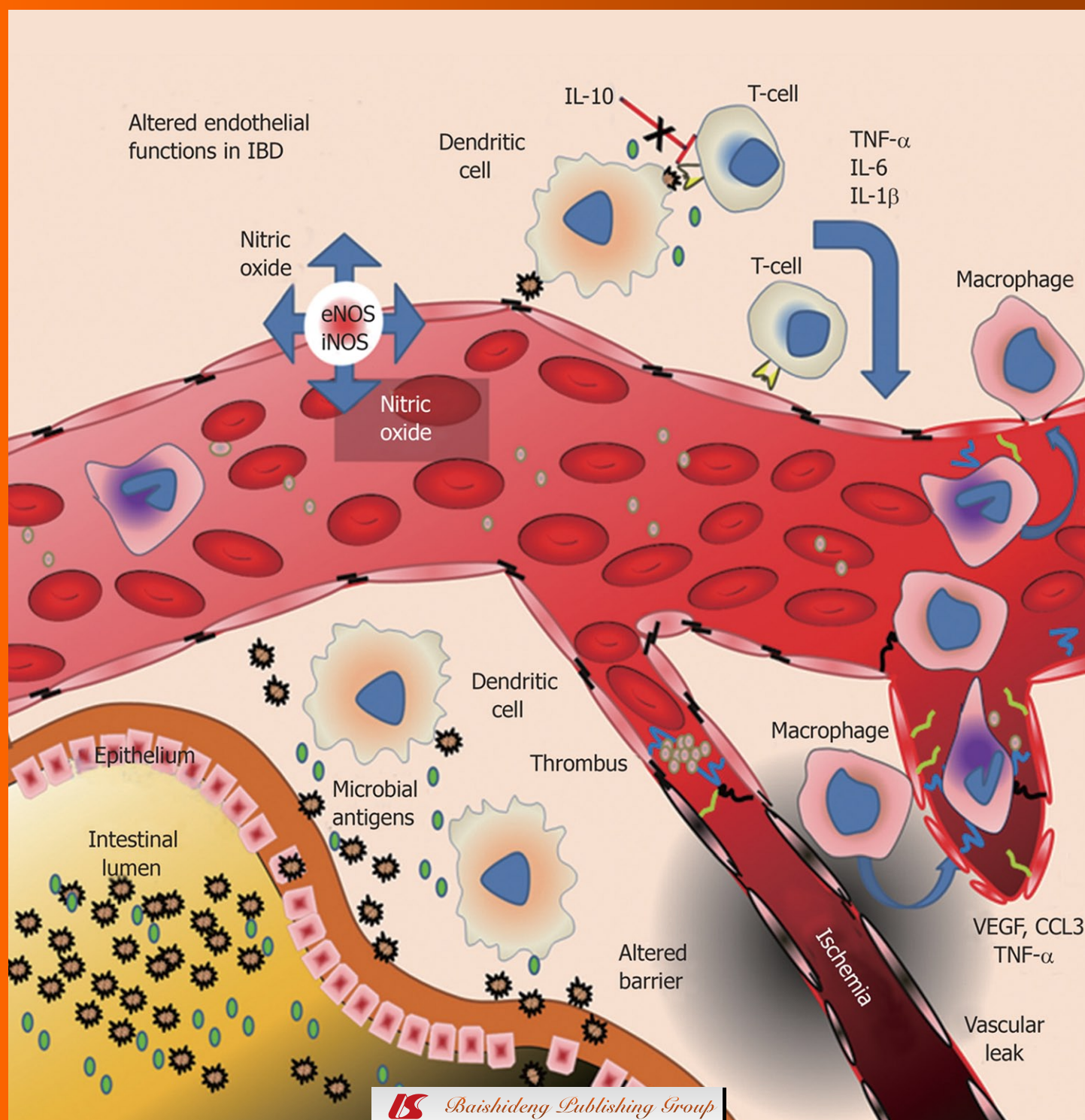
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings
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ABOUT COVER Cromer WE, Mathis JM, Granger DN, Chaitanya GV, Alexander JS.
Role of the endothelium in inflammatory bowel diseases.
World J Gastroenterol 2011; 17(5): 578-596
<http://www.wjgnet.com/1007-9327/full/v17/i5/578.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
February 7, 2011

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

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What's hot in inflammatory bowel disease in 2011?

Silvio Danese

Silvio Danese, IBD Center, Division of Gastroenterology, IRCCS Istituto Clinico Humanitas, Via Manzoni 56, 20089, Rozzano, Milan, Italy

Author contributions: Danese S contributed all to the paper.

Correspondence to: Dr. Silvio Danese, MD, PhD, IBD Center, Division of Gastroenterology, IRCCS Istituto Clinico Humanitas, Via Manzoni 56, 20089, Rozzano, Milan, Italy. sdanese@hotmail.com

Telephone: +39-2-82244771 Fax: +39-2-82244590

Received: January 4, 2011 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: February 7, 2011

Abstract

Ulcerative colitis and Crohn's disease (CD) are the two major forms of inflammatory bowel disease (IBD). In this highlight topic series of articles, the most recent advances in the IBD field are reviewed, especially the newly described cytokines, including the therapeutic implications for their manipulation. In addition, the interplay between the intestinal microbiota and the host is reviewed, including the role of defensins and dysbiosis in CD pathogenesis. Finally, the importance of the non immune systems such as endothelial cells and the hemostatic system are highlighted as new players in IBD pathogenesis.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Immunology; Pathogenesis

Danese S. What's hot in inflammatory bowel disease in 2011? *World J Gastroenterol* 2011; 17(5): 545-546 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/545.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.545>

(*WJG*), we have selected an expert group that is actively involved in the investigation of inflammatory bowel disease (IBD) pathogenesis.

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of IBD.

These diseases still pose major clinical and therapeutic challenges to the gastroenterological community.

It is now clear that CD and UC represent two distinct forms of chronic inflammation of the gastrointestinal tract and have different causes and pathogenic mechanisms. Still, the factors underlying the appearance of both CD and UC are roughly the same, and include a temporal association with progressive changes in the environment, an intrinsic genetic predisposition, the existence of a rich enteric flora, and an abnormal immune reactivity which is ultimately responsible for damaging the gut and causing clinical manifestations. Even though the categories of underlying factors are roughly the same, there are variations in each category as well as differences in how the underlying factors interact. The end result is two related but distinct disorders named CD and UC. In this special issue of *WJG*, differences and similarities of the etiopathogenic factors in each form of IBD will be illustrated and discussed in each review assessing the newly described cytokines^[1], the interplay between the intestinal microbiota and the host^[2], the role of defensins and dysbiosis^[3] and the importance of extraluminal factors^[4] and non immune systems such as endothelial cells^[5] and the hemostatic system^[6] as new players in IBD pathogenesis.

Since the recognition of IBD as a perplexing and challenging clinical entity, the investigation of its pathogenic mechanisms has gone through repeated cycles of new hopes, new knowledge, and new realities. Infectious, allergic, dietary, psychosocial, environmental, microbial, vascular, metabolic, immune and other basic theories have been put forward, most of them to be rebuked, if not ridiculed. At the moment, we appear to have settled down on a unifying but still wide-ranging hypothesis that IBD results from complex interactions between evol-

In this special issue of *World Journal of Gastroenterology*

ing environmental changes induced by society progress, a still undefined number of predisposing genetic mutations, an incredibly complex gut microbiota that may be constantly varying, and the intricacies of individual immune systems. The ability to integrate all these various components into a single cohesive and logical pathway of disease that explains all aspects of IBD appears still a bit distant at the moment. On the other hand, if we look back at where we stood only two or three decades ago, the progress achieved in our understanding of IBD pathogenesis and the way it has changed our approach to therapy is just short of spectacular.

Although we have made tremendous advances in disease pathogenesis, among the many diseases that exist, IBD is the one for which the exact etiology remains obscure and the mechanisms underlying tissue injury appear to be exceedingly complex. This certainly seems to be the case for the two main forms of IBD, namely CD and UC.

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Recent advances in cytokines: Therapeutic implications for inflammatory bowel diseases

Guillaume Bouguen, Jean-Baptiste Chevaux, Laurent Peyrin-Biroulet

Guillaume Bouguen, Inserm UMR991, Department of Hepato-Gastroenterology, University Hospital of Rennes, Pontchaillou, 35000 Rennes, France

Jean-Baptiste Chevaux, Laurent Peyrin-Biroulet, Inserm U954, Department of Hepato-Gastroenterology, University Hospital of Nancy, 54511 Vandoeuvre-les-Nancy, France

Author contributions: Bouguen G wrote the first draft of the paper; Chevaux JB and Peyrin-Biroulet L revised the manuscript.
Correspondence to: Laurent Peyrin-Biroulet, MD, PhD, Professor, Inserm U954, Department of Hepato-Gastroenterology, University Hospital of Nancy, Allée du Morvan, 54511 Vandoeuvre-les-Nancy, France. peyrin-biroulet@netcourrier.com
Telephone: +33-3-83153631 **Fax:** +33-3-83153633
Received: March 1, 2010 **Revised:** March 22, 2010
Accepted: March 29, 2010
Published online: February 7, 2011

Peer reviewer: Takayuki Yamamoto, MD, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan

Bouguen G, Chevaux JB, Peyrin-Biroulet L. Recent advances in cytokines: Therapeutic implications for inflammatory bowel diseases. *World J Gastroenterol* 2011; 17(5): 547-556 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/547.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.547>

Abstract

Inflammatory bowel diseases (IBDs) are complex and chronic disabling conditions resulting from a dysregulated dialogue between intestinal microbiota and components of both the innate and adaptive immune systems. Cytokines are essential mediators between activated immune and non-immune cells, including epithelial and mesenchymal cells. They are immunomodulatory peptides released by numerous cells and these have significant effects on immune function leading to the differentiation and survival of T cells. The physiology of IBD is becoming a very attractive field of research for development of new therapeutic agents. These include cytokines involved in intestinal immune inflammation. This review will focus on mechanisms of action of cytokines involved in IBD and new therapeutic opportunities for these diseases.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Cytokine; Pathophysiology; Biological therapy

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated disorders of the intestine^[1]. Accumulating data suggests that inflammatory bowel disease (IBD) results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host^[2]. Emerging evidence suggests that disease development implicates a dysregulated dialogue between the intestinal flora and components of both the innate and adaptive immune systems^[3,4].

Active IBD is defined as an infiltration of the lamina propria by innate immune cells [neutrophils, macrophages, dendritic and natural killer (NK) T cells] and adaptive immune cells (B and T cells). Increased numbers and activation of these cells in the intestinal mucosa enhance local levels of tumor necrosis factor- α (TNF- α) and several proinflammatory interleukins (IL)^[2,5]. Cytokines are essential mediators of the interaction between activated immune cells and non-immune cells, including epithelial and mesenchymal cells^[6,7].

Recent advances in the study of the regulation of key cytokines during major forms of IBD promise the development of more effective mechanism-based therapies^[8]. Given that many of these involve regulation of dynamic biological processes, it is likely that the most effective agents will fall within the broad rubric of biologic therapy.

The prototypic example of the ability of a biologic agent to effectively change the therapeutic landscape is provided by anti-TNF- α , first demonstrated through clinical validation of the prototypic agent infliximab^[8]. The advent of anti-TNF- α agents has changed the way of treating IBD refractory to standard medications^[3,9].

Advances in the understanding of IBD pathophysiology have become a very active area for the development of novel therapeutic agents. New targets for biologics include cytokines involved in intestinal immune inflammation that have led to new therapeutic opportunities^[10,11]. Although IBD etiology is unknown, some molecules which are involved in the physiopathology have been identified and can be targeted by biological therapies^[12]. This review will focus on cytokines involved in the dysregulated inflammatory response in IBD and targeted by biological therapies.

CYTOKINE NETWORK AND IMMUNITY

Cytokines (from greek cyto: cell; kinos: movement) are substances that are secreted by specific cells of the immune system and carry signals locally between cells, with extensive use in cellular communication. The term “cytokine” encompasses a large and diverse family of polypeptide regulators that are produced widely throughout the body by cells of diverse embryological origin. Basically, the term “cytokine” has been used to refer to the immunomodulating agent. Interferon was the first cytokine to be described in 1957^[13]. The clinical efficacy of targeting TNF- α indicates that cytokines are potential therapeutic targets in IBD^[6].

Cytokines have profound effects on immune functions^[14]. Beyond the classical T helper Th1/Th2 paradigm indicating predominant Th1-mediated responses dominated by the production of interferon- γ (IFN- γ) in CD and an exaggerated Th2-like inflammation in UC characterized by an increased production of IL-13^[2,15], there has been a surge of information with regard to the role of innate immunity in IBD pathogenesis. Thus new data on adaptive immunity are emerging, indicating that: (1) the mucosal Th1 and Th2 responses of CD and UC may be actually secondary to defects of the innate immune response; (2) the dysfunction of regulatory T cells may be contributing to mucosal immune abnormalities; and (3) the newly described Th17 cells are also prominently involved in the gut inflammatory response in both forms of IBD^[5,15].

The differentiation and survival of T cells depend on the relative amount of key regulatory cytokines produced mainly by macrophages and dendritic cells^[12]. In the presence of IL-12 and IFN- γ , naive CD4⁺ T cells adopt a Th1 phenotype which then activate macrophages that release IL-1, IL-6 and TNF- α . Thus this creates a positive feedback loop^[3,6,12]. In the presence of IL-4, naive CD4⁺ T cells adopt a Th2 phenotype^[12,16]. The Th17 development is triggered by both IL-6, IL-21, IL-23 and transforming growth factor- β (TGF- β), leading to secretion of the IL-17 cytokine family and IL-22^[6]. Although the function of Th17 cells is not clearly known, there is probably an important part of this T cell population which expresses

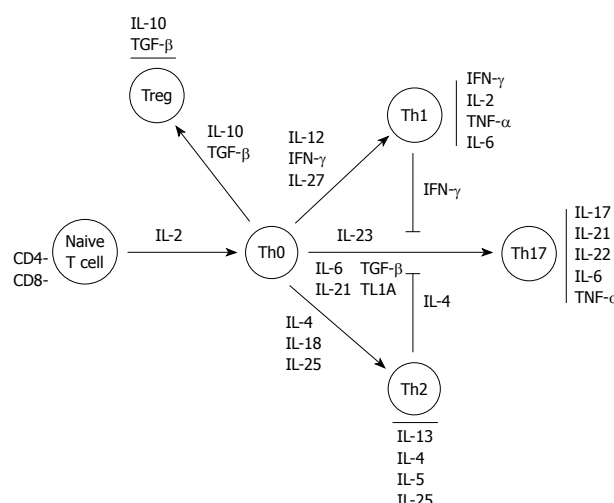


Figure 1 Overview of T cell differentiation and interleukin pathways. IL: Interleukin; IFN: Interferon; TGF: Transforming growth factor; TNF: Tumor necrosis factor; TL1A: TNF-like factor 1A.

IL-23 receptors. This has been recently demonstrated as an IBD susceptibility gene in genome-wide association studies.

In contrast, TGF- β and IL-10 modulate differentiation of naive T cells to T regulatory cell subgroups leading to high amounts of IL-10 and TGF- β , and are able to suppress bystander T cell activation. This could be defective in IBD^[17-20]. There is a complex network between these different cell populations in the case of inflammation as, for example, in the negative crossregulation of the differentiation of Th17 cells by Th2 cells (IL-4, IL-27) and Th1 cells (IFN- γ) (Figure 1)^[21].

PROINFLAMMATORY CYTOKINES

TNF family: TNF- α and TNF-like factor 1A

Mechanisms of action: TNF- α is a major mediator of inflammation in the gut^[22-25]. It is synthesized by several cells including intestinal epithelial cells but predominantly by cells of the monocyte line and T lymphocytes^[14]. TNF- α is a homotrimeric protein that mediates its diverse biologic effects through 2 distinct receptors known as TNF- α receptor type I expressed on all nucleated cells and TNF- α receptor type II restricted to cells of hematopoietic lineage^[26]. Through the activation of nuclear factor- κ B (NF- κ B), TNF- α induces the expression of various genes such as urokinase plasminogen activator, cyclooxygenase II (COX II) and vascular endothelial growth factor (VEGF)^[26]. By this method, TNF- α has multiple biological effects such as increasing leukocyte recruitment (induction of leukocyte adhesion molecules)^[27,28], modulation of nitric oxide (NO) production (increasing the vascular permeability)^[29,30], induction of secretion of proinflammatory cytokines^[31], and the proliferation and differentiation of immune cells^[26]. *TNFSF15* encodes TNF-like factor 1A (TL1A), which is a TNF-like molecule that mediates co-stimulation of Th1 and Th17 cells. It is required for optimal differentiation of

Table 1 Clinical efficacy and marketing approval for anti-tumor necrosis factor- α agents

Drug name	Efficacy (% of induction of remission/% sustained remission)			Approved (FDA/Europe)		
	Luminal CD	Fistulizing CD	UC	Luminal CD	Fistulizing CD	UC
Infliximab (Remicade®)	33/45	55/36	38.8/23.1	Yes/Yes	Yes/Yes	Yes/Yes
Adalimumab (Humira®)	36/36	No RCT	No RCT	Yes/Yes	No/No	No/No
Certolizumab (Cimzia®)	35/48	No RCT	No RCT	Yes/No	No/No	No/No

CD: Crohn's disease; UC: Ulcerative colitis; FDA: US food and drug administration; RCT: Randomized controlled trial.

Th17 cells^[21,32]. Variants in the *TNFSF15* gene contribute to overall CD susceptibility^[33,34] and an increased production of TL1A has been observed in CD^[35]. Interestingly, in mice, colitis was prevented and attenuated by an anti-TL1A antibody^[36].

Results of clinical trials (Table 1): Three anti-TNF agents, namely infliximab, adalimumab and certolizumab pegol have been approved by the US Food and Drug Administration for the treatment of luminal CD. In Europe, certolizumab has not yet received approval for IBD. Infliximab has also been approved for fistulizing CD and UC. In luminal CD, infliximab was effective in inducing clinical remission in 33% of patients compared with only 4% of a placebo group at week 4 ($P = 0.005$)^[37], and in maintaining clinical remission (45% in the infliximab group *vs* 21% in the placebo group, $P < 0.005$). Adalimumab was also significantly more effective than placebo in inducing clinical remission (36% *vs* 12%, $P < 0.001$)^[38], and more effective than placebo in maintaining clinical remission at week 56 (36% *vs* 16%). Infliximab and adalimumab have also been shown to be more effective than placebo in maintaining steroid-free remission at 1 year^[39,40]. Regarding certolizumab pegol, results from large randomized, placebo-controlled trials are more controversial, with no improvement at week 6 and different long-term response rates between trials^[41,42]. In fistulizing CD, 55% of the patients who received 5 mg/kg infliximab had complete fistula closure, as compared with only 13% of the patients assigned to placebo ($P = 0.001$)^[43]. In UC, 2 large randomized, placebo-controlled studies, namely the ACT 1 and ACT 2 trials, evaluated the efficacy of infliximab for induction and maintenance therapy in UC^[44]. In both trials, at week 8, nearly two-thirds of patients in the group receiving 5 mg of infliximab had had a clinical response, as compared with one-third of patients in the placebo group ($P < 0.001$).

Regarding the safety of anti-TNF agents, the Crohn's Therapy, Resource, Evaluation, and Assessment Tool registry, including 3179 CD patients who received infliximab, demonstrated that this agent was not an independent predictive factor of serious infections^[45]. In a meta-analysis of 21 placebo-controlled trials enrolling 5356 individuals, anti-TNF therapy did not increase the risk of death, malignancy or serious infection when compared to control arms^[9]. However, a longer duration of follow-up and a larger number of patients are required to better assess the safety profile of anti-TNF agents in CD.

Mechanisms of action of anti-TNF- α agents remain poorly known. Neutralization of TNF- α in the inflamed mucosa is unlikely to be a sufficient explanation. Antibody-dependent cytotoxicity also induces apoptosis or lysis of TNF- α -producing cells. This mechanism involves the Fc portion of antibodies that increases the pro-apoptotic factor caspase-3^[46].

IL-12, p40/IL-23, p40

Mechanisms of action (Figure 2): IL-12 is a key cytokine that drives the inflammatory response mediated by Th1 cells^[47,48]. As such, it underlies both normal host responses to a variety of intracellular bacterial, fungal and protozoan pathogens, and abnormal inflammatory responses linked to many autoimmune diseases, such as CD^[49]. Indeed CD is characterized by increased production of IL-12 by antigen-presenting cells in intestinal tissue^[50,51]. IL-23, secreted by antigen-presenting cells, is also a central cytokine involved in the differentiation and function of Th17 cells^[2]. The IL-23-Th17 interaction mediates microbial defenses and intestinal inflammation^[52,53]. Individual properties of IL-23 are also underscored by identification of the gene encoding the receptor for this cytokine as modifying host susceptibility^[8,54,55]. These 2 most potent Th1- and Th17-activating cytokines, IL-12 and IL-23 are both composed of a p40 subunit and therefore, a p40 antibody may have therapeutic potential in inhibiting both Th1-activating IL-12 and Th17-activating IL-23^[21].

Results of clinical trials (Figure 2 and Table 2): IL-12 and IL-23 are targeted by one humanized IL-12/23 antibody, ABT-874. It has shown promising results in a phase II dose-ranging study comprising 79 patients with CD^[49]. Seven weeks of uninterrupted treatment with 3 mg/kg ABT-874 resulted in higher response rates than placebo (75% *vs* 25%, $P = 0.03$). Another dose-ranging study comparing efficacy, safety and pharmacokinetic of intravenous infusions of ABT-874 *vs* placebo in subjects with active CD is ongoing. A double-blind, placebo-controlled, parallel-group, crossover study, assessing ustekinumab in 104 patients with CD has been completed^[56]. The clinical response to ustekinumab was significantly greater than the group given placebo at weeks 4 and 6 (52%-54% *vs* 22%-39%, $P < 0.05$) but not at week 8 (49% *vs* 40%, $P = 0.34$). Interestingly, the effect was most prominent in patients treated previously with infliximab at weeks 4, 6 and 8 (59% in the ustekinumab group *vs* 25%-26% in the placebo group, $P < 0.05$). A phase 2, randomized,

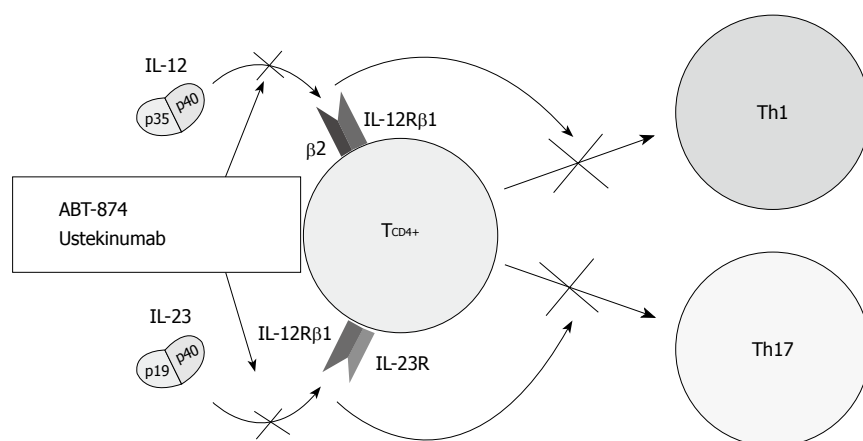


Figure 2 Therapeutic blockade of the interleukin-12/interleukin-23 pathway at the common p40 subunit of both cytokines. IL: Interleukin.

Table 2 Summary of safety and efficacy of anti-cytokine therapies in randomized, controlled trials

Study	Drug name	Targeted cytokine	Indication	No. of patients	Follow-up	Clinical response (%)	Clinical remission (%)	SAE (%)
Mannon <i>et al</i> ^[49]	ABT-874	IL-12/23	CD	79	8 wk	69	38	10
Sandborn <i>et al</i> ^[56]	Ustekinumab	IL-12/23	CD	131	8 wk	49	19	4
Sands <i>et al</i> ^[57]	Apilimod mesylate	IL-12/23	CD	220	168 d	25.7	NA	NS ^b
Ito <i>et al</i> ^[64]	Tocilizumab	IL-6	CD	36	12 wk	80	20	13
Hommes <i>et al</i> ^[68]	Fontolizumab	IFN- γ	CD	133	28 d	38-44	19-31	4.5
Reinisch <i>et al</i> ^[69]	Fontolizumab	IFN- γ	CD	201	29 d	31-38	21	13.6
Van assche <i>et al</i> ^[79]	Daclizumab	IL-2	UC	159	8 wk	25-33	2-7	4.3-12.5
Schreiber <i>et al</i> ^[85]	rhIL-10	IL-10	CD	320	29 d	NA	23.5	7
Schreiber <i>et al</i> ^[86]	rhIL-10	IL-10	UC	94	28 d	NA ¹	NA ¹	7.5
Colombel <i>et al</i> ^[87]	Tenovil	IL-10	Postoperative CD	65	12 wk	46% of patients with endoscopic recurrence		9
Sands <i>et al</i> ^[95]	rhIL-11	IL-11	CD	148	8 wk	31.5 ⁴	36.7 ⁴	NS ²
Herrlinger <i>et al</i> ^[90]	rhIL-11	IL-11	CD	51	12 wk	19 ³	4 ³	8
Pena-Rossi <i>et al</i> ^[96]	IFN- β 1a	IFN- β 1a	UC	194	12 wk	NA	20-29.2	12.3
Tilg <i>et al</i> ^[97]	PEG-IFN- α	IFN- α	UC	60	12 wk	NA	40	15

¹No difference between placebo and rhIL-10 treatment; ²More headache, edema and increased platelet count; ³Significantly inferior than prednisolone; ⁴Significantly superior than placebo at a dose of 15 microg/kg weekly. SAE: Severe adverse event; IL: Interleukin; IFN: Interferon; CD: Crohn's disease; UC: Ulcerative colitis; NA: Not available; NS: Not significant.

double-blinded, placebo-controlled study has evaluated the efficacy of apilimod mesylate, an oral IL-12 and IL-23 inhibitor in treating 220 patients with moderate-to-severe CD. The enrollment was closed early because it did not demonstrate efficacy over placebo^[57].

IL-6

Mechanisms of action: IL-6 is produced by various cells such as T cells, B cells, monocytes, fibroblasts, osteoblasts, keratinocytes, endothelial cells, mesangial cells and some tumor cells^[58]. This cytokine specifically binds to the IL-6 receptor (IL-6R) or a soluble IL-6R, forming the IL-6/IL-6R complex that binds to gp130 and activates intracellular pathways including JAK/STAT signaling, tyrosine phosphatase SHP2 and NF- κ B^[59]. Many cells express gp130, hence IL-6 is a pleiotropic multi-functional cytokine acting as both a proinflammatory and an antiinflammatory cytokine^[12,59]. It is involved in terminal differentiation of B cells, differentiation and activation of T cells, induction of a hepatic acute-phase response, hematopoiesis and fever^[60,61]. Thus activated IL-6 plays a major role in its own amplification and then in the chronic phase of inflammation helped by mononuclear cell accumulation at the site

of injury, through continuous monocyte chemoattractant protein-1 secretion, angioproliferation and antiapoptotic functions of T cells^[59,62]. Plasma soluble IL-6R is increased in patient with CD and IL-6 plasma concentrations increase in active CD^[63].

Results of clinical trials (Table 2): Tocilizumab binds to both the membrane-bound and the soluble forms of human IL-6R with high affinity and specificity^[3,64]. Tocilizumab has shown promising results in a small phase I / II study ($n = 36$) that met its primary endpoint. At 12 wk, the response rate was higher in patients given an 8 mg/kg infusion of tocilizumab every 2 wk than in those given placebo (80% *vs* 31%, $P = 0.019$) and is accompanied by a decrease in C-reactive protein concentration^[3,64]. However, only 2 of 10 patients went into remission, compared with none of 13 in the placebo group ($P = 0.092$), without significant improvement in mucosal healing^[3,64]. Improvement in disease activity in a patient with UC associated with Takayasu arteritis has been reported after treatment with tocilizumab^[65]. A placebo-controlled phase I study on the safety and biological effects of c326, an inhibitor of IL-6, in CD is ongoing.

IFN- γ

Mechanisms of action: Type II INF, also called IFN- γ , is a proinflammatory cytokine secreted by Th1-cells^[66]. IFN- γ drives expression of major histocompatibility complex class II on antigen-presenting cells, modulates lipopolysaccharide responsiveness in intestinal epithelial cells, and increases chemokine secretion. It also activates macrophages, Th1 lymphocytes in a positive feedback loop, NK cells and endothelial cells^[12,66,67]. Concentrations of IFN- γ are increased both in UC and CD.

Results of clinical trials (Table 2): Fontolizumab has been assessed in 3 phase I / II dose-ranging studies enrolling a total of 374 patients with moderate to severe CD^[68-70]. Fontolizumab at doses of up to 4 mg/kg improved endoscopic lesions and decreased concentrations of C-reactive protein^[68-70], but no study met its primary endpoint, which was defined as induction of clinical response at 1 mo^[68-70]; thus the development of fontolizumab for CD has been stopped^[3].

IL-2 family

Mechanisms of action: IL-2 is produced mainly by activated T cells^[71]. In addition to promoting T cell proliferation and activation, IL-2 increases cytokine production and modifies the functional properties of B cells, NK cells, and macrophages. Thus, it improves the activated macrophage microbicidal and cytotoxic activities and promotes secretion of hydrogen peroxide, TNF- α and IL-6^[72]. IL-2 signals through a heterodimeric ($\alpha\gamma$) or trimeric $\alpha\beta\gamma$ high-affinity receptor complex^[72]. Studies have proved a role for IL-2 in IBD pathogenesis, for example the calcineurin inhibitor cyclosporin, which inhibits IL-2 production, is effective in the treatment of severely active UC^[73]. IL-21, an IL-2 cytokine family member expressed by activated CD4+ T cells and NK T cells, is a key regulator in production of Th17 cells. It also increases the proliferation of Th1 cells, CD4+ and CD8+ lymphocytes and regulates the profile of cytokines secreted by these cells^[19,74]. Indeed, IL-21-deficient mice are protected from experimental colitis, possibly through the failure to generate the Th17 response^[75]. Furthermore, blockade of endogenous IL-21, with an antagonistic IL-21R/Fc, ameliorated dextran sulphate sodium colitis in mice^[75]. No studies have been performed in humans as yet.

Results of clinical trials (Table 2): Two antibodies against the α -chain of the IL-2 receptor (CD25), namely daclizumab and basiliximab, have been studied to mimic the activity of cyclosporine^[76-78]. Despite promising response rates observed in an uncontrolled trial, a randomized, double-blind, placebo-controlled, dose-ranging trial failed to demonstrate an increased remission or clinical response both at high (2 mg/kg intravenously at weeks 0, 2, 4, and 6) and low doses (1 mg/kg intravenously at weeks 0 and 4) in 159 treated patients with daclizumab for active UC^[79].

ANTIINFLAMMATORY CYTOKINES**IL-10**

Mechanisms of action: IL-10 is secreted by a wide variety of cells and has pleiotropic effects on T cells, B cells, myeloid cells, and other cell types^[80]. IL-10 has suppressive antiinflammatory activity on T cells, macrophages, and dendritic cells (among other cells) in humans, as well as in animal models of inflammatory diseases^[80]. In particular, mice deficient in IL-10 or the IL-10 receptor undergo spontaneous development of intestinal inflammation, similar to human disease^[81,82]. Even though IL-10 effectively treats colitis in mouse models and suppresses inflammatory cytokine production *in vitro* in intestinal cells from IBD patients^[83], unfortunately clinical trials using recombinant IL-10 to treat IBD in humans have been largely disappointing^[84].

Results of clinical trials: A placebo-controlled study was conducted in 329 patients with moderate-to-severe CD and in 94 patients with UC and did not demonstrate any significant improvement in response and remission rates compared to placebo^[85,86]. Also, no evidence of prevention of endoscopic recurrence in CD by subcutaneous IL-10 injections was observed in a placebo-controlled trial of 65 CD patients^[87]. Animal studies showed that local administration of IL-10 to the colon *via* genetically engineered *Lactococcus lactis* bacteria administered orally allowed for the achievement of high colonic mucosal concentrations of IL-10, potentially resulting in increased efficacy^[12,88].

IL-11

Mechanisms of action: IL-11 is a pleiotropic cytokine from mesenchymal cell origin^[89]. It exhibits potent antiinflammatory activity on macrophages and T cells by inhibiting the secretion of pro-inflammatory cytokines^[90-92] and has shown beneficial effects on intestinal mucosa in several animal IBD models^[89,90]. However one study suggested that the expression of the IL-11 receptor α -chain in the mucosa was restricted to epithelial cells, and although reducing apoptosis, it had no antiinflammatory effects on these cells^[93].

Results of clinical trials: In a placebo-controlled study in 76 active CD patients, subcutaneously administered recombinant human IL-11 was shown to be safe and well tolerated^[94]. In a second placebo-controlled study in 148 patients comparing 2 doses of subcutaneously administered recombinant human IL-11, it was significantly superior in inducing remission after 6 wk when compared to placebo^[95]. In contrast, a recent trial showed significant inferiority of recombinant human IL-11 when compared to prednisolone in inducing remission in active CD and in obtaining a clinical response^[90].

Type I IFNs

Mechanisms of action: Type I IFNs consist of 14 α

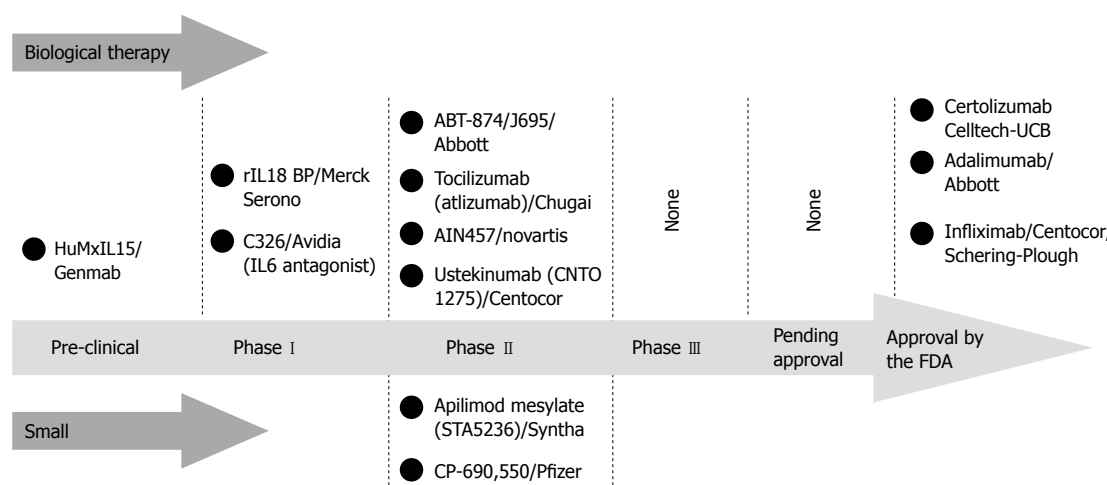


Figure 3 Cytokine therapies and inflammatory bowel disease: pipeline compounds.

isoforms and β , ϵ , ω , and κ isoforms^[66]. Immunoregulatory therapy with type I IFNs such as IFN- α or IFN- β can inhibit production of TNF- α and IFN- γ , antagonize the IFN- γ signaling pathway and increase production of the antiinflammatory cytokine IL-10. It has also been shown to be immunoregulated by enhanced regulatory T lymphocyte and NK cell activity^[66].

Results of clinical trials: Several type I IFNs have been studied in UC. A phase 2 placebo-controlled, dose-ranging trial, studied IFN- β 1a in 194 patients with moderately active UC. Clinical outcomes, including the proportion of patients achieving endoscopically confirmed remission, were not statistically significantly superior in the IFN- β 1a treatment groups over placebo^[96]. A randomized, placebo-controlled trial of pegylated IFN- α in 60 patients with active UC did not show any efficacy in clinical response and response rate despite a significant decrease in levels of C reactive protein^[97].

WHERE DO WE GO FROM HERE?

In 2010, infliximab represents the pinnacle of the therapeutic pyramid of IBD treatment. However, this anti-TNF agent has several limitations. First, despite its widespread use in IBD, 20% of patients still require surgery^[98]. Second, about 10% of patients are primary non-responders to infliximab and only one-third of IBD patients are in clinical remission at 1 year^[9,98]. Third, the annual risk of loss of response is 13% per patient-year^[99]. Finally, infliximab treatment optimization with combination therapy can be considered, but this must be weighed against the increased risk of serious infections and perhaps lymphoma. These data underscore the urgent need to develop new drug classes.

Humanized IL-12/23 antibodies seem the most promising therapy for the future: (1) IL-23 is an essential mediator for the differentiation and amplification of the proinflammatory Th17 pathway; (2) its role is underscored by the increased host susceptibility for IBD in cases of polymorphism of the gene encoding the receptor for this

cytokine; and (3) the effective results observed in a recent randomized, controlled trial, particularly in cases of infliximab withdrawal. Phase III trials are ongoing in IBD patients.

Recent advances in the pathophysiology of IBD have led to the identification of additional cytokine pathways representing potential therapeutic targets. Numerous other cytokines are currently under investigation: IL-27, produced mainly by dendritic cells, acting in the differentiation of both Th1 and Th2 cells; IL-32, produced by NK cell-activated lymphocytes and epithelial cells, providing a proinflammatory amplification pathway in the innate immune responses to bacteria^[7]; IL-31, preferentially produced by T cells skewed towards a Th2 phenotype, playing a role in the acute phase of inflammation by maintaining proliferation of B and T cells^[6]. Further studies are needed to fully explore their different roles in human IBD, and their biological significance, to eventually determine the therapeutic implications (Figure 3).

To overcome anti-TNF therapy failure in IBD, one way would be to develop more targeted therapy^[100]. A humanized TNF receptor-1 specific antagonistic antibody for selective inhibition of TNF action has shown interesting results in animal experiments^[100]. Avimer proteins or nanobodies look promising, offering multiple advantages with a low immunogenicity, a high ligand affinity, a high specificity, oral bioavailability and a low cost^[101]. Another way would be to use cytokine therapy in association with other anti-cytokine agents. The efficacy of TNF- α antagonist agents alone reflects probably the pleiotropic effects of TNF- α ^[2]. An effective treatment strategy for patients might therefore involve the blockade of multiple cytokines in order to intervene in several pathways^[102]. Animal studies in rheumatoid arthritis showed that anti-CD4 therapy acts synergistically with anti-TNF- α in improving established collagen-induced arthritis^[103]. In IBD, a safety study suggested several positive trends in improving efficacy when natalizumab was added to infliximab treatment^[104]. Further investigations are necessary to better evaluate the cost-effectiveness and long-term safety profile of these associations.

CONCLUSION

Despite recent advances in the pathophysiology of IBD, leading to the identification and understanding of several cytokine pathways, anti-TNF- α agents still represent the pinnacle of the therapeutic pyramid of IBD treatment. The humanized IL-12/23 antibodies appear to be the most promising therapy. Future directions could include the development of more targeted therapy or therapeutic blockade of multiple cytokines in order to intervene in several pathways.

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Silvio Danese, MD, PhD, Head, Series Editor

Intestinal microbiota in inflammatory bowel disease: Friend of foe?

Francesca Fava, Silvio Danese

Francesca Fava, Silvio Danese, Inflammatory Bowel Disease Unit, Istituto Clinico Humanitas, Via Manzoni 113, 20089 Rozzano, Milano, Italy

Author contributions: Fava F and Danese S both generated the ideas and contributed to the writing of this manuscript.

Correspondence to: Silvio Danese, MD, PhD, Inflammatory Bowel Disease Unit, Istituto Clinico Humanitas, Via Manzoni 113, 20089 Rozzano, Milano, Italy. silvio.danese@humanitas.it

Telephone: +39-2-82244771 Fax: +39-2-82245101

Received: June 28, 2010 Revised: August 18, 2010

Accepted: August 25, 2010

Published online: February 7, 2011

Abstract

Inflammatory bowel disease (IBD) arises from disruption of immune tolerance to the gut commensal microbiota, leading to chronic intestinal inflammation and mucosal damage in genetically predisposed hosts. In healthy individuals the intestinal microbiota have a symbiotic relationship with the host organism and possess important and unique functions, including a metabolic function (i.e. digestion of dietary compounds and xenobiotics, fermentation of undigestible carbohydrates with production of short chain fatty acids), a mucosal barrier function (i.e. by inhibiting pathogen invasion and strengthening epithelial barrier integrity), and an immune modulatory function (i.e. mucosal immune system priming and maintenance of intestinal epithelium homeostasis). A fine balance regulates the mechanism that allows co-existence of mammals with their commensal bacteria. In IBD this mechanism of immune tolerance is impaired because of several potential causative factors. The gut microbiota composition and activity of IBD patients are abnormal, with a decreased prevalence of dominant members of the human commensal microbiota (i.e. *Clostridium* IXa and IV groups, *Bacteroides*, bifidobacteria) and a concomitant increase in detrimental bacteria (i.e. sulphate-reducing bacteria, *Escherichia coli*). The observed dysbiosis is concomitant with defective

innate immunity and bacterial killing (i.e. reduced mucosal defensins and IgA, malfunctioning phagocytosis) and overaggressive adaptive immune response (due to ineffective regulatory T cells and antigen presenting cells), which are considered the basis of IBD pathogenesis. However, we still do not know how the interplay between these parameters causes the disease. Studies looking at gut microbial composition, epithelial integrity and mucosal immune markers in genotyped IBD populations are therefore warranted to shed light on this obscure pathogenesis.

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Key words: Microbiota; Inflammatory bowel disease; Microbial dysbiosis; Immune tolerance; Innate immunity; Mucosal barrier

Peer reviewers: Tauseef Ali, MD, Assistant Professor, Section of Digestive Diseases and Nutrition, University of Oklahoma Health Sciences Center, 920 SL Young Blvd, Oklahoma City, OK 73104, United States; Jürgen Büning, MD, Internal Medicine I, Department of Gastroenterology, University Hospital of Schleswig-Holstein, Ratzeburger Allee 160, D-23538 Lübeck, Germany

Fava F, Danese S. Intestinal microbiota in inflammatory bowel disease: Friend of foe? *World J Gastroenterol* 2011; 17(5): 557-566
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/557.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.557>

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory disorder affecting the gastrointestinal tract which involves an imbalanced host-commensal microbiota interaction. Crohn's disease (CD) and ulcerative colitis (UC) are commonly included in the collec-

tive term IBD, although the two diseases present with distinct pathogenesis, symptomatology, inflammatory profiles and gut microbiota composition. Inflammation associated with CD is discontinuous, may extend deeply into the submucosal regions and occurs anywhere along the alimentary canal. In UC, inflammation involves only the superficial layers of the intestinal mucosa and is localised to regions of the gut most highly colonized by bacteria, starting at the distal colon and moving proximally along the large bowel^[1]. CD is predominantly associated with a type 1 helper-T-cell (Th1) and type 17 helper-T-cell (Th17) immune responses, characterized by increased production of interleukin (IL)-12, IL-23, IL-27, interferon- γ (IFN- γ) and tumor necrosis factor (TNF)- α . Diversely, UC seems to be associated with a type 2 helper-T cell (Th2) immune response, mainly leading to raised levels of IL-5 and transforming growth factor- β (TGF- β)^[2]. The etiology of IBD is complex and multifactorial, where environmental, genetic and immunological components appear to play a role^[3].

A consistent body of evidence implicates the gut microbiota in the pathogenesis of IBD, including the consideration that inflammation mainly occurs in the intestinal sites with the highest bacterial concentration (in UC), that antibiotic treatment often results in amelioration of disease symptoms^[4], and that germ-free mice do not spontaneously initiate colitis^[5]. The most extensively investigated hypothesis is that IBD development might be due to an altered immune response and a disrupted mechanism of host tolerance to the non-pathogenic resident microbiota, leading to an elevated inflammatory response.

THE HUMAN INTESTINAL MICROBIOTA

The adult human gut contains around 10^{14} bacterial cells and up to an estimated 1000 different bacterial species, thus constituting the largest microbial community associated with the human body^[6]. Recent studies using culture-independent molecular microbiological techniques have shown that the most abundant bacterial phyla found in the healthy human large intestine are the Gram-negative *Bacteroidetes* and the Gram-positive, low GC% *Firmicutes*^[6,7]. *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla are relatively less abundant, but nonetheless are known to play important roles in human health^[6]. The same studies have described the vast diversity of bacterial species and identified the dominant bacterial groups to be *Clostridium coccoides* (*C. coccoides*)-*Eubacterium rectale*, *Clostridium leptum* (*C. leptum*), *Bacteroides-Prevotella*, *Bifidobacterium* species and *Atopobium* species^[8]. The gut microbial species composition varies greatly between individuals, with each individual harboring a unique collection of bacterial species, which is highly stable over time^[9]. Zoetendal *et al.*^[10] also showed that the gut microbiota composition of spouses, who were living in the same environment and had similar eating habits, showed the least degree of species similarity, while siblings showed increased similarity in species make-up. Interestingly, the gut microbiota profiles of identical twins showed a high degree of similarity, but

were yet distinct. These findings highlight that genetic factors play an important role in gut microbiota development, although environment also drives species acquisition. Studies have shown that the vast majority of intestinal bacteria are novel, new to science and so far resist cultivation using traditional culture techniques, necessitating the use of culture-independent molecular microbiology techniques, such as 16S rRNA gene probing and polymerase chain reaction (PCR)-based strategies.

Recently, the human body together with its gut microbiota has been referred to as a “superorganism” comprised of human and bacterial genes^[11]. It has been estimated that the human gut microbiome consists of 100 times more genes than the human genome. Therefore, the presence of the intestinal microbiota enriches the human organism with important functions, especially functions involved in deriving energy from nutrients which escape digestion in the upper gut and the metabolism of xenobiotics. The gut microbiota acts as a “metabolic organ”, through breakdown of complex indigestible dietary carbohydrates and proteins, with consequent generation of fermentation end-products (short chain fatty acids, ethanol and gas) and also through production of vitamins, ion absorption and conversion of dietary polyphenolic compounds into their active form^[12,13]. The commensal microbiota contribute to the “barrier effect”, which constitutes a real obstacle to pathogen invasion of the intestinal mucosa. Recent studies have shown that a modulation of the gut microbiota through dietary supplementation with a prebiotic (i.e. oligofructose) increases epithelial barrier integrity by increasing the expression of tight junctions proteins (i.e. ZO-1 and occludin), with a mechanism that is dependent on the augmented secretion of the GLP-2 gut hormone^[14]. The immune regulatory function of the intestinal microbiota consists of priming the mucosal immune system and maintenance of intestinal epithelium homeostasis. Studies in germ-free animals have demonstrated that the normal functioning of intestinal epithelial cells (IEC) and of the underlying immune cells are impaired in the absence of the gut microbiota. IEC expression of microbial recognition receptors, defensins and antimicrobial peptides are reduced in germ-free animals^[15,16]. Defective development of gut-associated lymphoid tissues, antibody production (i.e. sIgA) and maturation of isolated lymphoid follicles have also been shown in germ-free animals, together with reduced Peyer’s patches and mesenteric lymph node number and dimension^[17,18].

IMMUNE TOLERANCE TO THE COMMENSAL MICROBIOTA

In health, finely balanced mechanisms regulate the host’s immunological tolerance to the continuous stimulus of the resident gut microbiota and their metabolic end-products. Microbial recognition by antigen presenting cells (i.e. dendritic cells, DC) and epithelial cells is mainly carried out through sensing of conserved microbial-associated molecular patterns (MAMPs) by toll-like receptors (TLR), capable of detecting a variety of bacterial components, such

as lipopolysaccharide (LPS), lipoproteins, CpG DNA^[19], and by nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR), which recognise peptidoglycan molecules on the bacterial cell wall^[20]. In healthy hosts the pro-inflammatory pathways associated with TLR and NLR are suppressed by inhibitory molecules of both human and bacterial origin [i.e. cyclooxygenase-2 (COX-2) inhibitors; LPS; A20; peroxisome proliferator-activated receptor- γ (PPAR- γ); nuclear factor- κ B (NF- κ B) inhibitor I κ B- α ; interferon- α/β (IFN- α/β); interleukin-10 (IL-10); TGF- β ; eicosanoids]^[21,22]. Activated innate immune cells, such as mucosal DC, constantly sample luminal microbial antigens and present them to adaptive immune cells. Recent studies have shown that the intestine is home to specialised DC, whose function it is to induce a highly tolerogenic response from T and B cells, through induction of regulatory T cells (Treg) and secretion of IgA, respectively^[23,24]. Commensal bacteria actively coordinate the host tolerogenic response, either through DC-mediated conversion of naïve T cells into Treg, or through direct ligation of TLRs on the surface of Treg. Certain resident bacterial populations, often referred to as “beneficial bacteria” (i.e. lactobacilli and bifidobacteria) can influence DC differentiation towards a more undifferentiated and monocyte-like phenotype, which may account for DC immune tolerance^[25]. Moreover, incubation of monocyte-derived DC with probiotic bacteria was shown to induce DC maturation and cytokine secretion, with strain-specific cytokine secretory profiles^[26]. Repetitive TLR stimulation due to commensal bacterial exposure induces down-regulation of the NF- κ B pathway and stimulates production of antimicrobial peptides (i.e. defensins)^[27]. Also, chronic NOD-2 stimulation has been demonstrated to lead to down-regulation of pro-inflammatory cytokines (TNF- α , IL-8, IL-1 β) in primary human monocyte-derived macrophages after pre-treatment with muramyl dipeptide (MDP) and re-stimulation with NOD-2, TLR-2 and TLR-4 ligands^[28]. Therefore, the host’s mechanism of tolerance to the resident microbiota offers, at the same time, protection from unwanted inflammatory responses and from pathogen invasion. Microbial ligands have also been shown to modulate the expression levels of miR-155, a miRNA that is involved in immune homeostasis and whose absence causes a reduction in Treg numbers in miR-155-deficient mice^[29]. However, since commensal and pathogenic bacteria possess many common motifs that are immunologically recognised by the host, how the host can tolerate resident bacteria whilst being able to mount an effective inflammatory response to invading pathogens is still not fully understood. Nonetheless, pathogenic bacteria do differentiate themselves from commensals by their behaviour; breaching the intestinal epithelial barrier and, in healthy individuals, eliciting strong inflammatory reactions when they trigger MAMPs basolaterally on epithelial cells^[30].

In IBD, the homeostatic mechanisms that allow co-existence of the host organism and the commensal microbiota are disrupted. Polymorphisms in TLR (*TLR4* D299G associated with CD and UC; *TLR1* L80P and *TLR2* R753G, associated with pancolitis) and NLR (i.e.

three mutations in *NOD 2/CARD15* gene, *Arg702Trp*, *Gly908Arg*, and a frameshift deletion mutation at *Leu1007*, accounting for about 80% of all CD-associated mutations) have been implicated in increased susceptibility to IBD^[19,31-33]. However, not everyone who carries these mutations develops IBD, indicating that other etiologic mechanisms might underlie IBD pathogenesis.

INTESTINAL MICROBIOTA IN IBD

Evidence from several recent studies has highlighted that gut microbiota composition and activity in IBD patients are abnormal. In particular, several studies have demonstrated that IBD patients are characterized by a reduced abundance of dominant members of the gut microbiota. Through a combination of PCR of total bacterial genomic DNA with universal bacterial primers and clone sequencing of 16S rRNA genes, Frank *et al*^[34] showed that in mucosal biopsies taken from CD and UC patients there was reduced abundance of rRNA sequences associated with *Firmicutes* and *Bacteroidetes*, and a concomitant increase in 16S rRNA sequences of *Proteobacteria* and *Actinobacteria*, compared to non-IBD controls. In particular, the decreased relative abundance of the *Firmicutes* phylum was due to decreases in populations of *Clostridium* IXa and IV groups. As a consequence of this dysbiosis, the relative abundance of *Enterobacteriaceae* was increased in IBD patients compared to healthy controls, although their absolute numbers remained unaltered. No differences were observed in fecal and mucosal bacterial population numbers between CD and UC patients. These findings are common to several other studies, which also observed decreased clostridia concentrations in IBD^[35,36], although not always accompanied by a decrease in *Bacteroides*^[34,37].

Aberrancies in *Bifidobacterium* populations in IBD have also been previously observed in another study, where significantly lower counts of bifidobacteria were found in rectal biopsies of patients with UC compared to patients without UC^[38]. By employing fluorescent *in situ* hybridization (FISH), Macfarlane *et al*^[38] showed that bacteria belonging to the *C. leptum* phylogenetic group were significantly less abundant in fecal samples of CD patients compared to healthy individuals. Moreover, through a metagenomic approach, the same authors reported a conspicuous loss of microbial diversity in CD, mainly due to a reduction of operational taxonomic units (OTU) within the *C. coccoides* group and the *C. leptum* group. A reduction in bacterial diversity was also previously observed by Ott *et al*^[39] after analysis of mucosa-associated microbiota of CD and UC patients through a combination of single strand conformation polymorphism (SSCP) fingerprint, cloning and real time PCR. Additionally, Zhang *et al*^[40] more recently showed that bacterial diversity of lactobacilli and *C. leptum* group as determined by denaturing gradient gel electrophoresis (DGGE) analysis was also lower in ulcerated tissues compared to the non-ulcerated tissues within the same UC individual. These results suggest that microbial alteration in IBD patients might be caused by the physiological state of the intestinal mucosa. How-

ever, little is known about how inflammatory mediators (e.g. pro-inflammatory cytokines and chemokines) on the gut wall affect bacterial populations *in vivo*. We do know, however, that altered microbial composition may impact on important physiological processes in the intestinal environment. *Clostridium* and *Bacteroides* species are the main producers of short chain fatty acids (SCFA) in the human colon. Decreased clostridia of groups IV and XIVa, the main butyrate-producing bacteria in the gut, could therefore explain the decreased SCFA concentrations found in fecal samples of IBD patients. Among the SCFA produced upon carbohydrate fermentation, butyrate serves as a major source of energy for colonic epithelial cells^[41] and as an inhibitor of pro-inflammatory cytokine expression in the intestinal mucosa, through a mechanism that involves hyperacetylation of histones and suppression of NF- κ B signaling^[42]. Moreover, butyrate reinforces the mucosal barrier by inducing production of mucin and antimicrobial peptides, and by strengthening epithelial barrier integrity through directly increasing the expression of tight junction proteins^[43]. A decrease of butyrate levels could therefore be involved in the increased inflammatory state characteristic of IBD, and butyrate is already considered to be of possible therapeutic value in treating IBD^[44-46]. Stimulation of butyric acid production could be achieved through repopulation of clostridial clusters IV and XIVa, or even through probiotic therapy with lactic acid bacteria, by increasing butyrate production through enhancement of carbohydrate fermentation (i.e. by supplementation with butyrogenic prebiotics such as inulin or oligofructose). Lactic acid can be employed as substrate for the production of high concentrations of butyrate by clostridial cluster XIVa, in a process also known as cross-feeding^[47]. *Faecalibacterium prausnitzii* (*F. prausnitzii*), a prevalent member of the human gut microbiota belonging to clostridial cluster IV and an important butyrate producer, has been recently shown to be less abundant in the intestinal microbiota of IBD patients^[48,49]. *In vitro* and *in vivo* animal studies have also demonstrated the anti-inflammatory and anti-colitic properties of supernatants from *F. prausnitzii* cultures in peripheral blood mononuclear cells or in mouse models of colitis, respectively^[48]. This effect appeared to be due to an as yet unidentified metabolite produced by the microorganism, but was shown to be independent of butyrate production.

Overgrowth of a class of microorganisms referred to as sulphate-reducing bacteria (SRB) was also previously observed in IBD gut microbiota in concomitance with a decrease in clostridia of groups IV and XIVa, especially in UC and pouchitis patients^[50]. SRB metabolize sulphate into hydrogen sulphide, which is toxic to colonocytes, blocks butyrate utilization, induces cell hyperproliferation, and inhibits phagocytosis and bacterial killing^[51]. It was previously demonstrated that the presence of intestinal microorganisms is necessary for induction of dextran sodium sulphate (DSS) colitis in animal models, thus emphasizing the possible role of SRB in IBD, through their reduction of sulphate in DSS into the cytotoxic and inflammatory trigger molecule H₂S^[52]. SRB numbers or their metabolic activity were found to be significantly

higher in studies comparing UC patients to controls or to UC patients in remission^[53-55].

In the search for a putative microbial cause of IBD, the theory of bacterial pathogen-induced intestinal inflammation has also been put forward. A wide range of microorganisms have been suggested as etiologic agents of IBD, including mycobacteria, *Listeria monocytogenes* (*L. monocytogenes*), *Chlamydia*, *Enterobacteriaceae* [including strains of *Escherichia coli* (*E. coli*) and *Helicobacter*] and also reoviruses and paramyxovirus^[56-58]. However, when considering the diversity of IBD lesions and disease course, and the fact that no single pathogenic agent can routinely be isolated from diseased tissue, there is no conclusive evidence that a single pathogen is the cause of the disease. Among the *Enterobacteriaceae* genus, *E. coli* is the bacterium most commonly related to IBD. It was observed that IBD patients harbor increased *Enterobacteriaceae*, in particular *E. coli* belonging to the B2+D group (i.e. with increased virulent potential), compared to controls^[59]. Adherent invasive *E. coli* was commonly found in ileal CD patients, particularly associated with ileal mucosal lesions^[60,61]. On the other hand, *E. coli* isolated from UC patients was less invasive compared to CD^[62]. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an obligate intracellular pathogen that causes spontaneous granulomatous enterocolitis in cattle by evading phagocytosis. Therefore, MAP infection would be favored in those individuals with defective innate immunological defenses, such as CD patients. MAP presence was found with significantly higher frequency in CD patients compared to non-IBD controls, but not in all individuals^[63]. No significant correspondence was found between CD-associated *NOD-2* polymorphisms, especially in ileal CD, and MAP infection^[64,65]. Moreover, clinical studies failed to demonstrate the efficacy of antimycobacterium triple antibiotic therapy in inducing persistent response in CD patients^[66]. Detection of MAP by molecular techniques (i.e. detection of insertion element-900 (IS-900) by PCR) has the limitation of picking up environmental mycobacteria and presents high variability among laboratories^[67-69]. Hence, the etiologic role of MAP in IBD pathogenesis remains to be demonstrated.

Therefore, microbial dysbiosis consisting of a decrease in beneficial bacteria and their metabolic end-products, together with an increase of detrimental bacterial populations and their toxic metabolites, might alter gut luminal environment; thus contributing to the pathogenesis of IBD.

COMPROMISED EPITHELIAL BARRIER FUNCTION, DEFECTIVE INNATE IMMUNE RESPONSE TO BACTERIA AND LOSS OF IMMUNOTOLERANCE

Efficient functioning of the gut mucosa is achieved by means of a combination of intact epithelial barrier and effective bacterial killing through secretion of antimicrobial peptides (e.g. defensins), secretory IgA and phagocytosis.

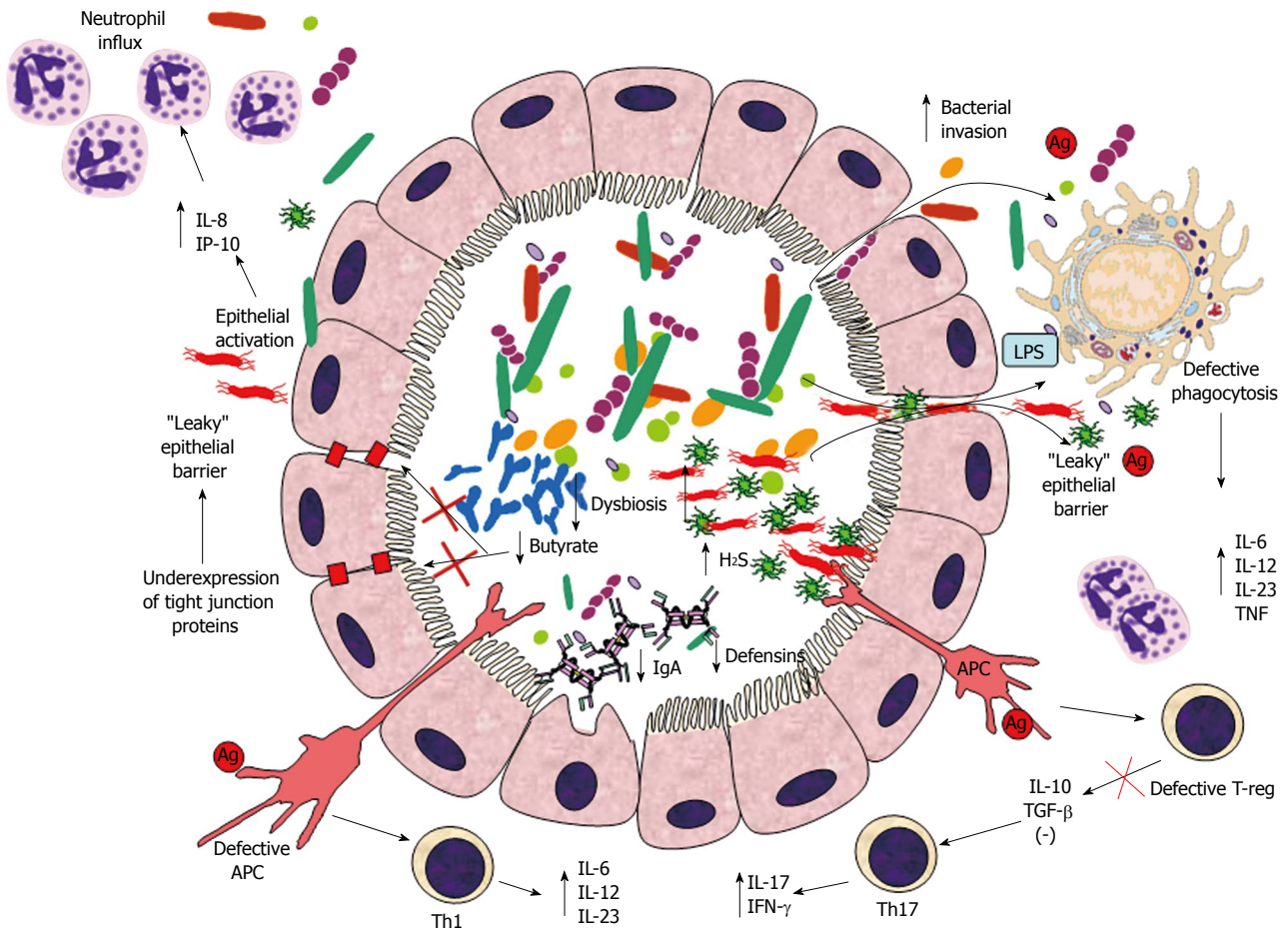


Figure 1 Suggested mechanism of inflammatory bowel disease pathogenesis. Intestinal dysbiosis in inflammatory bowel disease (IBD) consists of decreased prevalence of putative beneficial bacteria (e.g. bifidobacteria) and concomitant increase in detrimental bacterial (e.g. sulphate-reducing bacteria). This microbial imbalance causes reduced intraluminal levels of butyrate (because of decreased production through fermentation and decreased utilization due to increased H_2S levels), thus contributing to down-regulation of epithelial tight junction protein expression and increased epithelial permeability. Epithelial barrier dysfunction brings about increased bacterial translocation through the lamina propria, which is worsened by decreased luminal IgA and defensin concentrations. Killing of bacteria reaching the lamina propria through the "leaky" epithelium is also impaired by a genetically predisposed defective phagocytosis by macrophages. Ineffective bacterial clearance leads to excessive toll-like receptor (TLR) stimulation, secretion of pro-inflammatory cytokines and activation of innate and T-cell mediated immune responses. The disrupted mechanism of tolerance in epithelial cells and antigen presenting cells (APC) amplifies innate immune cell recruitment (i.e. neutrophils). Additionally, defective T-reg and APC cause excessive T-cell response (Th1 and Th17), with consequential intensification of the inflammatory response and granulomatous reaction. IL: Interleukin; IFN- γ : Interferon- γ ; TNF: Tumor necrosis factor; TGF- β : Transforming growth factor- β ; LPS: Lipopolysaccharide.

In IBD these mechanisms of mucosal defence are compromised at all levels and they all contribute to disease progression. A potential mechanism of pathogenesis of IBD is summarized in Figure 1. Disease arises from the initial epithelial barrier dysfunction that brings about increased bacterial translocation through the lamina propria, where microbial antigens elicit a strong inflammatory response, due to ectopic (i.e. basolateral) TLR stimulation, activation of the NF- κ B pathway and consequent induction of pro-inflammatory chemokine and cytokine secretion. This inflammatory process is aggravated by the decreased innate immune defense (i.e. reduced luminal defensin and IgA, defective phagocytosis in IBD), which amplifies the magnitude of bacterial translocation through the "leaky" epithelial layer. Disease progression mainly results from a more global defective immunoregulation and immunotolerance in response to the initial inflammatory insult, due to overaggressive T cell reaction, dysfunctional regulatory T cells and antigen presenting cells (APC) (Figure 1).

IBD, and especially CD, presents with a characteristic increased epithelial permeability, due to underexpression of certain tight junction proteins [e.g. claudins, junction adhesion molecule-A (JAM-A)] concomitant with up-regulation of other pore-forming proteins (i.e. claudin-2)^[70,71]. Defective bacterial clearance due to impaired defensin and IgA production contributes to increased bacterial translocation from the gut lumen across the lamina propria. α -Defensins (i.e. human defensin 5 and 6 (HD5 and HD6)) are antibactericidal compounds produced by Paneth cells efficacious against *Enterobacteriaceae* (e.g. *E. coli*, *Salmonella typhimurium*, *L. monocytogenes*) and *Bacteroides vulgatus*, and were found significantly reduced in association with ileal CD, in particular in patients with *NOD-2* mutations^[72,73]. On the other hand, colonic CD, but not UC, was observed to be associated with lower copy number of β -defensins 2 and 3, which are the main antimicrobial peptides found in the colon. This reduction in β -defensins was shown to be due to a chromosomal polymorphism,

since chromosome 8 presented with a lower copy number of β -defensin 2 in colonic CD^[74,75].

Microbial clearance can also be impaired because of reduced levels of protective secretory IgA (SIgA) in IBD. IgA constitutes the most abundant immunoglobulin phenotype present in the human body^[76]. In the gut, IgA is produced by lamina propria B cells, then translocates to the lumen by attaching to a basolateral receptor on epithelial cells, and finally is transported to the luminal surface of epithelial cells, where it forms SIgA clusters that elicit multiple roles in the intestinal lumen. Firstly, IgA in the mucus layer entraps bacteria and dietary antigens, down-regulates epitope expression on the bacterial cell surface and, therefore, regulates microbial intestinal colonization^[77-79]. Moreover, SIgA prevents pathogen attachment and invasion of epithelial cells and removes bacteria breaching the epithelial barrier by translocating them back to the lumen and by promoting their clearance by dendritic cells, neutrophils and phagocytes^[80-82]. In IBD, intestinal IgA is usually reduced and this is compensated for by increased secretion of IgG, which induces pro-inflammatory cytokine production and mounting of adaptive immune responses to the resident microbiota^[83]. Mucosal secretory IgG was found to be significantly higher in UC and CD patients compared to control patients with irritable bowel syndrome^[84]. In addition, the same study showed that both CD and UC patients presented with increased mucosal IgG bound to fecal bacterial cytoplasmic antigens compared to control patients with irritable bowel syndrome and to non-IBD controls with intestinal inflammation^[84].

Malfunctioning bacterial killing in IBD has also recently been linked to dysfunctional autophagy. Autophagy is a constitutive pathway of cellular homeostasis and organelle turnover. However, it has recently been demonstrated that autophagy plays a key role in innate and adaptive immunity. Macrophages use autophagy to capture and effectively kill intracellular and extracellular invading bacterial pathogens, including *Legionella*, *E. coli*, *Streptococcus* and *Mycobacterium* species, by fusion of the phagocytic compartment with the lysosome^[85,86]. Epithelial cells also employ autophagy to kill invading bacteria and the gene *ATG 16L1* has been shown to be necessary for starting the autophagic process against the cytoplasmic invasion of *Salmonella typhimurium*^[87]. Mutations in *ATG 16L1* have recently been associated with CD, thus implicating defective bacterial killing by autophagy in IBD^[87]. Autophagy impairment might also influence the adaptive immune response to bacteria, since autophagy is involved in major histocompatibility complex (MHC) class II loading in the lysosome, where the autophagic cytoplasmic content is also delivered^[88]. Therefore, a defect in the autophagy pathway could influence antigen presentation by APC, epithelial cells and immune surveillance. Finally, autophagy has been implicated in the regulation of T cell death and proliferation, and *ATG 16L1* is central to these autophagy-regulated processes^[89]. Alteration of *ATG 16L1* in CD might therefore, at least in part, explain the pathologic behaviour of T cells in IBD. In IBD the coexistence of compromised epithelial barrier and defective

innate immunity aggravates the impaired mechanism of tolerance to the resident microbiota and causes inflammatory granulomatous reaction (Figure 1). Defective interaction between regulatory T lymphocytes in the lamina propria and epithelial cells is central to the process of loss of tolerance, through a mechanism that involves NF- κ B signaling. Epithelial NF- κ B activation in healthy hosts is normally suppressed by anti-inflammatory cytokines produced by the underlying T lymphocytes, such as TGF- β and IL-10, while in IBD Th1- and Th17-type immune responses are predominant and lead to chronic inflammation and worsening of the epithelial layer damage^[90]. Perpetuation of the epithelial damage causes increased basolateral as opposed to physiological apical stimulation of TLR-9 receptors, thus causing activation, rather than blockade, of NF- κ B signaling^[30]. This leads to a vicious cycle of aberrant immune response, mucosal inflammation, altered microbiota composition and/or activity and increased mucosal permeability, which would explain the persistent and recurrent nature of IBD.

THERAPEUTIC IMPLICATIONS OF GUT MICROBIOTA-HUMAN HOST INTERACTION

The increasing understanding of the gut microbiota-host immune system interaction has recently drawn interest towards a modulation of intestinal bacterial communities as a novel potential adjuvant in IBD therapy. Although antibiotic therapy constitutes an established therapeutic tool for the treatment of specific IBD-associated symptoms (e.g. abscesses and fistulae), as well as a possible preventive measure, research studies that demonstrate antibiotic efficacy in IBD are still limited^[91]. Promising outcomes have been observed after gut microbiota modulation through probiotic, prebiotic and synbiotic supplementation in CD and UC to change IBD-associated dysbiosis. Treating CD patients with the probiotic strain *E. coli* Nissle 1917 has been shown to induce remission more rapidly than untreated control patients, although it did not influence the number of patients achieving remission^[92]. In UC, *E. coli* Nissle 1917 was proven as effective as mesalazine in maintaining remission^[93,94]. Maintenance of remission after probiotic supplementation was observed in a study with the yeast probiotic *Saccharomyces boulardii* (reduced percentage of relapses in probiotic + mesalamine-treated CD patients, compared to control mesalamine-treated CD patients), although the significance of the study is somewhat restricted because of the low number of subjects involved ($n = 32$)^[95,96]. Positive results were also observed in a double-blind, randomised controlled trial with *Bifidobacterium breve* and *Bifidobacterium bifidum* fermented milk supplementation in 20 UC subjects for 12 wk, where a significant decrease of clinical indices was observed compared to unsupplemented controls^[97]. The probiotic mixture VSL#3 showed convincing effects in the maintenance of remission in UC patients^[98-100], and it was later shown to prevent the onset of pouchitis^[101]. On the other

hand, the data with regard to VSL#3 supplementation in CD are still preliminary. VSL#3 supplementation did not result in a reduction in post-surgical relapse when administered to pediatric CD patients, compared to control mesalamine-treated patients^[102]. In general, it appears that this probiotic supplementation is more effective in reducing disease onset or recurrence, rather than diminishing active inflammatory symptoms.

Prebiotic supplementation with inulin was shown to improve clinical condition in pouchitis patients, and to increase tolerance (i.e. through decreased TLR-2 and TLR-4 expression on DC) and fecal bifidobacteria levels in CD patients^[103-105]. Synbiotics (i.e. a synergy of pro- and pre-biotics in a single preparation) also showed potential therapeutic effect, although the number of studies in IBD is still limited. Supplementation of the inulin-derived prebiotic, Synergy-1, together with *Bifidobacterium longum*, in 18 UC patients for 4 wk significantly decreased rectal pro-inflammatory cytokine levels and down-regulated the expression of inflammation-associated β -defensins^[106].

In summary, some evidence has already indicated a promising therapeutic effect of pro-, pre- and synbiotics in IBD. However, the studies are still very few, underpowered and their design and selection of active agent are sometimes less than optimal. Indeed, this topic deserves further investigation in studies using an adequate number of subjects and employing functional food products targeting the gut microbiota, that have been specifically selected for their anti-inflammatory properties from preliminary *in vitro* and animal studies.

CONCLUSION

Despite the observation that IBD is associated with an abnormal gut microbiota composition, the question as to whether the altered gut microbial dysbiosis is a cause of disease or a consequence of the inflammatory state of the intestinal environment still remains unanswered. Although several studies implicate the gut microbiota in IBD pathogenesis, so far no pathogenic/infectious microorganism has been identified as sole disease causing agent. It is more likely that microbial dysbiosis and lack of beneficial bacteria, together with genetically predisposed increased epithelial permeability, bacterial translocation into the lamina propria, defective innate immunity and loss of tolerance to the resident microbiota, may lead to the abnormal inflammatory response and granulomatous reaction characteristic of IBD. A modulation of the gut microbiota through pro-, pre- and synbiotics, specifically designed to reduce IBD-associated dysbiosis and inflammation, constitutes an interesting approach in the field of novel therapeutic approaches for IBD.

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Defensins couple dysbiosis to primary immunodeficiency in Crohn's disease

Mathias Chamaillard, Rodrigue Dessein

Mathias Chamaillard, Rodrigue Dessein, Institut Pasteur de Lille, Center for Infection and Immunity of Lille, F-59019 Lille, France

Mathias Chamaillard, Rodrigue Dessein, CNRS, UMR 8204, F-59021 Lille, France

Mathias Chamaillard, Rodrigue Dessein, Institut National de la Santé et de la Recherche Médicale, U1019, Team 7, Equipe FRM, F-59019 Lille, France

Mathias Chamaillard, Rodrigue Dessein, Univ Lille Nord de France, F-59000 Lille, France

Rodrigue Dessein, C.H.R.U. de Lille, F-59000 Lille, France

Author contributions: Chamaillard M and Dessein R wrote the paper.

Correspondence to: Mathias Chamaillard, PhD, Institut Pasteur de Lille, Center for Infection and Immunity of Lille, 1, rue du Professeur Calmette, F-59019 Lille, France. mathias.chamaillard@inserm.fr

Telephone: +33-3-59317427 Fax: +33-3-59317480

Received: May 8, 2010 Revised: June 4, 2010

Accepted: June 11, 2010

Published online: February 7, 2011

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Key words: Crohn's disease; Defensins; Primary immunodeficiencies

Peer reviewer: Yuji Naito, Professor, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

Chamaillard M, Dessein R. Defensins couple dysbiosis to primary immunodeficiency in Crohn's disease. *World J Gastroenterol* 2011; 17(5): 567-571 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/567.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.567>

INTRODUCTION

So far, *in silico* mining of human genomic databases have identified or predicted more than one hundred defensin-related sequences^[1,2]. Based on their amino-acid sequences, these human peptides are divided into two categories, the α - and β -defensins (DEFA and DEFB, respectively). Although some defensins are not fully characterized, these molecules are cationic polypeptides smaller than 4 kDa. DEFA and DEFB possess six conserved cysteine residues that are linked in a 1-6, 2-4 and 3-5 pattern for DEFA and in a 1-5, 2-4 and 3-5 pattern for DEFB. Structural studies showed that the tertiary configuration of both DEFA and DEFB consist of triple-stranded β -sheet structures that are stabilized by three intramolecular disulfide bonds. DEFA and DEFB are stored within proteinaceous granules and are secreted through molecular mechanisms that still remain to be elucidated.

Of at least six human DEFA that are expressed within the gut mucosa, DEFA1-4, also referred to as Human Neutrophil Peptide 1 to 4, are predominantly secreted from azurophilic granules of polymorphonuclear leukocytes, while DEFA5 and DEFA6 are primarily contained in the apically-oriented secretory granules of Paneth cells.

Abstract

Antimicrobial peptides, including defensins, are essential effectors in host defence and in the maintenance of immune homeostasis. Clinical studies have linked the defective expression of both α - and β -defensin to the reduced killing of certain microorganisms by the intestinal mucosa of patients suffering from ileal and colonic Crohn's disease (CD), respectively. Only recently have the events leading to defective expression of defensins in CD been further elucidated, and are discussed herein. These events may account for CD-associated alterations in the microbiome and may subsequently precipitate the development of granulomatous inflammatory lesions in genetically-predisposed patients. We also address how these discoveries may pave the way for the development of a molecular medicine aimed at restoring gut barrier function in CD.

The latter is one of the four major epithelial cell lineages that reside at the base of the crypt of Lieberkühn in the small intestine. In addition to DEFA, only four human β -defensins (DEFB1 to DEFB4) have been studied in the past few decades. DEFB1 to DEFB4 are primarily expressed by epithelia of diverse gastrointestinal tissues, including stomach, small intestine and colon^[3].

Biologically-active defensins are released upon the proteolytic processing of their proforms by certain enzymes^[4], including trypsin for DEFA5 and DEFA6 in humans^[5] and matrilysin for cryptidins in mice^[6]. This suggests that appropriate control of homeostatic quantities of both defensins and defensin-activating proteases may ultimately dictate the outcome of the gut immunological response to intruding pathogens and to commensal microorganisms that are permanently present. Our understanding of the regulatory mechanisms that maintain appropriate expression of these antimicrobial factors, which are discharged within the intestinal lumen, is at an early stage. However, recent experimental and clinical findings, which are discussed hereafter, provide us with preliminary answers on how failure to maintain optimal defensin function may lead to dysbiosis and to the development of chronic inflammatory lesions.

THE ANTIMICROBIAL ACTIVITY OF DEFENSINS

The membrane integrity of a broad spectrum of microorganisms, including enveloped viruses, protozoa, bacteria and some fungi, is sensitive to the amphiphilic nature of defensins (Figure 1). Over the past decade, several models have been proposed to explain how defensin may induce non-oxidative killing of microorganisms. Notably, the Shai-Matsuzaki-Huang model provides a reasonable structure-function explanation for the antimicrobial character of defensins^[7], whereby the cationic property of these antimicrobial effectors may disrupt the phospholipid bilayer as a detergent. Defensins may promote the formation of micelles by electrostatic forces to negatively-charged components of the microbial membrane, including lipopolysaccharide from Gram negative bacteria and lipoteichoic acids from Gram positive bacteria. Conversely, the nosocomial Gram positive bacteria *Staphylococcus epidermidis* and *Staphylococcus aureus* are thought to repulse the killing activity of defensins by expressing a membrane-bound molecule with a high density of negative charge^[8] and by modifying its lipid membrane through Mprf^[9], respectively. Similarly, both the two-component system PhoP/PhoQ and lipopolysaccharide are involved in resistance to defensins in the facultative intracellular bacterium *Salmonella typhimurium* (*S. typhimurium*)^[10], providing a mechanism whereby certain pathogens may circumvent innate immune mechanisms. Additional investigations are now eagerly awaited to determine whether luminal secretion of certain defensins may influence immune homeostasis in any part of the gastrointestinal tract.

Genetically engineered mice that express the human DEFA5 showed oral resistance to salmonellosis^[11]. Con-

versely, matrilysin deficiency is linked to enhanced susceptibility to oral infection by *S. typhimurium*^[6]. Similarly, genetic ablation of the Crohn's disease (CD) predisposing NOD2 gene leads to reduced expression of certain cryptidins and to enhanced susceptibility to orogastric *Listeria monocytogenes* infection^[12]. More importantly, molecular analyses point to profound changes in the composition of the gut microbiome from DEFA5-transgenic mice^[13]. As a consequence, an abnormal T cell homeostasis, including fewer interleukin 17 (IL-17)-producing lamina propria T cells, and an overt resistance to colonization of the ileum by segmented filamentous bacteria were observed in DEFA5-transgenic mice^[13]. The gastrointestinal tract, in a large proportion of the human population, is colonized by Gram positive anaerobic segmented filamentous bacteria that are thought to regulate both Th17 differentiation^[14,15] and secretory immunoglobulin A production^[16,17]. Besides DEFA5, a physiological role for mouse β -defensin 1 (mDefB1) in host defence has been primarily unveiled in the pulmonary and urinary tract. Genetic ablation of mDefB1 expression failed to impede the outcome of lung infection by *Haemophilus influenzae*^[18], but also resulted in enhanced bacterial burden of *Staphylococcus* in the bladder when compared to controls^[19]. However, even if certain pathogenic microorganisms inhibit the secretion and/or killing activity of defensins^[20], the latter still provide partial protection against infection, suggesting another prophylactic role for these key elicitors of mammalian immunity.

THE IMMUNOREGULATORY FACET OF DEFENSINS

In essence, defensins are regarded as "natural antibiotics", but accumulating immunological investigations revealed that these long-held antimicrobial peptides may also educate the gut immune system through multiple mechanisms (Figure 1). Indeed, it has been known for some time that DEFA1 is not only involved in the attraction of monocytes to inflammatory sites^[21], but also of T cells and immature dendritic cells^[22]. Recent immunological investigations have now revealed that this phenomenon is not restricted to α -defensins. Notably, β -defensins were chemotactic for T cells^[23], dendritic cells^[23], monocytes^[24,25] and mast cells^[26]. Interestingly, the presence of a disulfide bridge was required for DEFB2 to recruit immunocytes through the C-C chemokine receptor 6^[27]. Besides their direct chemoattracting function, certain defensins may also contribute to the recruitment of immune cells by eliciting the expression of co-stimulatory molecules and secretion of a subset of cytokines and chemokines by a myriad of cell types through receptor-dependent mechanisms^[28]. Notably, DEFB2 and DEFB3 have been characterized as signalling ligands *per se* for some membrane-bound pathogen-recognition receptors, including the toll-like receptors 4 and 2, respectively^[29,30]. The expression of the co-stimulatory molecules CD80, CD86 and CD40 is consistently enhanced after exposure of monocytes and dendritic cells to recombinant DEFB3 in a MyD88-dependent manner^[29]. Likewise, treatment of immature

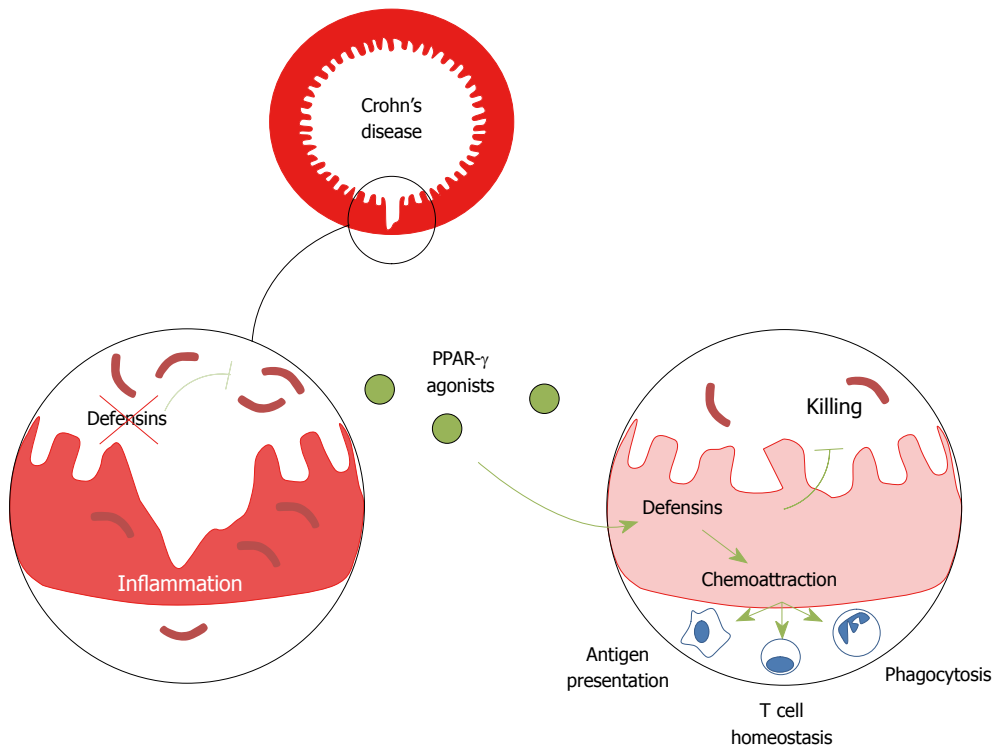


Figure 1 Model of defensin-mediated control of luminal microbiota and maintenance of immune homeostasis in the gut. PPAR: Peroxisome proliferator-activated receptor.

dendritic cells with exogenous recombinant DEFA1 and DEFB1 enhanced the expression of CD80, CD86, CD40, the maturation marker CD83, and HLA-DR^[31]. Moreover, both neutrophil-derived defensins and DEFB2 have been involved as positive regulators of neovascrogenesis and wound healing^[32-34], but our understanding of the underlying mechanisms still remains incomplete.

TOWARDS A CAUSAL LINK BETWEEN DEFENSINS AND CROHN'S DISEASE DEVELOPMENT

In recent years, defensins have been observed to be negative regulators of both infectious diseases and of chronic inflammatory diseases, including CD. As many as 4 million adults are affected by CD in Europe and North America, and there is no cure for this relapsing-remitting granulomatous illness. CD is traditionally characterized by the development of transmural inflammatory lesions that may affect any part of the bowel. The ileal and colonic mucosa of patients with CD show impaired antimicrobial activity against major components of the microbiota, which is not found in biopsies of healthy subjects or patients with ulcerative colitis, another inflammatory bowel disease^[35,36]. In a clinical study by Wehkamp and collaborators, decreased expression of both DEFA5 and DEFA6 was linked to the development of CD-associated lesions in the small intestine^[35,37]. More recently, decreased expression of DEFB1 was observed in CD patients with colonic involvement^[38]. While the transcript level of the neutrophil chemoattractant IL-8 is a surrogate marker of inflammation, it failed

to correlate with *DEFA5* and *DEFB1* transcript levels in Crohn's ileitis and colitis, respectively^[35,38]. Similarly, levels of DEFA5 and DEFB1 were not modulated by most current therapies for CD^[39]. Collectively, these findings reinforce the notion that decreased expression of defensins may result in excessive inflammation which is the basis of CD. Furthermore, consistent with the chemoattracting function of defensins, trauma to intestinal or cutaneous epithelia in CD patients is also associated with impaired neutrophil attraction when compared to controls^[40]. Decreased expression of defensins in CD may account for defective local microbial killing and for reduced attraction of immunocytes to mucosal breaches, but what causes the impaired expression of defensins?

To determine the mechanisms underlying the defect in defensins in ileal and colonic CD, the promoter of both DEFA5 and DEFB1 were screened for potential disease-associated variants. Genetic errors in a regulatory region downstream of the human *DEFA5* gene were found to predispose to ileal CD development^[41]. In the ileum, both NOD2 and the Wnt-signalling pathway transcription factor Tcf7l2 were characterized as positive regulatory molecules of DEFA5 expression in human and of certain cryptdins in mice^[12,35,42]. Notably, patients carrying the 1007fs NOD2 mutation and those with lowered expression of Tcf7l2 showed a significantly decreased transcript level of DEFA5^[43]. We recently showed in the colon, that engagement of the peroxisome proliferator-activated receptor peroxisome proliferator-activated receptor (PPAR)- γ with rosiglitazone triggered epithelial DEFB1 expression *in vitro* and *in vivo*^[38]. Dysregulated PPAR- γ production consistently results in reduced antimicrobial activity of the

mucosa against major components of the microbiota in mice^[38]. Furthermore, the single nucleotide polymorphism rs1800972, which is located within the promoter region of the human *DEFB1* gene, was also found to be protective towards colonic CD development^[38,44] and to positively regulate the expression of *DEFB1*^[45].

CONCLUSION

At present, the therapeutic management of CD is far from optimal. Approximately 25% of patients fail to respond to current biologics and/or to immunosuppressive drugs. Given the essential role of defensins in maintaining gut homeostasis, defensin deficiency requires to be corrected in CD. Recent experimental investigations provided preliminary answers to this problem by identifying the PPAR- γ agonist rosiglitazone as a potent inducer of a subset of β -defensin in mouse colon^[38], opening the way for the potential correction of gut barrier function in CD. The development of a mucosal-delivery system for PPAR- γ agonists is now eagerly awaited to improve their therapeutic efficacy, while avoiding potential systemic side effects. Finally, it is worth noting the regulatory role of additional nuclear receptors, including vitamin D and PPAR- β , in regulating the expression of other antimicrobial peptides, including certain defensins^[46-49]. The potential synergistic effects of these regulatory factors on the expression of intestinal defensins remains to be addressed. Drugs and diets which can modulate the expression of defensins may thereby protect against colitis and colitis-associated cancer by maintaining sufficient levels of these versatile antimicrobial peptides.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

Silvio Danese, MD, PhD, Head, Series Editor

Extraluminal factors contributing to inflammatory bowel disease

Arvind Batra, Thorsten Stroh, Britta Siegmund

Arvind Batra, Thorsten Stroh, Britta Siegmund, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Medical Department I (Gastroenterology/Rheumatology/Infectious Diseases), Hindenburgdamm 30, 12200 Berlin, Germany
 Author contributions: Batra A, Stroh T and Siegmund B contributed equally to this work.

Supported by SFB 633 of the Deutsche Forschungsgemeinschaft

Correspondence to: Britta Siegmund, MD, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Medical Department I (Gastroenterology/Rheumatology/Infectious Diseases), Hindenburgdamm 30, 12200 Berlin, Germany. britta.siegmund@charite.de

Telephone: +49-30-84454039 Fax: +49-30-84454017

Received: February 3, 2010 Revised: April 7, 2010

Accepted: April 14, 2010

Published online: February 7, 2011

Peer reviewer: Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Batra A, Stroh T, Siegmund B. Extraluminal factors contributing to inflammatory bowel disease. *World J Gastroenterol* 2011; 17(5): 572-577 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/572.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.572>

INTRODUCTION

The genome-wide association studies in recent years have contributed significantly to the understanding of the pathogenesis of inflammatory bowel disease (IBD)^[1]. The results obtained from these studies have not only confirmed the relevance of earlier characterized pathways, but equally have opened novel avenues. One possible hypothesis for the etiology of IBD is that the mucosal immune system is hyper-responsive to luminal antigens (e.g. dietary factors, commensal bacteria) in genetically predisposed individuals^[2]. This hypothesis is limited to the intestinal lumen and wall. Focusing on Crohn's disease, the inflammation is not restricted to the luminal side of the intestinal wall. Rather, transmural inflammation presents as the dominant phenotype, which leads to the question of whether extraintestinal/extraluminal structures contribute to the inflammatory process. In the present overview, three extraluminal structures are discussed, which have been demonstrated to play a role in the regulation of intestinal inflammation, namely the mesenteric fat tissue, microvasculature and lymphatics (Figure 1).

MESENTERIC FAT TISSUE

Historic view

Crohn BB himself provided the first evidence that the mesenteric fat tissue might play a role in the pathogen-

Abstract

Many identified and yet unknown factors contribute to the pathogenesis of inflammatory bowel disease (IBD). The genome-wide association studies clearly support the earlier developed concept that IBD occurs in genetically predisposed individuals who are exposed to distinct environmental factors, which together result in dysregulation of the mucosal immune system. Thus, the majority of previous studies have focused on the immune response within the intestinal wall. The present review aims to emphasize the contribution of three extraluminal structures to this inflammatory process, namely the mesenteric fat tissue, the lymphatics and the microvasculature. Broadening our view across the intestinal wall will not only facilitate our understanding of the disease, but will also us to identify future therapeutic targets.

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Key words: Inflammatory bowel disease; Extraluminal structures; Mesenteric fat tissue; Lymphatics; Microvasculature

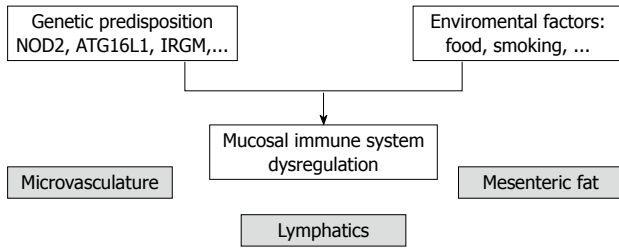


Figure 1 Extraluminal structures contributing to Crohn's disease. The figure illustrates the potential contribution of the extraluminal structures of mesenteric fat tissue, lymphatics and microvasculature to the dysregulation of the mucosal immune system.

esis of Crohn's disease, by describing local hypertrophy of the mesenteric fat adjacent to inflamed intestinal segments^[3]. This phenomenon, which is also called "creeping fat" or "fat wrapping" is restricted to Crohn's disease, and is not observed in ulcerative colitis or other forms of chronic intestinal inflammation.

Anatomical view

The characteristic "fat wrapping" seen only in Crohn's disease represents fat hypertrophy that results in partial cover of the intestinal circumference, which is defined as > 50% coverage of the intestinal surface by adipose tissue and occurs in both the large and small bowel. The localization of this "creeping fat" correlates with transmural inflammation, ulceration, and stricture formation^[3]. These observational results have been underlined by magnetic resonance imaging that quantified the amount of intra-abdominal fat in relation to total body fat, which indicates that the intra-abdominal fat but not total body fat increases^[4]. Adipocytes within this hypertrophied fat are significantly smaller, but a fourfold increase in the total number of adipocytes is present in the mesentery of Crohn's disease patients as compared to controls^[5]. How can we explain this observation and what might be the possible contribution to disease?

Adipocytes and chronic inflammation

Each lymph node in our body is in close proximity to adipose tissue. Once the lymph nodes are activated, the number of adipocytes increases, which allows for the supply of sufficient energy for a functional immune system^[6-8]. However, is the role of the mesenteric fat tissue restricted to energy supply? In the first studies to analyze the expression of pro-inflammatory mediators in fat tissue, an increase of tumor necrosis factor (TNF)- α and the adipokine leptin was demonstrated in the fat tissue of Crohn's disease patients, in comparison to non-inflammatory controls^[4]. In addition, adipocytes express C-reactive protein, and there is a significant correlation between serum C-reactive protein levels and increased mesenteric fat density in Crohn's disease^[9]. What is the relevance of these mediators released by the adipose tissue?

Adipokines

Various adipokines are released by adipose tissue. The relevance of adipokines in IBD has been summarized

recently in broad detail^[10]. The adipokines characterized best with regard to intestinal inflammation are leptin and adiponectin, respectively.

Leptin is a 16-kDa peptide predominantly produced by adipocytes, which signals the status of satiety to the hypothalamus^[11]. Leptin deficiency or non-function of the long isoform of the leptin receptor (OB-Rb) is associated with massive obesity in mice and humans. From a structural point of view, leptin can be classified as a helical cytokine^[12]. Thus, the structure of leptin suggests a regulatory function within the immune system. In humans, leptin deficiency is rare, but results in impaired T-cell proliferation and is associated with increased mortality in childhood due to infection^[13,14]. In mice, leptin deficiency has been associated with protection from dextran sodium sulfate (DSS)-, oxazolone- and trinitrobenzene sulfonic acid (TNBS)-induced colitis. In addition, results from the transfer model of colitis indicate that leptin serves as crucial T-cell stimulator in intestinal inflammation^[15-17]. In addition, leptin stimulates the proliferation of naive CD4⁺ T cells and affects T-cell polarization^[15,18,19]. In Crohn's disease, increased expression of leptin mRNA as well as protein in the hypertrophic mesenteric fat has been reported^[20,21]. Together with data from animal studies, a pro-inflammatory role for leptin in Crohn's disease has been suggested.

Adiponectin, a 30-kDa polypeptide, contributes 5-10 $\mu\text{g/mL}$ to 0.01% of plasma proteins, and hence is the most abundant adipokine in the circulation^[22]. Adiponectin has a high affinity to form trimers that can further multimerize to polymers, which results in various high and low molecular isoforms. The biological significance of the different high and low molecular forms is not finally understood. In Crohn's disease patients, adiponectin mRNA and protein release is upregulated in hypertrophied adipose tissue, as compared to normal adipose tissue from the same subjects, or mesenteric adipose tissue from ulcerative colitis patients and controls^[23]. Data concerning the effects on disease severity in experimental models of colitis are conflicting. Whereas one group has observed increased susceptibility to the chemically induced model of DSS colitis^[24], another has reported protection against DSS- as well as TNBS-induced colitis in adiponectin-deficient mice^[25]. To confuse the issue even more, a third study has reported that adiponectin deficiency does not affect the outcome of disease in interleukin (IL)-10-deficient mice that develop colitis spontaneously^[26]. In the model of chronic TNBS-induced colitis in rats, the size of mesenteric adipocytes is decreased, and production of adiponectin, besides other mediators, is increased in perinodal mesenteric fat^[27]. As a result of the conflicting effects mediated by adiponectin on immune cells, both pro- and anti-inflammatory consequences of altered adiponectin production in IBD are possible. However, adiponectin does seem to modulate immune responses, and abnormal production could thus be involved in the altered responsiveness of immune cells that occurs in IBD.

Recent data from genetic studies have added independent support for such dysregulated production of adiponectin and leptin in Crohn's disease. In mice, deficiency

of the autophagy gene Atg16L1 results in upregulation of leptin as well as adiponectin mRNA expression^[28]. In humans, the ATG16L1 risk allele is associated with an increased risk of developing Crohn's disease^[28,29]. Further cross-population studies are needed to ascertain whether this mutation is the cause of the altered leptin and adiponectin production in the hypertrophic fat of Crohn's disease patients. However, so far, it is tempting to speculate that the ATG16L1 risk allele and the subsequent altered production of adipokines might contribute to the predisposition to Crohn's disease.

The data described above indicate that adipokines are able to regulate the acquired immune response and that the production of some is altered in mesenteric fat of IBD patients. What kind of stimulus is required to modify the production of these adipokines in patients with Crohn's disease? Translocation of luminal antigens (e.g. bacteria) from the intestinal lumen to the adipose tissue could offer this stimulus, presuming that adipocytes and pre-adipocytes express innate receptors.

Adipocytes as cells of the innate immune system

The release of free fatty acids by adipocytes following lipopolysaccharide (LPS) stimulation, and hence responsiveness of fat cells to bacterial components, was first detected over 30 years ago^[30]. In line with these historic data, the expression of toll-like receptor (TLR)4 and TLR2 was described in adipocytes generated from the 3T3-L1 cell line^[31]. Furthermore, our group and others have demonstrated that adipocytes and their precursors from mice and humans express TLR1-TLR11 and that specific stimulation induces secretion of immune regulatory mediators^[32-34]. In addition, data from our group indicate expression of functional nucleotide oligomerization domain (NOD) proteins-1 and -2 on pre-adipocytes^[35]. Expression of these NOD proteins in adipocytes and pre-adipocytes is further regulated by TNF- α or LPS (NOD2), respectively, IFN γ (NOD1)^[35]. This observation is of particular interest, since mutations in the NOD2 gene have been associated with an increased risk of developing Crohn's disease^[36-38]. Thus, adipocytes and pre-adipocytes share functional properties of immune cells, which suggest an active role in defense against bacterial and viral antigens *in vivo*. Hence, adipocytes and pre-adipocytes could represent a yet ignored population of innate immune cells.

A working model could be that primary increased production of pro-inflammatory mediators in the mesenteric fat due to genetic predisposition might contribute to the development of Crohn's disease. Additionally, the massive cytokine production in the inflamed colon, in addition to translocating bacteria, could further induce the production of pro-inflammatory mediators in the adjacent adipose tissue, thus inducing a vicious cycle, in which inflammatory conditions in the intestine and the mesenteric fat support each other.

et al^[39] reminded us of their presence in a concise review. They reviewed the pathological descriptions of Crohn's disease in the era before antibiotic, corticosteroid, immunomodulatory and biological therapy. These pathologists described lesions in the basal portion of the lamina propria, in the superficial and deep submucosa, and in the subserosa, which suggested lymphatic disease. These lesions comprised lymphocytic thrombi within the lymphatics and multiple large aggregates of lymphocytes with or without multi-nucleated giant cells, a picture consistent with chronic lymphangitis^[39]. The granulomas of Crohn's disease appear to be in and around the very thin-walled lymphatics that are found adjacent to small vessels^[40]. This further supports the idea that lymphatics might directly contribute to the pathogenesis of this disease.

Almost more intriguing are the rat and pig models in which regional lymphatics of the small intestine were obstructed with sclerosing agents^[41,42]. These animals subsequently developed segmental intestinal disease that was characterized by many of the alterations that occur in Crohn's disease, including lymphocytic and granulomatous changes. Remarkably, enterocutaneous as well as enterocutaneous fistulas developed in these models, which are not seen in animal models routinely used today^[41,42]. Additional observations have pointed out that the distribution and character of these lesions represent obstructive lymphocytic lymphangitis^[43]. In these older studies, the connection between the shorter segments of Crohn's disease in the jejunum and the longer segments in the ileum, with the shorter vasa recta of the jejunum and the longer lymphatic collecting ducts of the ileum, was emphasized^[39,43].

Very recent work by Vetrano *et al*^[44] has provided further experimental data underlining the relevance of lymphatics in IBD^[44]. They have concentrated on the expression of D6, a promiscuous decoy receptor and scavenger for CC chemokines that plays a non-redundant role in the control of the inflammatory response in various organs. Vetrano *et al*^[44] have demonstrated upregulation of D6 in human colitis. The expression could be localized to lymphatic vessels and leukocytes in the mucosa. D6-deficient mice showed an increased susceptibility to experimental colitis when compared to wild-type mice. *Via* bone-marrow chimeras, the regulatory function of D6 in colitis has been tracked to the stromal/lymphatic compartment, and a contribution of hematopoietic cells could be excluded. Thus, these data further emphasize the regulatory role of the lymphatic system in intestinal inflammation.

In line with these observations, Van Kruiningen *et al*^[39] have suggested recently to focus again on the lymphatic damage in Crohn's disease, and the identification of possible harmful agents that cause lymphangitis and lesions in the lymphatic endothelium. Although the lymphatics are not completely separated from the intestine, they represent the second structure that should be reevaluated in Crohn's disease.

LYMPHATICS

The lymphatic system is closely connected to and within the intestine, and is a neglected structure. In 2008, Van Kruiningen

MICROVASCULATURE

In similar close proximity to the intestinal wall is the

microvasculature that is embedded in the mesenteric fat tissue, which thus provides an additional link as outlined below.

Whether increased vascularization as assessed by mesenteric angiography or Doppler ultrasound reflects Crohn's disease activity is disputed^[45]. Recent evidence for angiogenesis playing a role in IBD pathogenesis has prompted interest in anti-angiogenic therapies for IBD^[46,47]. Remarkably, angiogenesis plays a crucial role in various chronic inflammatory disorders such as atherosclerosis, rheumatoid arthritis, peptic ulcer, IBD, psoriasis, and Alzheimer's disease^[48]. Growth of new blood vessels is intrinsic to inflammation and is associated with structural changes, including activation and proliferation of endothelial cells and capillary and venule remodeling, all of which result in an expansion of the tissue microvascular bed^[49-51]. As inflammation evolves, vessels expand to supply nutrients that sustain the accumulation of activated immune cells, and in the chronic phase, local immune cells overproduce endothelial cell growth factors^[49].

This expansion of the vascular network facilitates several mechanisms. The influx of inflammatory cells increases the nutrient supply that allows the metabolically active immune response to take place, and the activated endothelium contributes to the local production of cytokines, chemokines, and matrix metalloproteinases^[52,53]. In chronic inflammatory disorders, infiltration by macrophages and lymphocytes, tissue damage and repair occur concurrently, and the newly formed vessels become permanent^[51,54]. The anatomical expansion and increased activation of the remodeled microvascular bed foster further influx of immune cells, and angiogenesis and inflammation become co-dependent processes^[55]. Both innate as well as adaptive immune responses promote angiogenesis.

Thus, the endothelium, and more specifically, the endothelial cells of the microvasculature seem to assume a central function, because they are not only capable of generating a range of mediators, but also display distinct adhesive molecule patterns, to activate a unique sets of genes and form capillaries^[56-58]. In addition, endothelial cells act depending on the body compartment heterogeneity^[56,59-61]. An example is the expression of mucosal addressin cellular adhesion molecule-1 by Peyer's patches and high endothelial venules to recruit $\alpha 4\beta 7$ homing receptor-positive naïve lymphocytes^[62]. Similarly, endothelial cells from brain, liver, and other organs express distinct surface markers, protein transporters, and intracellular enzymes^[61-63]. This heterogeneity becomes of particular interest when considering the regulation of organ-specific inflammation; in our case, intestinal inflammation. The distribution and infiltration of leukocytes is tightly regulated by numerous homing and adhesion molecules on the surface of microvascular and immune cells^[64]. At inflamed sites, endothelial cells still control the type and number of immune cells that extravasate into the interstitium in a dysregulated fashion^[65,66].

An additional cell population has been identified to play a crucial role in the process of cell infiltration *via* the endothelium in areas of inflammation, namely platelets. They normally circulate without attaching to the endotheli-

um, but do so when the endothelial cells become activated, and platelet adherence triggers inflammation^[67]. Activated platelets produce massive amounts of pro-inflammatory mediators and interact with various other cell populations^[68,69]. In inflamed areas, the microvasculature can recruit leukocytes through a platelet-dependent mechanism, but at the same time, platelet recruitment is leukocyte dependent^[70]. The details of this crucial interaction have been summarized by other reviews^[71].

A number of animal studies have proven that the process of angiogenesis can be taken advantage of as a therapeutic approach. Hence, in models of DSS-induced inflammation, as well as spontaneous colitis in IL-10-deficient mice, angiogenesis occurs. However, when this angiogenesis is inhibited, clinical severity and the signs of histological inflammation decrease significantly^[47]. Furthermore, vascular endothelial growth factor A that induces angiogenesis has been recently shown to be up-regulated in samples from patients with IBD, and in mice with colitis. The overexpression of VEGF-A in mice exposed to DSS was followed by deterioration of disease and an additional increase in angiogenesis when compared to DSS-exposed wild-type mice^[72].

PERSPECTIVE

Considering the factors that contribute to intestinal inflammation, particularly in Crohn's disease, we should avoid restricting ourselves to the luminal site and immune-cell infiltration, but rather include the extraluminal structures discussed in this review. This broadened view might help us in understanding the disease, and more importantly, in identifying novel therapeutic targets.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH

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Role of the endothelium in inflammatory bowel diseases

Walter E Cromer, J Michael Mathis, Daniel N Granger, Ganta V Chaitanya, J Steven Alexander

Walter E Cromer, J Michael Mathis, Department of Cellular Biology and Anatomy, LSU Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932, United States
Daniel N Granger, Ganta V Chaitanya, J Steven Alexander, Department of Cellular and Molecular Physiology, LSU Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932, United States

Author contributions: Cromer WE wrote the paper; Mathis JM, Granger DN and Chaitanya GV significantly contributed to the editing of this paper; Alexander JS significantly contributed to the outline, design and editing.

Supported by National Institute of Health, NIH DK 43785

Correspondence to: J Steven Alexander, PhD, Department of Cellular and Molecular Physiology, LSU Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932, United States. jalex@lsuhsc.edu

Telephone: +1-318-6754151 Fax: +1-318-6754156

Received: March 8, 2010 Revised: June 29, 2010

Accepted: July 6, 2010

Published online: February 7, 2011

IBD pathology and distinctive features of the intestinal endothelium contributing to these conditions.

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Key words: Microvasculature; Endothelium; Inflammation; Nitric oxide; Adhesion molecules; Crohn's disease; Ulcerative colitis; Cytokines; Chemokines; Growth factors

Peer reviewers: Tauseef Ali, MD, Assistant Professor, Section of Digestive Diseases and Nutrition, University of Oklahoma Health Sciences Center, 920 SL Young Blvd, Oklahoma City, OK 73104, United States; John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent, ME20 7NH, United Kingdom

Cromer WE, Mathis JM, Granger DN, Chaitanya GV, Alexander JS. Role of the endothelium in inflammatory bowel diseases. *World J Gastroenterol* 2011; 17(5): 578-593 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/578.htm>
DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.578>

Abstract

Inflammatory bowel diseases (IBD) are a complex group of diseases involving alterations in mucosal immunity and gastrointestinal physiology during both initiation and progressive phases of the disease. At the core of these alterations are endothelial cells, whose continual adjustments in structure and function coordinate vascular supply, immune cell emigration, and regulation of the tissue environment. Expansion of the endothelium in IBD (angiogenesis), mediated by inflammatory growth factors, cytokines and chemokines, is a hallmark of active gut disease and is closely related to disease severity. The endothelium in newly formed or inflamed vessels differs from that in normal vessels in the production of and response to inflammatory cytokines, growth factors, and adhesion molecules, altering coagulant capacity, barrier function and blood cell recruitment in injury. This review examines the roles of the endothelium in the initiation and propagation of

INTRODUCTION

Inflammatory bowel diseases (IBD) include Crohn's disease (CD), ulcerative colitis (UC) (and indeterminate colitis), which share several inflammatory characteristics with other chronic immune disturbances including immune activation, leukocyte infiltration into tissues and increased vascular density^[1]. In UC, the colon shows a continuous, superficial inflammation, while CD occurs as patchy transmural inflammation which may affect any region of the gastrointestinal tract. Genetic susceptibilities may play an important role in the development of IBD^[2-6] with polymorphisms in CARD15/NOD2 haplotypes (especially in Caucasians) and HLA-DR haplotypes (especially in Asian IBD) and possible defects in interleukin (IL)-23, IL-2, and IL-10 signaling^[2,7-10]. IBD is more prevalent in developed nations^[11], with several mechanisms being considered to explain disease pathology including environment, hygiene

and altered gut flora^[11-13]. These different contributing causes may underlie divergent forms and patterns of IBD, which ultimately may lead to a redefinition of different sub forms of UC and CD.

While the mechanisms initiating and sustaining IBD may differ, both UC and CD may reflect dysfunction within antigen-presenting cells (e.g. dendritic cells) or excess activation of CD4⁺ T-cells (resembling T-cell disturbances in psoriasis). Reduced activation of T-cells in some forms of CD appear to allow gut microbiota that have breached the gut epithelium to trigger microvascular inflammation^[1,5,9,14-16]. The activation of immune responses in IBD release inflammatory cytokines [e.g. tumor necrosis factor (TNF)- α] and growth factors [e.g. vascular endothelial growth factor (VEGF)-A] into gut tissues provoking gut inflammation and injury^[5,17]. Antibodies produced against “normal” gut antigens (e.g. anti-colon, anti-mucin, anti-tropomyosin) have been found in IBD and are suggested to activate cytotoxic T lymphocytes, further increasing inflammation^[7]. As IBD progresses, cytokine-mediated inflammation and epithelial apoptosis disturb the intestinal barrier, to allow penetration of gut flora beyond the lamina propria causing intense inflammatory responses^[18] while also provoking endothelial microvascular permeability^[19].

Another key event in IBD progression is the expansion of the intestinal microvasculature. Angiogenesis in IBD sustains inflammation through alterations in the endothelial lining of these vessels. The endothelium regulates recruitment of inflammatory cells, tissue damage (e.g. vasogenic edema), and production of inflammatory mediators^[19-22]. In this review we describe the key roles of the endothelium in mediating and aggravating inflammation in IBD (Figure 1).

ENDOTHELIAL CELLS IN IBD

Endothelial cells (ECs) are the major constituent of the microvasculature that line blood and lymphatic vessels. ECs during IBD undergo rapid and remarkable changes in response to elevated levels of cytokines and growth factors often producing injury to gut tissues. Normally ECs provide an anti-adhesive and selectively permeable exchange barrier^[23]. Even though ECs have long been recognized as participants in inflammation their roles in intestinal inflammation during IBD are not yet clear. The unique physiological and molecular characteristics of gut microvessels may help explain several characteristics of IBD. The close relationships between gut metabolism, tissue perfusion, microvascular expansion and immune cell infiltration are unclear but suggest that microvascular alterations may be maladaptive in IBD. Intestinal vascular ECs basally exhibit unique properties which may contribute to IBD. Haraldsen *et al.*^[24] first described characteristics of human intestinal ECs (HIMECs) in long-term cultures and differences from ECs of different origin. For example lipopolysaccharide (LPS) only transiently increases HIMEC adhesion molecule ex-

pression, while causing long-lasting increases in human umbilical vein ECs (HUVECs)^[25]. Nilsson *et al.*^[26] found that HIMEC cultures produce different cytokines (IL-1 β , IL-3 and IL-6) upon stimulation with inflammatory cytokines (e.g. TNF- α , IL-1) compared to HUVECs. Binion *et al.*^[27,28] have shown distinctive HIMEC properties such as constitutive inducible nitric oxide (NO) synthase (iNOS) as well as unique adhesive determinants, and that these properties were altered in IBD and may underlie endothelial dysfunction in IBD development.

ENDOTHELIAL NO IN IBD

Endothelial-derived NO reduces leukocyte and platelet adhesion to the endothelium^[29,30], mediates flow-dependent and agonist-dependent vasodilatation, and couples VEGF-A signaling with NO-dependent permeability^[31,32]. NO-mediated endothelial permeability involves 2 separate mechanisms: (1) increased guanylate cyclase and phospholipase C activity which increases intracellular Ca²⁺; and (2) permeability mediated by Erk1/2 *via* Ras/Raf/PKC causing increased actin contractility^[29,33,34]. Increased p38 mitogen-activated protein kinase (MAPK) signaling, Rho-GTPase activity and increased Ca²⁺ release mediated by upregulated cytokines and growth factors may also represent possible mechanisms for increased endothelial permeability^[35-37].

Endothelial nitric oxide synthase (eNOS)-derived NO is a radical scavenger not only absorbing O₂ but also generating the potent oxidant ONOO⁻. eNOS expression is reduced in IBD; eNOS deficiency in IBD is exacerbated by arginase-mediated depletion of substrate as well as eNOS uncoupling^[38-40]. Decreased eNOS activity in IBD reduces endothelium-dependent vasodilatation, leading to uncontrolled oxidant formation, prominent in IBD^[41]. Deletion of eNOS (eNOS^{-/-}) increases severity of experimental IBD^[42,43] consistent with protective roles of NO against inflammation. NO may prevent development of endothelial inflammatory and hyper-adhesive phenotype in IBD by suppressing cytokine-induced EC adhesion molecules (ECAMs) and matrix metalloproteinases (MMPs)^[44]. Increased endothelial oxidant stress (e.g. in IBD) also disturbs tight junctional organization *via* p38, p42/44 MAPK^[45-47].

Sera from patients with CD reduce, while UC sera increase eNOS in HUVECs; both UC and CD sera increase iNOS^[48]. This may reflect differences in anatomic origins of the endothelium i.e. venous *vs* intestinal. HIMEC iNOS expression appears to be a unique feature of gut microvessels. In HIMECs, iNOS appears at least as important a source of NO as eNOS. Binion *et al.*^[30] have shown that HIMECs persistently express iNOS, and that iNOS-derived NO limits leukocyte adhesion in normal HIMECs. Paradoxically iNOS inhibition increases binding of leukocytes. Thus, while leukocyte-derived iNOS may drive inflammation, HIMEC expression of iNOS limits the inflammatory responses (leukocyte adhesion, permeability, vasodilatation) in the gut, and decreased endothelial iNOS abundance and activity in IBD may represent an

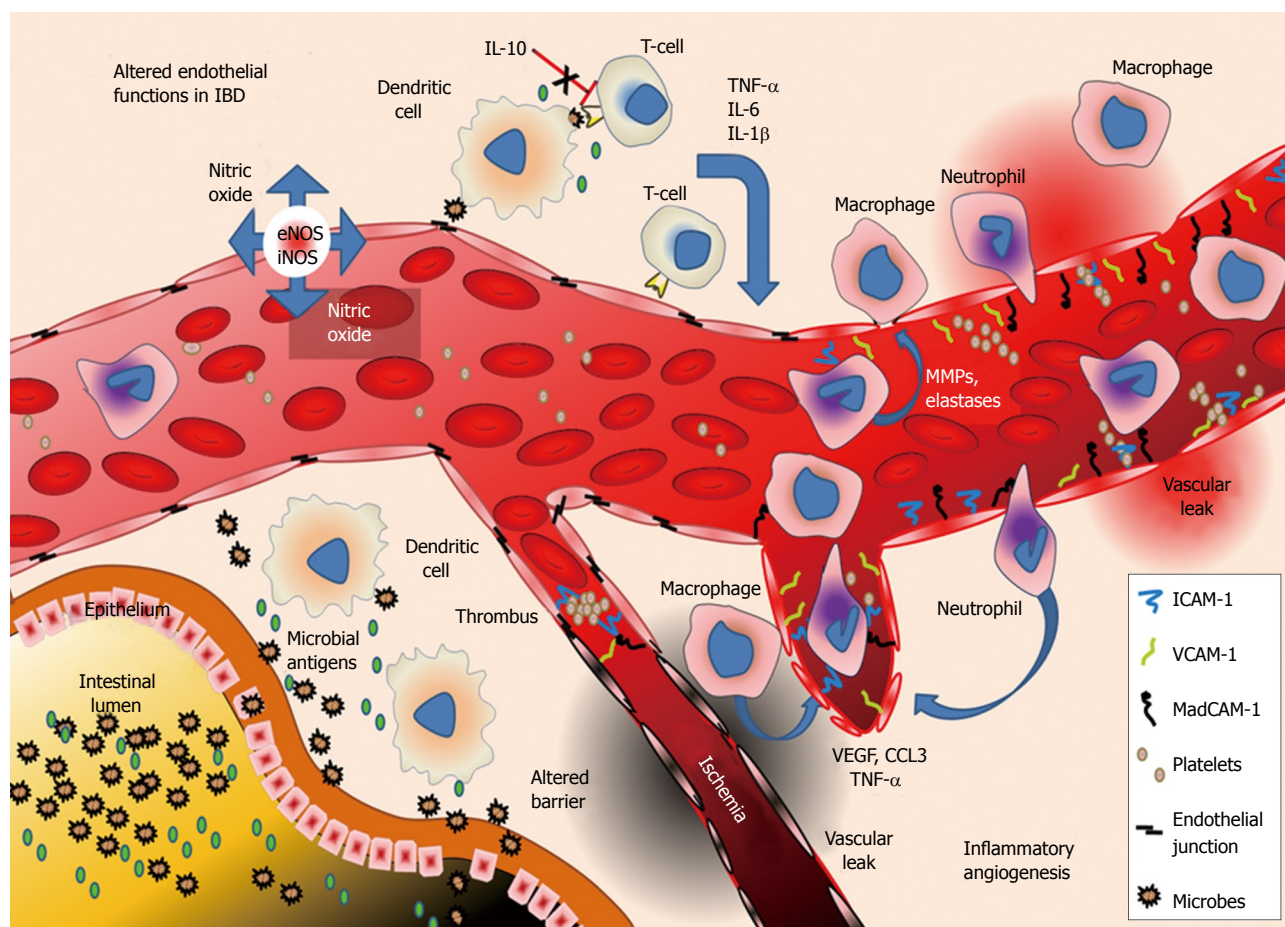


Figure 1 Inflammation triggers a change in the endothelium of the intestinal vasculature in response to the cytokines, chemokines and growth factors released by immune cells leading to increased angiogenesis, adhesion molecule expression, leukocyte extravasation, decreased endothelial barrier function and increased coagulation. TNF: Tumor necrosis factor; IL: Interleukin; iNOS: Inducible nitric oxide synthase; eNOS: Endothelial nitric oxide synthase; VEGF: Vascular endothelial growth factor; MMPs: Matrix metalloproteinases; VCAM: Vascular cell adhesive molecule; ICAM: Intracellular adhesive molecules.

underrated basis of IBD pathology^[27,30]. HIMECs derived from CD patients also show a persistent loss of iNOS expression^[27]. Interestingly, iNOS can be decreased by injury to normal HIMECs (opposite to most tissues which mobilize iNOS in response to injury^[27]) suggesting that during injury, reduced iNOS might trigger inflammatory responses. Even with the loss of endothelial iNOS, there is often increased NO in tissues surrounding the area of inflammation. Despite decreased endothelial iNOS derived-NO, IBD frequently exhibits increased leukocyte recruitment and activation of gut epithelial cells to increase overall NO production^[44]. Kriegstein *et al.*^[49] found that tissue-derived iNOS, and to some extent leukocyte iNOS, mediate colitis injury, but could not specifically distinguish between tissue and endothelial contributions of iNOS in colitis. Aoi *et al.*^[50] have suggested that iNOS-derived NO plays an important role in gut healing after injury through induction of VEGF, necessary for angiogenesis in wound healing. We have previously shown that excess NO may play an important role in IBD exacerbation. Using STAT-6^{-/-} mice (which have high iNOS levels) in dextran sulfate sodium (DSS) colitis, we found more severe IBD in STAT-6^{-/-} mice correlate with extraordinary NO flux suggesting that excess NO may also drive gut injury^[51].

Despite elevated NO abundance, downstream guanylate cyclase signaling appears to be depressed in DSS colitis leading to decreased cGMP in the inflamed intestine^[52]. Under these conditions, cGMP dependent protective NO effects may be masked by pro-oxidant effects of NO metabolites. Conner *et al.*^[53] and Grisham *et al.*^[54] revealed an important role of the 26S proteasome in the regulation of endothelial nuclear factor-κB (NF-κB) and cumulative iNOS NO production and adhesion molecule expression. Cumulatively, these studies suggest that intestinal homeostasis is controlled by distinctive and compartmentalized NO sources, and that excess NO formation may support the pathophysiology of IBD.

ENDOTHELIAL TOLL-LIKE RECEPTORS AND IBD

The gut is an organ supporting a high bacterial load; despite physical and chemical barriers, some bacterial antigens will ultimately penetrate the gut wall to activate gut microvascular ECs through Toll-like receptor (TLR) signaling^[18,55]. The intestinal microvascular endothelium also differs from ECs of other origins in TLR responses. For example, repeated exposure and activation of TLR4 in HIMECs

leads to development of lipopolysaccharide tolerance; however HUVECs lack such a mechanism, indicating the importance in controlling endothelial-dependent inflammation and host commensal interactions^[25,56,57]. Protease activated receptors activate transforming growth factor (TGF)- β to induce TLR4 and lead to increased disease severity in IBD^[58,59]. TLR5 is constitutively expressed in all ECs, and is of particular interest in gut pathophysiology. TLR5, a receptor for flagellin^[60], is constitutively expressed on the basolateral surface of the gut endothelial (an epithelial) layers^[61]. TLR5 signaling induces endothelial intercellular adhesion molecule-1, TNF- α production and leukocyte binding and emigration^[61]. Loss of TLR5 activity in murine models leads to the development of infectious as a result of deficient and improper responses to normal flora and pathological microorganisms^[61,62]. Conversely, endothelial TLR3 has been shown to be protective in the DSS model of acute colitis. This process is mediated by interferon (IFN) type 1 induction of IL-10, a potent anti-inflammatory cytokine^[63]. However, Heidemann *et al.*^[64] in 2007 found that IL-12 expression and its associated gene products were also induced by TLR3 signaling in addition to increased adhesion and transmigration of leukocytes and TLR functions in the gut remain complex, and requires further study.

IBD-ASSOCIATED CYTOKINES AND CHEMOKINES EFFECTS ON GUT ECs

During inflammation there is an increase in plasma levels of inflammatory cytokines, including IL-6, IL-23, IL-12 and TNF- α , in both human IBD and animal IBD models^[1,2,15]. Kawachi *et al.*^[65,66] examined cytokine alterations in the adoptive T-cell transfer and the IL-10^{-/-} IBD models and found IL-1, IL-6, IL-18 and TNF- α were upregulated in both models. Many of the inflammatory cytokines that are upregulated in IBD are pro-angiogenic, the best examples being IL-17 (produced by invasive Th17 cells) and TNF- α produced by several tissue types, including infiltrating immune cells (macrophages and monocytes)^[67,68] and the endothelium^[69]. EC produce inflammatory mediators in response to activation by immune cells and alterations in the tissue microenvironment^[64,70].

TNF- α is one example of a cytokine with pleiotropic effects on the endothelium in IBD, ranging from adhesion molecule induction [vascular cellular adhesion molecule (VCAM)-1 and mucosal addressin cellular adhesion molecule (MAdCAM)-1], promoting interaction of platelets with ECs and inducing expression of pro-angiogenic growth factors such as VEGF-A^[25,44,71-73]. Defects in the activity of the anti-inflammatory cytokines such as IL-10 may play a role in the establishment of some IBD, and IL-10 deficient mice (IL-10^{-/-}) develop IBD spontaneously, while other animal models of colitis show reduced injury when treated with exogenous IL-10^[2,74-76]. Interestingly Oshima *et al.*^[19] observed that pretreatment of ECs with IL-10 prevented IFN- γ mediated endothelial barrier

disruption, indicating that an important role of IL-10 may be to prevent cytokine mediated EC barrier disturbances which initiate and exacerbate disease. This is supported by the finding that several EC adhesion molecules such as intercellular adhesion molecule (ICAM)-1, VCAM-1 and MAdCAM-1 are increased in IL-10^{-/-} mouse colitis and may mediate leukocyte recruitment in this model^[66].

Over 40 chemokines in 4 separate families interact with as many as 19 receptors to regulate trafficking of leukocytes. Of these, several chemokines may mediate leukocyte trafficking to the gut and colon dysfunction in IBD. Papadakis *et al.*^[77] showed that CCL2 and CCL5^{-/-} mice are protected from colitis. Interestingly, Barcelos *et al.*^[78] and Wu *et al.*^[79] showed that CCL5 and CCL3 can induce inflammatory angiogenesis in a murine sponge model and promote angiogenesis in murine tumors. Eyman *et al.*^[80] have also shown that CCL5 upregulates pro-angiogenic genes. CCL25 interacting with its receptor on CCR9⁺ leukocytes plays a major role in the early stages of experimental IBD pathogenesis^[81]. CXCL8 (IL-8) another pro-angiogenic chemokine, is known to be stored in EC Weibel-Palade bodies, can be rapidly secreted, and induces HIMEC proliferation in culture *via* binding to CXCR2^[82,83]. Although angiogenesis may support injury IBD, IL-8 may be dysregulated in some forms of IBD. IL-8 seems to be downregulated in leukocytes and in the endothelium of patients with CD. There appears to be no upregulation in the endothelium of UC patients, suggesting a possible link to TGF- β 1 over expression in IBD^[84-86]. In contrast, Scaldaferri *et al.*^[87,88] found that intestinal fibroblasts treated with TNF- α produce IL-8 and monocyte chemoattractant protein-1 *via* p38/p42/44 mitogen-activated protein kinase.

CX3CL1/fractalkine is a chemokine expressed by EC, can be upregulated by TNF- α , IL-1, LPS and IFN- γ , and is highly upregulated in IBD^[89,90]. CX3CL1 can function as an endothelial adhesive determinant to recruit a subpopulation of dendritic cells and macrophages that have high CX3CR1 expression. CXCL1 can be shed from the surface of the ECs (in response to increased IL-1 β in IBD). This form of CX3CL1 acts as a chemoattractant for CD4⁺ and CD8⁺ T-cells^[90]. Sans *et al.*^[91] reported that in fact there is enhanced recruitment of CX3CR1 expressing T-cell to the gut *via* interactions with CX3CL1. CXCR4/SDF-1 α and its ligand CXCL12 is an important chemokine/receptor pair in angiogenesis, but have received very little attention in IBD. Heidemann *et al.*^[92] reported that blocking this CXCR4/CXCL12 interaction is sufficient to inhibit migration and proliferation of HIMECs in response to VEGF-A. CXCR4/SDF-1 α plays an important role in the recruitment of EC precursors to sites of angiogenesis, and may be impaired in IBD, leading to the conclusion that this pathway may be interrupted^[93-95]. Midkine, another chemokine of great interest is increased in serum and is associated with tumor drug resistance and poor cancer prognosis^[96-98]. Midkine is also upregulated in IBD serum, and has prognostic value like VEGF, TNF- α , sVCAM and VCAM^[20,99-102]. Midkine has a pronounced

angiogenic effect, like some other inflammatory factors, and also increases the levels of surface glycosaminoglycans on ECs to favor recruitment of circulating leukocytes in IBD^[103].

INCREASED ENDOTHELIAL ADHESION MOLECULE EXPRESSION IN IBD

Inflammation in IBD is characterized by increases in both blood and lymphatic vessels in the intestine. This increase in endothelial surface area provides a powerful means of increasing leukocyte recruitment with the mobilization of ECAMs including selectins^[28]. Animal models of IBD (IL-10^{-/-}, IL-2^{-/-}, SAMP1/Yit and T-bet^{-/-}), like human IBD, all show ECAM upregulation is linked to disease severity^[66,104-106], allowing use of adhesion antagonists in IBD therapy^[55,102,107]. Endogenous endothelial-derived inhibitors of leukocyte binding (e.g. sVCAM-1) may also be downregulated in IBD^[21,108-111] and could provide new diagnostic or anti-adhesive strategies.

P and E-selectins, glycoproteins expressed on the surface of platelets and other leukocytes, are also expressed on the surface of activated or inflamed endothelium in IBD. P-selectin can interact with ECAMs such as VCAM-1/ICAM-1, as well as with O-glycans collectively referred to as peripheral lymph node addressins (PNAds) containing sialyl Lewis X moieties^[112,113]. P-selectin at least partially mediates rolling and recruitment of gut-infiltrating leukocytes in IBD, with approximately 50% increase in gut P-selectin in UC *vs* control groups; serum levels of soluble P-selectin, an inhibitor of selectin binding, are decreased in IBD patients^[109,114,115]. Increased platelet P-selectin, with the enhanced prothrombotic surface of the gut EC in IBD increases thrombus formation and tissue damage by ischemic injury^[115]. E-selectin, a relative of P-selectin is expressed solely on the surface of activated ECs during inflammation and is a major contributor to leukocyte rolling injury. E-selectin is not stored in Weibel-Palade bodies and must be produced in response to inflammatory stimuli such as IL-1, TNF- α and VEGF-A^[116,117]. In contrast to sP-selectin, sE-selectin is not downregulated in IBD and in CD, and actually increases in comparison to controls^[109].

High endothelial venules (HEV) are specialized post-capillary venules that allow trafficking of leukocytes between immune (e.g. Peyer's patches) and vascular compartments, and are increased in IBD^[113]. L-selectin expressed on leukocytes (after activation) binds PNAd on HEV and recruits leukocytes expressing L-selectin in IBD. The gut and brain selective adhesion determinant, MAdCAM-1 is also expressed on HEV, and in UC MAdCAM-1 O-glycosylation increases, allowing greater L-selectin binding^[118]. MAdCAM-1 interacts with $\alpha 4\beta 7$ integrins on the surface of a subset of naive CD4⁺ T-cells^[119,120]. MAdCAM-1 induction is found only in chronically inflamed gut endothelium and suggests that in IBD there is a fundamental alteration in the phenotype and gene expression pattern in the inflamed intestinal EC^[28]. Mizushima *et al.*^[121] dem-

onstrated that inhibition of angiotensin-II type 1 receptors reduced TNF- α dependent MAdCAM-1 expression and reduce the severity of DSS-induced colitis, possibly linking vasoregulation and inflammation. In HIMEC MAdCAM-1 is also expressed inversely with cell density, with proportionally greater levels of MAdCAM-1 found at low densities. This indicates that in newly formed vessels, larger amounts of MAdCAM-1 may be available to recruit leukocytes to these "leaky and permissive" vessels^[122,123].

ICAM-1, another important ECAM in IBD binds LFA-1 (aLb2), Mac-1 (aMb2) and $\alpha 4\beta 2$ integrins, and is expressed by inflamed ECs to mediate the firm adhesion of leukocytes to activated ECs^[124,125]. ICAM-1 has a unique relationship with VEGF-A; Goebel *et al.*^[125] reported that HIMECs constitutively express ICAM-1, which is significantly upregulated following treatment with 50 ng/mL VEGF-A, linking inflammation and angiogenesis. In addition to direct activation and upregulation of ICAM-1 by VEGF, Zitterman *et al.*^[117] found that VEGF treatment also sensitizes cells to TNF- α induced ICAM-1 mobilization. Normally ICAM-1 concentrates at EC junctions, but is redistributed to apical surfaces of ECs under inflammatory conditions where it supports firm adhesion of leukocytes^[25]. In the adoptive T-cell transfer model of murine IBD, Ostanin *et al.*^[126] found that T-cells that lack LFA-1, (a T-cell ICAM-1 ligand), fail to induce disease, revealing a critical role for EC modulated immune responses. ICAM-1 was the one of the first clinical targets in IBD, but an antisense IBD therapy showed limited success^[21].

VCAM-1, an ECAM highly expressed on the luminal surface of activated ECs in IBD, mediates the adhesion of $\alpha 4\beta 1$ expressing lymphocytes. In HIMECs, the expression of VCAM-1 is regulated by the PI3K/NF- κ B signaling pathway and its stimulation by mediators can be inhibited by curcumin^[127]. Like ICAM-1, VCAM-1 can also up regulated by VEGF-A *via* NF- κ B^[117,128]. Studies in the picrylsulfonic acid model of UC using radiolabeled anti-VCAM-1 antibodies show that leukocyte infiltration and histological damage are proportionate to VCAM-1 expression in the gut microvasculature^[129]. In addition, in the DSS model of UC, there is an upregulation of VCAM-1 which if blocked (by specific antibodies) attenuates disease activity, while ICAM-1 and MAdCAM-1 blockade do not protect in this manner^[129,130].

CD31/PECAM-1 expressed by ECs and leukocytes mediates homophilic binding between activated ECs and leukocytes especially during extravasation. CD31 is found on the endothelial surface and in endothelial junctions. Work by Romer *et al.*^[72] found that CD31 is not upregulated in response to inflammatory cytokines but is redistributed from cellular junctions. CD31 blockade inhibits leukocyte transmigration, and CD31 inhibition in IBD reduced leukocyte rolling and firm adhesion suggesting a unique role for CD31 in IBD or in the function of the gut microvasculature^[131].

Originally considered a mesenchymal stem cell marker^[132-134], CD146 is now described as a novel immunoglobulin super family adhesion molecule which is increased in

gut tissue of IBD patients^[108]. The function of CD146 in IBD is not completely understood, but has potential roles in inflammation since it supports rolling and invasion of natural killer T-cells^[135]. The upregulation of CD146 in IBD, like ICAM-1 and VCAM-1, may be driven by VEGF-A overexpression during IBD^[100]. Additionally, the soluble form of CD146, regulates endothelial and leukocyte CD146 interactions with their ligands, and is reduced in IBD, enhancing leukocyte extravasation^[100,108,135]. Interestingly Tsiolakidou *et al.*^[100] determined that new vessels formed in IBD are disproportionately CD146⁺. Inflamed ECs from CD and UC patients show an increased ability to recruit naïve T-cells and macrophages to the intestinal immune compartment after stimulation with several inflammatory cytokines, but not with LPS^[28,120]. These data are consistent with IBD not being initially driven by immune cells, but rather by the endothelial response to an increased inflammatory mediatory load.

PLATELETS AND COAGULATION IN IBD

Platelet and leukocyte aggregation as well as activation of the coagulation cascade increase during IBD, reflecting loss of the non-thrombogenic EC phenotype in IBD. Thrombi aggravate inflammation by binding of micro infarcts to the endothelial surface often leading to ischemic inflammation in the intestinal microvasculature^[136]. Mesenteric venous thrombosis has been observed in a fraction of IBD cases, and thrombotic processes are being recognized in altered perfusion, inflammation and tissue injury in IBD^[137]. Indeed, subclinical thrombosis is common in IBD, and is a major source of morbidity in approximately 25% of IBD deaths^[136]. Increased markers of coagulation include thrombin anti-thrombin complex, tissue factor and fibrinopeptide B^[55], and can be described early in IBD. Factor XIIIa, a fibrin-stabilizing coagulation factor (and agonist for VEGFR-2), is increased in IBD, while factor XIII T_T has an increased number of mutations in IBD patients compared to controls suggesting links between thrombosis, angiogenesis and inflammation. However, Bernstein *et al.*^[138], Dardik *et al.*^[139] and Vrij *et al.*^[140] reported that factor XIII activity is reduced in IBD patients.

In addition to increased levels of coagulation cascade proteins in IBD, CD40, CD40L and soluble CD40L are increased in IBD. CD40, expressed on several cell types (including ECs) is involved in inflammatory and immune activation, and interacts with CD40L on T-cells. Danese *et al.*^[141] suggested that the primary source of sCD40L was from activated platelets. CD40 signaling increases production of pro-inflammatory cytokines and chemokines by ECs and surrounding tissue^[142]. CD40L release also leads to binding of platelets and immune cells to ECs by increasing tissue factor, ECAM expression and pro-thrombotic phenotype in HIMECs^[141-143]. Danese *et al.*^[71] suggested that a possible therapeutic benefit of TNF- α blockade was downregulation of CD40/CD40L signaling in IBD. A still unanswered question is whether coagulation is a secondary or initiating event in inflam-

mation. It is worth mentioning that individuals with coagulation cascade disorders (e.g. hemophilia, factor V deficiency and von Willebrand disease) rarely develop IBD^[55]. The opposite of the previous observation is also true; patients with IBD have an increased likelihood of having genetic pro-thrombotic disease Factor V Leiden^[144]. This evidence strongly links thrombus formation as a possible trigger of IBD and suggests prognostic factors which may increase risk of IBD development.

ENDOTHELIAL BARRIER DYSFUNCTION IN IBD

The maintenance of normal vascular barrier supports nutrient and O₂ exchange, osmotic balance and leukocyte abundance in the extracellular compartment. In IBD, increased vascular permeability leads to tissue edema and damage in both human IBD and animal models of IBD^[19]. This alteration in solute permeability of the vasculature is not restricted to the gut microcirculation but is widespread affecting the vasculature of other organs including the brain^[145]. Several classes of mediators in IBD alter both solute permeability and angiogenic balance, including angiogenin (an angiogenic peptide with ribonuclease activity), chemokines (e.g. IL-8, IL-10), coagulation factors (thrombin), cytokines (IFN- γ , IL-13), and growth factors, most notably VEGF, the most potent and important blood vascular angiogenic growth factor and an important inflammatory mediator^[19,36,37,47,146-148]. Tolstanova *et al.*^[149] found that VEGF-A inhibition by neutralizing antibodies reduced vessel permeability in the iodoacetamide model of colitis. Downregulation of anti-inflammatory cytokines e.g. IL-10 may play an equally important role in increasing endothelial permeability. Oshima *et al.*^[19] have shown increased vascular permeability in the IL-10^{-/-} colitis model due to loss of IL-10 inhibition of IFN- γ induced junctional degradation; also IL-10 protects against IFN- γ mediated loss of human microvascular barrier.

Leukocytes, e.g. neutrophils and monocytes, can degrade endothelial junctions through protease secretion and upregulation. Cytokines and growth factors also induce MMP-9, MMP-3 and MMP-1^[150,151], resulting in degradation of junctional and matrix targets^[152]. Neutrophil elastase is elevated in IBD and can degrade vascular endothelial cadherin, important in maintaining junctional apposition, adhesion and barrier function^[153-156]. Endothelial junctional adhesion molecule-A is also dysregulated in IBD, and is closely linked to disease activity in DSS colitis^[57,157].

ANGIOGENESIS IN IBD

Angiogenesis (increased blood vessel density) in IBD increases the area of endothelium available for exchange, but also for extravasation of blood constituents into surrounding tissue to increase disease severity in IBD^[158]. Increased vessel formation in IBD may represent recruitment of endothelial progenitor cells, vascular intussuscep-

tion (splitting) and extension from existing vessels^[159]. Increased angiogenesis is observed in animal (2,4,6-trinitrobenzene sulphonic acid (TNBS), DSS and iodoacetamide) colitis models and in human colitis. However, inflammatory angiogenesis in IBD does not simply match increased tissue mass. Vessels formed during inflammation are different from those formed during normal development. These vessels are immature, lacking investment with pericytes. They express ECAMs, leak, are hypoperfused, often stenose and are hyperthrombotic, with an elevated ability to respond to growth factors^[160-163] actively supporting IBD progression^[149,164-168]. Spalinger *et al.*^[158] and Maconi *et al.*^[169] concluded that there is an increased blood vessel density in the intestines of CD and UC patients and that increased vascular density in IBD was directly correlated with increased IBD disease severity. This is also true in animal models of IBD like TNBS- and DSS-induced colitis models^[166,170].

Growth factors, especially VEGF-A, dramatically alter several aspects of the colon microvascular endothelial phenotype, resembling a de-differentiation (loss of maturity) of the vessels which can reflect changes in vascular support cells, e.g. pericytes/smooth muscle, that surrounds the capillaries. Inflamed tissues display increased vascular density resulting from the formation of new vessels during angiogenesis. These changes result in decreased perfusion, increased solute permeability (*via* cytokines and VEGF-A induced junction remodeling) and contractility, as well as increased leukocyte and platelet adhesiveness^[161,171,172]. Ganta *et al.*^[163] have demonstrated that in angiotensin-2 knockout mice (using the DSS model of UC), loss of the pericytes around vessels resulted in diminished angiopoietin-1 signaling that destabilized the endothelial layer, increased leukocyte recruitment to the tissue, increased vessel permeability and induced vessel hyper-proliferation. Blood and lymphatic vessels are hyperstabilized by angiopoietin-2 deficiency, and show diminished inflammatory remodeling as well as decreased capacity to recruit leukocytes suggesting a link between maturity and inflammatory capacity^[163].

ENDOTHELIAL CELL AND ANGIOGENIC GROWTH FACTOR INTERACTIONS IN IBD

VEGF-A is the first described and best known VEGF, which controls developmental angiogenesis, wound healing and pathology^[173,174]. Bousvaros *et al.*^[175], Kapsoritakis *et al.*^[101] and Ozawa *et al.*^[176] all found elevated VEGF-A levels in plasma and tissue during active human and animal IBD, often twice normal^[101,109,166,175,176]. However, Chidlow *et al.*^[166] have reported that DSS diminishes levels of VEGF-A as well as VEGF-C and VEGF-D, suggesting complex, concentration-dependent and inhibitor-regulated effects of VEGF in different animal models of IBD. Danese *et al.*^[177] and Scaldaferrì *et al.*^[167] have shown that inhibition of VEGF signaling can attenuate disease activity in the DSS model of UC while overexpression of VEGF-A in-

creases disease severity in the same model^[167,177]. VEGF-A is released by several cell sources (e.g. neutrophils, platelets, macrophages, pericytes, fibroblasts, ECs, and colonic epithelial cells) and is transcriptionally activated by hypoxia through hypoxia inducible factor 1 α , and message stabilization *via* eukaryotic translation initiation factor 4e^[70,178-183]. Interestingly Birmingham *et al.*^[184] have shown that activated colonic epithelium represents an important source of VEGF-A, and injury or inflammation of the colon epithelium may provide a local stimulus for blood vessel growth. Invasive leukocytes, specifically neutrophils, granulocytes, macrophages and platelets, are increased in tissue during active IBD, and are also important sources of VEGF-A in inflamed tissues^[178,179,185-187]. Salivary secretions also contain high levels of VEGF-A and VEGF-C, which have been suggested as important sources of these growth factors in IBD^[188] released site-specifically during denudation. Apart from VEGFs, other angiogenic growth factors, e.g. basic fibroblast growth factor (bFGF), TGF- β and platelet-derived growth factor (PDGF) are upregulated in IBD and may be of clinical relevance^[86,189].

TGF- β is an important regulator of the cell cycle and apoptosis, especially in mucosal immune cells. The expression of TGF- β and its 2 receptors (TGFR1 and TGFR2) are increased in IBD, specifically UC; however, it appears that the levels are decreased in CD^[190]. In IBD either tachyphylaxis develops for TGF- β (UC), or the lack of TGF- β (CD) allows mucosal immune cells to proliferate when they would have undergone apoptosis^[190,191]. Early studies on the role of TGF- β in IBD indicated a protective role; more recent studies may point to a pathological role of TGF- β signaling in IBD^[191,192]. In fact, TGF- β is important in the formation of fibrosis in the colon of IBD patients by stimulating the transition of many cell types to fibroblasts^[193]. Over one-third of the fibroblasts responsible for inflammatory fibrotic injury may actually originate from the transformation of ECs to fibroblasts (not counting contributions of pericytes to fibroblast formation). Therefore the vasculature may provide a significant proportion (if not the majority of fibroblasts) and associated fibrosis in IBD^[194,195]. bFGF, a potent mitogen for the cells of mesodermal origin, stimulates EC proliferation, activates MMPs resulting in proteolysis of extracellular matrix, and increases cellular motility^[191]. Even though levels of bFGF are elevated in IBD there is no correlation with the stage or severity of the disease. However, the contribution of bFGF in the initiation or maintenance of IBD should not be discounted^[196]. PDGF is a close relative to VEGF and is upregulated in IBD. PDGF is predictive of both oxidative stress and angiogenesis in the intestine^[189]. PDGF is released in response to inflammatory and thrombotic stimuli. PDGF increases P-selectin expression on ECs and induces histamine secretion which induces other effects such as increased vascular leakage^[197,198].

ENDOTHELIAL PROGENITOR CELLS AND VESSEL SPROUTING IN IBD

Recruitment of endothelial progenitor cells (EPCs) may

contribute to angiogenesis in IBD, although reduced numbers of VEGFR2⁺, CD34⁺, CD133⁺ cells (endothelial, bone marrow, and stem cell markers) have been reported in IBD^[199], and EPCs from IBD have reduced antigenic activity^[95]. These findings suggest that recruitment of EPCs is unlikely to be a source of increased vessels, however, these findings are from patients with established disease as initial angiogenesis in early stages of IBD may rely on EPCs. Apart from EPC recruitment, angiogenic sprouting is active in IBD; sprouting ECs referred to as “tip” cells, are highly motile with distinct gene expression compared to that in quiescent ECs^[200]. VEGF-A induces the tip cell phenotype and also guides vessel sprouting, indicating that in IBD, VEGF might induce new vessel formation in this way^[201]. Normally, not all sprouts survive, many undergoing apoptosis, (vessel “pruning”) suggesting that high levels of VEGF prevent endothelial apoptosis resulting in increased numbers of surviving sprouts in IBD^[201].

INHIBITORS OF VASCULAR ENDOTHELIAL EXPANSION IN IBD

While increased pro-angiogenic growth factors increase angiogenesis, reductions in anti-angiogenic factors (seen in the DSS model of colitis) may be as important for permitting expansion of the vascular endothelium^[166,167]. Angiopoietin-1 a competitive inhibitor of Ang-2, binds to the Tie-2 receptor and inhibit vascular remodeling. Angiopoietin-2 is upregulated during inflammation and angiogenesis^[163,202] and competes with angiopoietin-1, to allow ECs to maximally respond to cytokines and growth factors. Work by Ganta *et al.*^[163] found that angiopoietin-2 signaling also appears to be necessary for neutrophil infiltration, and blood and lymphatic vessel proliferation in DSS colitis. Interestingly angiopoietin-2 can be upregulated by both bFGF and VEGF, potent pro-angiogenic growth factors also upregulated in IBD^[203-205].

Angiostatin, a fragment of plasminogen generated by MMPs has anti-angiogenic and anti-proliferative effects on ECs and blocks vessel maturation^[206]. During IBD, levels of MMPs are elevated and generate angiostatin^[153]. In fact 2 models of experimental colitis (iodoacetamide, TNBS) show increased angiostatin, and may represent a feedback control for angiogenesis^[207]. Interestingly, the effect of angiostatin hinges less on inhibition of EC proliferation, but more on inhibiting final vessel maturity^[208]. Much like angiostatin, endostatin results from the cleavage of collagen type XVIII yielding an anti-angiogenic fragment that is upregulated in experimental colitis^[207,209]. Endostatin reduces EC migration and proliferation; however like angiostatin, endostatin fails to block angiogenesis in the TNBS model, but may play a role in disease progression and maintenance by impairing vessel maturity and tissue healing by antagonizing VEGF-A induced tissue repair^[207]. Interestingly Deng *et al.*^[210] showed mesalamine treatment of iodoacetamide colitis restored levels of endogenous angiogenesis inhibitors, endostatin and angiostatin helping reduce disease severity.

Soluble VEGF receptors (sVEGFRs) are truncated forms of VEGFR1 or VEGFR2 genes^[211] that under normal physiological conditions maintain tissue avascularity (e.g. in the cornea) and might be dysregulated in IBD. During inflammation, sVEGFR1 inhibition seems to be lost (e.g. in the case of an alkali burn)^[211,212]. sVEGFR2 seems to play an important role in the inhibition of lymphangiogenesis compared to sVEGFR1, but sVEGFR2 blocks transplant rejection which points out its greater immunomodulatory effect^[213]. Additionally, Scaldaferrri *et al.*^[167] found that over expression of sVEGFR1 reduced disease severity in the DSS model of colitis, suggesting that loss of this molecule in IBD would be detrimental. Interestingly the anti-angiogenic VEGFs, alternate splice variants of VEGFs, are downregulated in several inflammatory diseases, and are linked to the alteration of the cytokine milieu in the tissues^[214-217]. These inhibitory VEGFs make up a majority of the VEGF load in the normal intestinal micro-environment with approximately 20 times greater levels in the healthy gut^[217]. Currently, the levels of these inhibitory VEGFs are unknown in IBD, but may provide a new avenue for anti-angiogenic therapies, we are pursuing this possibility which is currently showing great promise (unpublished data).

IBD THERAPIES

It is increasingly clear that IBD therapies affect the microvasculature, and that the microvasculature is a central target in IBD, coordinating cell infiltration, solute permeability, cytokine/chemokine production and gut immunological responses. An increasing number of drugs that show efficacy in treating IBD have now been found to affect the endothelium. Accumulating evidence suggests inhibition of angiogenesis as a secondary mechanism of action for many IBD therapies including anti-TNF- α antibodies, and some immunosuppressive agents (cyclosporine A)^[218-220]. Scaldaferrri *et al.*^[167] found TNF- α mediated lymphocyte adhesion and chemotaxis across intestinal microvascular ECs depends on expression of ICAM-1, VCAM-1 and fractalkine in the affected ECs mediated by p38 MAPK, p42/44 MAPK and JNK. Danese *et al.*^[71] found that anti-TNF- α therapies can reduce thrombus formation and adhesion to the endothelium by interfering with CD40/CD40L signaling. Integrin-blocking antibodies have been used in the treatment of IBD, but not without a controversial side effect. Natalizumab (Tysabri), an α 4-integrin blocking monoclonal antibody originally developed for use in the treatment of multiple sclerosis, but has recently been approved for the treatment CD^[21,221]. AJM300, a peptide blocker for α 4 integrins, successfully blocked α 4-VCAM-1 and MAdCAM-1 adhesion and prevented exacerbation in IBD models^[20,21]. However, recent preclinical trials using AJM300 failed to inhibit disease progression^[20,21]. Rafiee *et al.*^[222] found that 2 drugs used in IBD, thalidomide and cyclosporine-A, are anti-angiogenic; thalidomide targets TNF- α and VEGF-A, while cyclosporin-A targets VEGF-A alone^[222,223]. Studies by Ogawa *et al.*^[224] determined that HIMEC expression of the inflammatory mediators IL-6 and cyclooxygenase (COX)-2

by LPS were inhibited by butyrate, and that butyrate also inhibited HIMEC angiogenesis^[224-226]. Despite its anti-inflammatory properties^[223,227,228], cyclosporin-A increases leukocyte binding, unlike thalidomide which reduces leukocyte binding to HIMECs^[227,228].

IBD-INDUCED ANGIOGENESIS AND COLORECTAL CANCER

The risk of developing cancer is elevated by inflammation, and the link between IBD and colorectal cancer (CRC) is convincing^[229-231]. Inhibition of angiogenesis in CRC by bevacizumab (anti-VEGF monoclonal antibody) improves clinical outcomes, revealing the importance of angiogenesis in the progression from IBD to CRC^[232]. As stated before, IBD in human disease and animal models is associated with an increase in vascular density, and it is possible this vascular endothelial expansion may enable CRC^[158,163,169]. CRC incidence may depend on COX expression (seen in adenomatous polyposis coli, pre-cancerous lesions enriched in COX^[233-235]). COX-2 is increased in human IBD and IBD models, and may promote CRC through angiogenesis^[236,237]. COX-2 promotes EC proliferation by prostaglandin induction of VEGF-A, important for tumor angiogenesis^[237,238]. Chan *et al.*^[239] reported that the regular use of aspirin, a non-selective COX inhibitor, significantly reduced the risk of COX-2⁺ CRC, which constitutes approximately 67% of human CRC^[239,240]. Additionally COX-2 inhibition reduces tumor growth and increased tumor apoptosis, and is associated with reduced tumor angiogenesis^[238,241,242]. Conversely, Ishikawa *et al.*^[243] found that COX-deficient animals were not protected from tumor formation in azoxymethane (tumor promoter)-induced colorectal cancer, and concluded that COX expression was not a major determinant of tumor formation in UC. While COX expression may not be necessary for tumor formation in UC, COX-2 upregulation is only one mechanism for increased angiogenesis in IBD^[86,166,189]. VEGF-A and other angiogenic factors are upregulated independent of COX-2 in IBD; therefore, while COX-2 may be important in CRC in the absence of IBD, expansion of the vasculature in IBD through other mechanisms may contribute to the development and growth of CRC^[166,244].

CONCLUSION

A unique combination of genetic and environmental factors may contribute to development of IBD. ECs are now recognized as central and fundamental elements in IBD pathophysiology. ECs are indirectly affected by many IBD medications, which are increasingly targeting ECs directly. As treatments for IBD are developed and refined there will be an increased interest in inhibiting functions of ECs in IBD such as immune cell recruitment and inflammatory angiogenesis, and improving beneficial lymphatic function. Use of endogenous inhibitors of leukocyte binding (sVCAM) and peptides (AJM300) may become novel therapies which supplement or replace current anti-

adhesion treatments. Additional studies on the interactions between the gut microvasculature, platelets and their regulation of inflammatory angiogenesis may provide new avenues for treatments that not only reduce thrombosis but also several clinical manifestations of IBD. Inhibition of inflammatory growth factors, cytokines and chemokines that promote angiogenesis by the use of “traps” or decoy receptors, alone or in combination, in addition to current treatments could provide greater anti-inflammatory effects by reducing endothelial expansion in IBD. More importantly, work in our laboratory suggests that endogenous angiogenic inhibitors (VEGF164b) have great potential in the treatment of IBD. Future studies promoting therapeutic intervention by combining anti-angiogenic, anti-immune and anti-inflammatory agents as treatment options focusing on the endothelium as core/vital for IBD pathogenesis will provide greater specificity and efficacy for treating CD and UC patients.

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Haemostatic system in inflammatory bowel diseases: New players in gut inflammation

Franco Scaldaferri, Stefano Lancellotti, Marco Pizzoferrato, Raimondo De Cristofaro

Franco Scaldaferri, Marco Pizzoferrato, Department of Internal Medicine and Medical Specialties, Gastroenterology Division, Catholic University of Rome, 00168 Rome, Italy

Stefano Lancellotti, Raimondo De Cristofaro, Department of Internal Medicine and Medical Specialties, Haemostasis Research Center, Catholic University of Rome, 00168 Rome, Italy

Author contributions: All authors performed the literature revision and writing; Scaldaferri F and De Cristofaro R also revised and edited the paper.

Supported by Italian Ministry of University, No. PRIN-2007; and Catholic University School of Medicine, No. Linea D1-2009

Correspondence to: Raimondo De Cristofaro, MD, Professor, Department of Internal Medicine and Medical Specialties, Haemostasis Research Center, Catholic University of Rome, Largo Gemelli 8, 00168 Rome, Italy. rdcristofaro@rm.unicatt.it
 Telephone: +39-6-30154438 Fax: +39-6-30155915

Received: January 25, 2010 Revised: March 25, 2010

Accepted: April 1, 2010

Published online: February 7, 2011

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Key words: Activated protein C; Coagulation; Crohn's disease; Inflammation; Inflammatory bowel disease; Platelets; Ulcerative colitis

Peer reviewer: Kenji Okajima, Professor, PhD, MD, Department of Translational Medical Science Research, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Scaldaferri F, Lancellotti S, Pizzoferrato M, De Cristofaro R. Haemostatic system in inflammatory bowel diseases: New players in gut inflammation. *World J Gastroenterol* 2011; 17(5): 594-608 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/594.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.594>

Abstract

Inflammation and coagulation constantly influence each other and are constantly in balance. Emerging evidence supports this statement in acute inflammatory diseases, such as sepsis, but it also seems to be very important in chronic inflammatory settings, such as inflammatory bowel disease (IBD). Patients with Crohn's disease and ulcerative colitis have an increased risk of thromboembolic events, and several abnormalities concerning coagulation components occur in the endothelial cells of intestinal vessels, where most severe inflammatory abnormalities occur. The aims of this review are to update and classify the type of coagulation system abnormalities in IBD, and analyze the strict and delicate balance between coagulation and inflammation at the mucosal level. Recent studies on possible therapeutic applications arising from investigations on coagulation abnormalities associated with IBD pathogenesis will also be briefly presented and critically reviewed.

INTRODUCTION

Inflammation and coagulation are two crucial systems in mammals. They constantly influence each other and are constantly in balance. In particular, inflammatory processes can promote coagulation which, in turn, can also sustain inflammation. The inter-dependence of the two processes is confirmed by clinical settings where the inherited or acquired deficiency of natural anticoagulants is associated with an increase in inflammatory processes^[1].

This observation is particularly relevant in acute inflammatory diseases, such as sepsis^[1], but it also seems to be very important in chronic inflammatory conditions, such as inflammatory bowel disease (IBD).

Patients with Crohn's disease (CD) and ulcerative colitis (UC) have an increased risk of thromboembolic events^[2], which appears to be more frequent when IBD is in an active phase^[3-5] and is affecting the whole colon^[3,6,7]. However, it is worth noting that, in a large study, one-third of thromboembolic complications occurred during

disease quiescence, supporting the hypothesis of a greater prothrombotic tendency in IBD, independent of disease activity^[3].

The incidence of thromboembolic events in patients with IBD has been reported to be 1%-8%^[4,8,9]. Patients with IBD have a 3-fold increased risk for deep vein thrombosis and pulmonary embolism compared with the general population^[8,10,11]. In addition, IBD patients experience more thromboembolic events at a younger age than the general population or patients affected by rheumatoid arthritis or celiac disease^[4,8].

Finally, indirect evidence that vascular thrombosis may be involved in the pathogenesis of IBD was provided by an epidemiological study performed on a large cohort of subjects with hemophilia or von Willebrand's disease^[3,12]. In this population, in which more than 9000 patients were included (6000 patients with hemophilia and more than 3000 with von Willebrand's disease), IBD occurred less frequently than expected, and it was suggested that inherited hemorrhagic disorders might be protective against IBD^[3].

Most available reports tried to explain the increased thromboembolic risk in IBD by analyzing different components of the coagulation cascade, such as serological/phenotypical markers and genetic pro-thrombotic mutations/polymorphisms. Several studies exist on major pro-thrombotic genetic predispositions and IBD. Most published data demonstrate that there is no difference in the prevalence of Factor V Leiden between IBD patients and healthy controls^[3,13-15], as well as PT gene G20210A mutation^[3,15-17]. Polymorphisms of Methylenetetrahydrofolate reductase, the enzyme involved in the re-methylation pathway of homocysteine metabolism, have been found to have discordant results in IBD patients compared to controls^[3,15,18]. Other studies looking at the prevalence of Antithrombin III deficiency^[17,19], Protein C^[20] and Protein S deficiencies^[21] in IBD have been contradictory or equivocal^[22,23], suggesting that these factors, although possibly related to IBD pathogenesis, are not genetically related to IBD^[23]. Finally, the inherited Val34Leu factor XIII polymorphism, which is protective against thrombosis, has been evaluated in IBD patients^[3,24]. Available data demonstrated no significant difference in the prevalence of this polymorphism in IBD patients with respect to the general population^[25].

Taken together, the information on genetic factors does not explain the greater risk of venous thrombosis in CD and UC^[26,27]. On the contrary, a pathogenesis-oriented approach suggests that the coagulation abnormalities occurring in IBD are very likely the result of the biological and biochemical effects exerted by the activation of the inflammatory machinery (cells, cytokines, *etc.*) in these disorders. Furthermore, activation of the coagulation cascade can in turn sustain activation of inflammatory reactions, promoting the vicious circle between chronic inflammation and thrombosis.

In this review, we will firstly describe single quantitative abnormalities of coagulation factors observed in IBD and cellular components closely involved in the co-

agulation pathway. We will then describe the mechanisms by which these abnormalities interfere with intestinal mucosa homeostasis. Finally, the possible therapeutic implications emerging from the unraveling of coagulation abnormalities associated with IBD pathogenesis will also be briefly presented and critically reviewed

QUANTITATIVE ALTERATIONS OF HEMOSTATIC FACTORS IN IBD

Coagulation is a complex system, which can be schematically divided into different pathways, referred to as "intrinsic", "extrinsic" and "common" pathways^[28]. An important role is also played by the fibrinolysis system, which controls clot dissolution, and the family of serine protease inhibitors, which inhibits many coagulation enzymes. We will use this classification to better summarize findings concerning the linkage between the coagulation system and inflammation associated with IBD.

The extrinsic pathway

The extrinsic pathway is initiated by tissue damage that exposes tissue factor (TF) to blood, causing the formation of TF/FVIIa, which circulates at low levels in the bloodstream. It is thought that high factor VIIa activity is associated with an increased risk of ischemic myocardial events in men over 40 years of age^[8,29]. It is the main determinant of the laboratory assay referred to as prothrombin time (PT). No definitive data are available on changes of the extrinsic pathway in IBD. Most of the data available report no significant difference in PT among active UC and/or CD and control patients^[3,8,30]. Other studies reported different findings showing that PT values and platelet levels are predictors of CD activity index in female patients^[2]. Levels of factor VIIa seem to be higher in active IBD compared to controls^[3,31,32]. This finding suggests the existence of a pro-thrombotic tendency in IBD patients, arising from activation of the extrinsic pathway.

The intrinsic pathway

Activation of the "intrinsic" pathway of coagulation leads to formation of factor Xa (FXa)^[28]. This process stems from previous activation of Factor IX and FVIII, with formation of the tenase complex, that is FVIIIa-factor IXa on the membrane of platelets and endothelial cells^[28]. As FVIII strongly accelerates the formation of FXa, recent studies showed that inherited high levels of FVIII (> 140%) can be considered a risk factor for venous thromboembolic disorders^[33]. No significant difference in APTT value or FVIII level was observed in IBD patients compared to controls^[8,34]. However, higher APTT levels were found in other reports, although this finding may be the expression of mere consumption of some coagulation factors upon their activation^[2]. Other investigations found that factor XIa and XIIa levels, were higher in active IBD patients compared to patients in the quiescent phase^[8,35]. This finding may be in agreement with studies showing that higher levels of FXIa and FXIIa may be considered coagulation

markers associated with increased risk for thromboembolic disorders^[36-38].

The common pathway

FXa thus occupies a central position in the coagulation cascade as a convergence point between the intrinsic and extrinsic pathway. In fact, in the presence of its cofactor FVa, FXa converts prothrombin to thrombin^[28]. The common pathway is considered the main determinant of both PT and APTT assays. In observational studies, FXa and FVa levels were significantly elevated in active IBD patients compared to those in patients with disease remission^[3,8,39]. The protease-mediated stimulus for inflammatory reactions, particularly for FXa and thrombin, is mediated by cleavage of membrane cleavable receptors and will be discussed below.

The thrombin-generating system

Markers of the thrombin generating system directly involve zymogen prothrombin but also other side-products of prothrombin cleavage such as prothrombin fragment 1+2 (F₁₊₂) and the thrombin-antithrombin complex (TAT). Prothrombin levels in active IBD patients were significantly higher than those in patients with inactive disease or control patients^[8,30,32,39-41]. The same observation was also made for F₁₊₂ and TAT, suggesting that thrombin generation might be an early event in IBD^[8,40,42,43].

Factor XIII

Coagulation factor XIII is a plasma transglutaminase involved in the crosslinking of fibrin, the last step of the coagulation cascade and a connective tissue factor contributing to the wound healing process. It circulates as a heterotetrameric molecule consisting of two identical proenzyme subunits (factor XIIIa) and two carrier protein subunits (factor XIIIs).

A study by Chiarantini *et al.*^[30] reported decreased factor XIII (FXIII) levels, especially in acute phases of the disease, and deposits of FXIII have been detected in both affected and macroscopically normal bowel mucosa^[30,41,43-45]. Those results were confirmed by several other reports, comparing IBD patients to control subjects as well as patients affected by diverticulitis and rheumatoid subjects^[46,47]. As anticipated above for other coagulation factors, this finding does not play an etiopathogenetic role in IBD but may represent only the result of chronic consumption of this enzyme associated to deposition of fibrin at the level of inflamed vessels in enteric mucosa.

The system of natural coagulation inhibitors

The system of coagulation inhibitors is composed of a family of proteins, globally referred to as serpins, a typical example of which is antithrombin (AT), and by the protein C pathway, in which a series of different proteins (such as protein C and protein S) and membrane receptors [thrombomodulin (TM), endothelial protein C receptor (EPCR)] are involved. AT is the physiological inhibitor of thrombin, factor Xa, FIXa, FXIa and FXIIa. There is a growing body of evidence that AT is not only an inhibitor

of blood coagulation, but it is also able, when present at high concentrations, to reduce the inflammatory responses of endothelial and other cells^[48-50]. Thus, AT was shown to reduce the mortality of patients with severe sepsis in a recent clinical trial^[49]. AT-induced attenuation of inflammatory responses might be linked to endothelial production of prostacyclin and inhibition of leukocyte and endothelial cell expression of pro-inflammatory mediators *via* suppression of nuclear factor (NF)- κ B activation^[48,50,51]. Furthermore, AT prevents water-immersion restraint stress-induced gastric mucosal injury in rats by promoting the endothelial release of PGI₂^[52]. In addition, non-uniform information exists on the quantitative expression of these components in IBD. Overall, it seems that no differences in the levels of protein S^[8], protein C^[3,53] and ATIII^[34,41] exist among IBD patients compared to controls. However, in studies comparing the levels of these molecules in the active *vs* inactive form of the disease or in controls, lower levels of protein C, ATIII and protein S were observed^[21,30,34,53,54]. In a single study, higher levels of protein S and C in IBD patients compared to controls were also reported^[30]. These conflicting results indicate that changes in systemic levels of these inhibitors do not necessarily reflect the local loss of inhibition of coagulation occurring within enteric mucosa in IBD. Hence, a new approach has tried to correlate an enhanced production of thrombin in IBD with a possible loss of function in natural anticoagulants mostly occurring on the endothelium of enteric mucosa. This issue will be addressed in the section below.

The fibrinolytic system

In normal hemostasis, the fibrinolytic system allows removal of a fibrin clot when the damaged vessel wall is restored. Activation and regulation of fibrinolysis occurs by multiple proteins and results in the generation of plasmin. Plasminogen may be activated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). The latter binds to a cellular receptor [urokinase plasminogen activator receptor (uPAR)] resulting in enhanced activation of cell-bound plasminogen and its main role is the induction of pericellular proteolysis^[55]. tPA is the most potent activator of plasminogen in plasma and the main regulator of fibrinolysis. After stimulation, tPA is locally released into the circulation from the endothelial cells where it is produced. tPA-mediated plasminogen activation is facilitated by a fibrin surface, which restricts fibrinolysis to the site of thrombus formation^[56]. Moreover, once bound to fibrin, tPA is protected from inhibition by plasminogen activator inhibitor 1 (PAI-1), its principal inhibitor in plasma^[57]. The level of PAI-1 in blood usually exceeds that of tPA; thus, in general, no active tPA circulates in plasma^[58].

α_2 -Antiplasmin is the primary physiological inhibitor of plasmin, as it can very rapidly inhibit plasmin in plasma^[59]. However, plasmin is partly protected from α_2 -antiplasmin inhibitory activity when the enzyme is bound to fibrin^[60]. During thrombus formation, α_2 -antiplasmin is cross-linked to fibrin by factor XIIIa, facilitating local inhibition of fibrinolysis^[61].

Another important player in the fibrinolytic system is

thrombin-activatable fibrinolysis inhibitor (TAFI), which directly connects coagulation and fibrinolysis. It is activated by thrombin, but its activation is over 1000-fold enhanced by the thrombin-TM complex. Activated TAFI removes carboxyl-terminal lysine residues from partially degraded fibrin. Consequently, the binding of plasminogen and tPA to fibrin clots is decreased, which attenuates clot lysis^[62].

Reduced activity of the fibrinolytic system has been described in IBD^[63-65]. Indeed, a reduction in activators (such as tPA) and an increase in inhibitors (such as PAI and TAFI) of the fibrinolytic system have been described in IBD patients^[40,63-66]. This condition would favor pro-thrombotic mechanisms.

Cellular elements involved in the coagulation pathway

The haemostatic system is composed not only of soluble proteins and enzymes but also of different cell types. Platelets and endothelial cells play a central role in the maintenance of a physiological balance between pro- and anti-coagulant mechanisms. Moreover, accumulating evidence indicates that platelets and endothelial cells, besides their well-known haemostatic functions, play a role in inflammation and its resolution mechanisms^[67].

Platelets

Platelets can release a number of mediators of the inflammatory response, including cytokines, chemokines, nitric oxide (NO) and eicosanoids. Furthermore, they interact with polymorphonuclear cells (PMN) and monocytes, regulating their extravasation and recruitment at sites of inflammation. Along these lines, platelets have the enzymatic machinery to synthesize both pro- and anti-inflammatory eicosanoids. As an example, platelets contain 2-lipoxygenase (12-LO), a key enzyme in the biosynthesis of the lipoxins (LXs), arachidonic acid metabolites with potent anti-inflammatory properties^[68]. LX formation during platelet/PMN interactions occurs *in vivo* and represents a main stop signal of inflammation^[69]. Thus, a sustained inflammatory response, as it occurs in IBD may originate by both increased formation of pro-inflammatory mediators, and reduction in counter-regulatory signals.

Platelet integrin GPIb α is a ligand of P-selectin, a transmembrane adhesion molecule present on endothelial cells, and supports platelet rolling on activated endothelium. P-Selectin binding to P-Selectin Glycoprotein Ligand 1 (PSGL-1) stimulates the release of microparticles bearing tissue factor on their surface by leukocytes^[70]. P-selectin is split as a soluble form (sP-selectin) that stimulates expression and exposition on the monocyte surface of further tissue factor^[71]. Microparticles, together with sPselectin, are considered the main factors responsible for the high procoagulant status of blood in inflammation^[72]. During adhesion to endothelium, platelets release pro-inflammatory cytokine CD40-ligand (CD40L) (CD154, gp39) that can stimulate the endothelium, stabilize platelet aggregates, their binding to blood cells and vascular wall cells and promote stable thrombus formation^[73]. CD40L is expressed on activated platelets and also on immune system cells activated during inflammation (activated

CD4⁺ T cells, basophils, and mast cells)^[74]. This factor is a transmembrane protein related to tumor necrosis factor (TNF)- α . The inducible CD40L on platelets binds to the CD40 receptor on endothelial cells and on monocytes, macrophages, and smooth muscle cells (SMC)^[74]. The CD40/CD40L interaction plays an important role in inflammation and atherothrombosis^[73]. Through binding to the ligand, CD40 induces the inflammatory response independent of cytokines. The pro-inflammatory activity of CD40L also occurs on platelets and other cells by stimulation of the expression of chemokines [monocyte chemoattractant protein 1 (MCP-1)], interleukins (IL-6, IL-8), pro-inflammatory adhesive molecules [vascular cell adhesive molecule-1 (VCAM-1)], intracellular adhesive molecules (ICAM-1, CD54), and P-/E-selectins.

Notably, the activity of CD40L induces the expression of tissue factor, which, as mentioned above, is the major inducer of blood coagulation, and suppresses the expression of TM, which is a thrombin cofactor in activation of the protein C anticoagulant system^[75]. Upon binding of CD40L to the CD40 receptor, intracellular signaling results in activation of the transcriptional factor NF- κ B and its translocation into the nucleus. This event induces the expression of new molecules of CD40L and CD40. The interaction of CD40 expressed by endothelial cells with CD40L exposed on activated platelets stimulates the synthesis of a powerful pro-inflammatory mediator, platelet activating factor (PAF), which induces platelet aggregation with leukocytes and also contributes to remodeling of vessels, stimulating neo-angiogenesis^[76].

Platelet activation is associated with the metalloprotease-mediated split of a soluble fragment of the CD40-ligand (sCD40L)^[76]. Soluble CD40L was shown to promote blood coagulation by two mechanisms: induction of tissue factor expression on monocytes and activation of platelets *via* interaction with integrin α II b/ β 3. The sCD40L binding to integrin α II b/ β 3 activates platelets at high shear stress and stabilizes arterial thrombi^[74]. Increased levels of CD40L on platelets and sCD40L in circulating blood were found in clinical settings characterized by thrombosis associated with inflammation, such as unstable angina, myocardial infarction and other cardiovascular diseases^[77,78].

Thus, a realistic scenario shows that platelet activation during inflammation and expression of adhesive proteins, P-selectin and integrins, leads to their aggregation with leukocytes and the release of contents of intracellular granules. In conclusion, platelet-platelet and platelet-leukocyte aggregates produce a cell surface, which provides activation of both blood coagulation and inflammation.

The association between active IBD and thrombocytosis was first recognized in 1968 and it became clear that patients with IBD also have increased numbers of circulating platelet aggregates and activated platelets compared with healthy controls^[79-81]. More recently, it was demonstrated in studies from different groups that supranormal platelet-leukocyte aggregates are frequently present in patients with IBD compared with both healthy and inflammatory controls^[82]. In other studies, significant changes in platelet volume were also observed^[83,84]. In particular, an

increase in platelet count^[83] and a statistically significant decrease in MPV was noted in patients with colitis compared with healthy controls. Moreover, MPV of active colitis patients was significantly lower than that of the inactive phase of the disease. It is, however, difficult to correlate this finding with the functional alterations that could be responsible for the platelet-mediated thrombotic mechanisms summarized above. It may be hypothesized that in IBD the reduction in MPV may be associated with a peripheral platelet activation responsible for an exalted formation of platelet-platelet, platelet-PMN and platelet-endothelium adducts. This process would mainly involve younger platelets which have a bigger volume. Thus, the overall reduction of MPV could reflect the relative prevalence in circulating blood of less reactive platelets, which are older and smaller^[85]. In a very recent study, an enhanced expression of CD40/CD40L in intestinal epithelial cells was demonstrated^[86]. In particular, endoscopy biopsies taken from CD and UC patients showed a positive immunofluorescence staining for CD40 in intestinal epithelial cells of inflamed ileal or colonic mucosa, while no staining was observed in uninvolved intestinal segments^[86]. These findings provide, for the first time, direct evidence for the epithelial expression and modulation of CD40 in IBD-affected mucosa and indicate its involvement in the pro-inflammatory and platelet-activating function of inflamed intestinal cells.

Finally, another paper from our group showed that *in vitro* activated platelets directly increase CD40L expression by intestinal endothelial cells, leading them to interact with other immune cells and sustain intestinal chronic inflammation. This pathway has been proposed as a new mechanism of chronic inflammation, as a result of the complex interplay among different cell types in the intestinal mucosa^[87].

Endothelium

In a normal artery, endothelium creates a non-thrombogenic surface that acts as a selectively permeable barrier. Endothelium plays a key role in response to vascular injury, regulating leukocyte adhesion, platelet activation and adhesion and blood coagulation. Endothelium expresses and responds to multiple active substances, including cell adhesive molecules, cytokines and chemokines, to accomplish these functions^[88,89]. Injury to a vessel wall results in the triggering and propagation of inflammatory and coagulation events. The cell adhesion molecules (CAM) are expressed onto the surface of activated endothelial cells and attach leukocytes and platelets. Adhesive proteins provide for the binding and spreading of leukocytes, their rolling, and their further transmigration across endothelium. There are three major classes of CAM: selectins, the immunoglobulin superfamily CAM and integrins. Some integrins in turn can be receptors of CAM and the endothelial adhesion molecule, von Willebrand factor (VWF), which binds platelets.

Weibel-Palade bodies in endothelial cells and platelet α -granules contain and release platelet P-selectin (CD62P, GMP140) responsible for adhesion of leukocytes, their

rolling, and for stabilization of platelet aggregates^[90,91]. The lectin-containing N-terminal domain of P-selectin binds to PSGL-1 on monocytes, neutrophils and platelets^[91,92].

E-selectin (CD62E) is another molecule exposed on the endothelial cell surface that can bind to PSGL-1 in response to mechanical injury and inflammatory mediators as IL-1 β , TNF- α , bacterial toxins and oxidants^[89]. P- and E-selectin mediate rolling of activated and quiescent platelets on activated endothelium similar to the mechanism of leukocyte rolling^[93].

The immunoglobulin superfamily CAM includes ICAM-1 (CD54), ICAM-2 and VCAM-1, which are expressed by many cell types including endothelial and SMC. In response to vascular injury, these cells upregulate expression of ICAM-1 and VCAM-1^[89], engaged in leukocyte adhesion. Adhesion of platelets to injured endothelium is controlled by VWF, a multimeric protein, whose molecular weight ranges from 0.5 to 20 million Da^[94] and is stored and released from Weibel-Palade bodies in endothelial cells^[95]. Hence, VWF is considered a marker of endothelial injury. The VWF molecule contains domains responsible for binding blood coagulation factor VIII and platelet integrins such as glycoprotein transmembrane complexes GPIb/IX/V and integrin α IIb β 3 (GP IIb/IIIa), as well as collagen^[94]. VWF binds subendothelial collagens and after immobilization attaches to platelets *via* the membrane complex GPIb α -IX-V^[96]. VWF may be involved in the pathogenesis of acute thrombotic occlusion of stenosed arteries, where high shear stress promotes the formation of "stretched" VWF conformers, which are suitable for binding to platelets and subendothelial components^[88]. P-Selectin could serve as an anchor site for the ultra large VWF multimers on the surface of activated endothelium, to facilitate their cleavage at the Tyr1605-Met1606 peptide bond by the disintegrin and metalloproteinase with thrombospondin motif-13 (ADAMTS-13)^[97]. Microvascular dysfunction has been clearly demonstrated in IBD patients and involved several aspects of endothelium biochemical physiology^[98,99]. In particular, such dysfunction involves an alteration in nitrogen and reactive oxygen species balance, where the microvascular endothelium fails to generate \cdot NO, a potent vasodilator and anti-aggregating agent, forming instead elevated levels of superoxide anion^[54]. However, the mechanism responsible for the loss of endothelial nitrogen oxide in IBD gut microvessels also involves additional biochemical pathways. Previous studies showed an acquired deficient transcription of nitric oxide synthase 2 (NOS2) in chronically inflamed IBD endothelium^[100]. Furthermore, more recently, it was demonstrated that decreased production of nitrogen oxide in IBD endothelial cells can also arise from the induction by many inflammatory cytokines (IL2, TNF- α) of the enzyme arginase (isoform I and II)^[101]. This enzyme converts L-Arg into urea and L-ornithine, precursors for polyamines and L-proline compounds, which are vital to tissue homeostasis and wound repair^[102]. Arginase I and II compete with inducible NOS (iNOS, NOS2), the most relevant inducible pathway for the production of \cdot NO, for L-Arg, which is their common substrate in endothelial cells^[103]. Thus, an

increased arginase activity in IBD may contribute to inhibit the production of a potent antithrombotic agent such as nitric oxide. The increased production of reactive oxygen species in inflamed endothelium may also contribute to oxidative stress in VWF molecules, which become unresponsive to proteolysis by ADAMTS-13 and the accumulation of ultra large VWF multimers^[104]. The latter are the most haemostatically active forms of VWF and, favoring platelet adhesion and aggregation, may contribute to microvascular thrombosis in IBD.

To conclude, endothelium plays an essential role in inflammation due to its central “gatekeeper” function, which controls the quality and quantity of leukocytes that transmigrate from the vasculature into the interstitial space, regulates vascular tone and promotes platelet adhesion and aggregation. The latter function directly affects the haemostatic system and may clearly favor thrombotic phenomena. Several papers reviewed over the last few years suggest an activated status of endothelium in the course of IBD^[98,99].

A COHERENT SCENARIO FOR UNBALANCED HAEMOSTASIS IN IBD

At this point a question arises as to whether the haemostatic and inflammatory alterations briefly described in the above paragraphs could be functionally linked in a coherent framework.

Globally, the coagulation system in IBD patients seems to sustain pro-thrombotic mechanisms, involving both soluble factors and cells, such as platelets, endothelium and leukocytes. This conclusion is supported by results obtained from new laboratory assays. The conventional and global coagulation tests such as PT and APTT both have low sensitivity and specificity and do not contain sufficient amounts of TM or glycosaminoglycans. Thus, these assays do not automatically reflect the coagulation reactions and their inhibition as they occur *in vivo*^[105,106]. In contrast, the latest generation of methods that monitor the tissue factor induced thrombin generation in the presence of TM are credited as better laboratory tools to represent the balance of pro- and anti-coagulant forces operating in plasma^[105-108]. In a recent study from Saibeni *et al*^[105] endogenous thrombin potential, a parameter of the thrombin generation curve, was significantly higher in IBD patients than controls only when the test was performed in the presence of TM. This new assay strongly suggests that in IBD, as anticipated above, the increased generation of thrombin is mainly linked to a partial loss of function of natural anticoagulant pathways, and particularly of the TM-PC system.

Thus, systemic coagulation alterations in IBD may be recognized using more sophisticated techniques, which better reflect the *in vivo* setting. Furthermore, pathogenic considerations suggest that the coagulation imbalance in IBD could be particularly relevant in the vasculature of enteric mucosa, where inflammation shows the majority of destroying effects.

THE INFLAMMATION-COAGULATION INTERPLAY WITHIN THE INTESTINAL MILIEU

In addition to the demonstration that coagulation abnormalities and thromboembolic complications are clinically relevant events in IBD, they have been shown to exert effects at the mucosal level, where a coagulative imbalance exists^[3].

In fact, one of the earliest abnormalities in CD mucosa is the presence of platelet thrombi cross-linked with fibrin in the mucosal microvasculature^[109]. This feature, however, is not specific for CD and can be found in other idiopathic IBD^[110]. The involvement of the microcirculation in IBD pathogenesis is underlined by the analysis of a segment of the small and/or large bowel during active IBD which reveals vasodilatation, venocongestion, edema, infiltration of large inflammatory cells and ulcerations^[111]. This picture is the result of an unregulated intestinal inflammation with a consequent abnormal immune response and production of inflammatory cytokines, which, in turn, sustain the activation of the microvascular endothelium and subsequent recruitment of more leukocytes into the intestinal wall. This uncontrolled inflammatory response produces dramatic alterations in gut microvascular function which contributes greatly to perpetuating the inflammatory damage observed in IBD^[98,112].

Coagulation factors mainly interact with local endothelium, although this interaction is potentially conditioned by many features of the mucosal immunity.

The principal link between endothelium and the coagulation cascade is determined by Protease-activated receptors (PARs)^[113]. PARs, and in particular PAR-1 and PAR-2, are cellular receptors activated after proteolytic cleavage by enzymes^[114], mainly thrombin and activated factor X. Only a single study reports over-expression of PAR1 in patients with IBD^[115], and there are no data available on animal models addressing its role in IBD. The expression of PAR2, which is greatly increased in patients with UC and CD^[116-118], and the functional consequences of its activation in animal models are more widely documented.

Next to expression in the intestine, PAR expression in enteric neurons might be highly relevant for IBD because PARs can mediate gut inflammation *via* neurogenic mechanisms. Interestingly, PAR activation on submucosal and myenteric neurons causes severe edema in rat models. Moreover, the local activation of PAR2 but not PAR1 in the gut causes colitis through a neurogenic mechanism^[116]. Taken together, these results point towards PAR2 expression/cleavage as a cardinal factor in IBD.

The APC-TM system is the natural pathway by which the pro-inflammatory activity of PAR-1 and 2 signaling is contra-balanced. In the following section major findings on how thrombin, factor X and APC contribute to IBD, will be shown and briefly discussed.

Thrombin

Once thrombin is sequentially activated through the in-

trinsic and extrinsic pathway, it not only amplifies the coagulant process but it can also favor inflammation induced by other stimuli, either through ischemia (consequent upon thrombosis), indirectly through the generation of downstream mediators or directly *via* signals through protease-activated receptors (PAR)^[119].

Thrombin activates PARs, thereby establishing a link between activation of coagulation and pathophysiology of IBD. Indeed, thrombin signals through PAR1, PAR3 (in mouse) and PAR4, while tissue factor (TF)/FVIIa activates PAR2, and FXa activates PAR1 and PAR2^[116,120].

In addition to promoting platelet activation, thrombin exerts influence over monocytes, macrophages^[121] and neutrophils in processes related to tissue repair at the site of injury^[122,123]. Thrombin also interacts through an equilibrium high affinity binding with the N-terminus of GpIb α of platelets and endothelial cells^[124,125]. Notably, on platelet membrane, binding of thrombin to GpIb, accelerates cleavage of PAR1 by the enzyme^[126]. Thrombin also affects endothelial cells through various pathways including NF- κ B, early growth response factor-1 and GATA binding proteins^[127]. Thrombin signaling might result in post-transcriptional changes, including calcium influx, cytoskeletal reorganization, and release of soluble mediators, growth factors, and matrix metalloproteinases. In addition, thrombin signaling results in changes in downstream gene transcription, for example increasing the expression of genes involved in cell proliferation, inflammation, leukocyte adhesion, vasomotor tone, and hemostasis^[128-130].

Factor Xa

Borensztajn *et al*^[116] suggested that Factor Xa signaling through PAR2 contributes to the progression of IBD and fibro-proliferative responses. Because FXa is a well-known PAR2 agonist, FXa-induced PAR2 activation is gaining attention in intestinal pathology. Accordingly, in a variety of endothelial *in vitro* systems, FXa induces an array of pro-inflammatory responses and the deposition of connective tissue growth factor^[116,131,132]. It also leads to the activation of NF- κ B, and the release of IL-6, IL-8, and MCP-1 on endothelial cells as well as fibroblasts^[120,133]. Moreover, on endothelial cells, FXa induces the expression of E-selectin and both intracellular ICAM-1 and VCAM-1, resulting in leukocyte adhesion^[116,134,135]. In synergy with tumor necrosis factor, FXa induces TF expression *via* inhibition of its negative regulators I κ Ba and A20^[116,136]. Most of these responses are mediated *via* PAR2 activation, although some studies showed minor involvement of PAR1^[136,137].

Although the potential pro-inflammatory role of FXa on epithelial cells of the gastrointestinal tract is not fully investigated, studies on Hela cells showing that FXa induces activation of the pro-inflammatory transcription factor NF- κ B, suggest that it plays an important role^[116,138]. Finally, FXa also affects immune cells inducing the production of IL-2 by lymphocytes^[139]. Evidence that FXa may mediate inflammatory responses *in vivo* has come from several studies. In particular, Cirino *et al*^[140] demonstrated that FXa induces the formation of edema when

injected subcutaneously in a rat paw inflammation model, *via* local recruitment of mast cells.

The PC pathway

Traditionally described as a major anti-coagulant system, the protein C (PC) pathway, consisting of TM, the EPCR and activated PC (APC), is gaining increasing attention as an important regulator of microvascular inflammation, and in particular intestinal inflammation observed in IBD^[141]. The anticoagulant function of the PC pathway has been reviewed extensively^[142-144]. The main components of the PC system are the cell membrane receptor for PC, referred to as EPCR, the integral membrane glycoprotein TM, and two vitamin K-dependent plasma proteins, the zymogen PC, and the cofactor protein S. Upon cleavage of a dodecapeptide from the N-terminus of the light chain of PC by the thrombin-TM complex, the zymogen PC is activated to PC. Protein C *per se* is a poor substrate for thrombin. Allosteric binding of free thrombin to TM enhances, by several orders of magnitude, the thrombin-PC interaction and subsequent conversion of PC zymogen into its proteolytically active form, APC. The rate of PC activation by the TM-thrombin complex is further enhanced when the substrate PC zymogen is bound to its receptor, EPCR, which is able to reduce the K_m value of the catalytic interaction with thrombin. The extent of *in vivo* PC activation is therefore greatly linked to the bioavailability of PC, thrombin and, critically, by the density of TM and EPCR molecules expressed on endothelial cells. With the exception of disseminated intravascular coagulation, consumptive coagulopathy and defective biosynthesis, thrombin bioavailability in first approximation reflects the intensity of coagulation activation. Within such limits, it was shown that thrombin formation and generation of APC are strongly correlated^[145]. APC is formed mainly within the microcirculation, where endothelial cells express high levels of TM. As a consequence, due to the very small intravascular volume, the concentration of TM may be > 100 nmol/L, greatly exceeding the K_a value of the thrombin-TM interaction. Under these conditions, any amount of formed thrombin is rapidly and completely bound to TM. At variance with this situation, expression of TM is much lower in the endothelium of larger arteries and veins. Notably, EPCR expression is inversely related to that of TM, as it is more abundant in large vessel endothelium than in microcapillary beds. Thus, the efficiency and extent of APC formation differs considerably between different organs. The anticoagulant activity of the PC pathway includes the limited proteolysis by APC of the activated forms of coagulation factors V and VIII (FVa and FVIIIa), thereby limiting active thrombin generation. Protein S, in turn, cooperates with APC in inactivating FVa and FVIIIa, exerting an accelerating effect^[146]. The current model of APC suppression of excessive thrombin generation assumes that the EPCR-bound pool of endothelial cell-associated APC plays a more important role for FV inactivation than the circulating plasma pool of APC. In part, this may be explained by the fact that binding of APC to cell surfaces is

mediated only by EPCR, implying that the site of EPCR expression largely dictates the site of the anticoagulant function of APC. This model is fully consistent with the finding that FVa is highly susceptible to proteolytic degradation by APC when it is associated with the endothelial cell surface. FVa is instead refractory to APC cleavage in the platelet-associated prothrombinase complex^[147]. Other mechanisms can potentially limit the anticoagulant activity of APC on, or close to platelets, i.e. inhibition of APC by the vitronectin-plasminogen activator inhibitor-1 complex, secondary to local release of plasminogen activator inhibitor-1 from activated platelets, and the inhibition of protein S activity by platelet factor 4 released from platelet α -granules^[148-150]. Notably, platelet factor 4 can inhibit the anticoagulant function of APC alone, but not its ability to cleave and activate PAR-1. Thus, the interaction of platelet factor 4 may potentially redirect APC function toward anti-inflammatory and cytoprotective signaling pathways.

Overall, the relatively poor ability of APC to suppress thrombin generation in forming platelet aggregates might support effective and localized platelet-dependent hemostasis while sustaining the systemic anticoagulant potential. Thus, the APC activity in the presence of platelets may be considered another example of the compartmentalized haemostatic system.

Recently, it was demonstrated that surface-immobilized PC supports in a GPIIb α - and apolipoprotein E receptor 2-dependent manner the adhesion and aggregation of platelets under flow conditions^[151]. Thus, the ability of zymogen to engage these receptors raises the question as to whether PC immobilization occurs *in vivo* and whether changes in PC plasma levels are associated with altered platelet adhesion and aggregation.

Protein S circulates in blood in complex with a carrier protein, C4b-binding protein^[152]. The level of C4b-binding protein increases in clinical settings characterized by inflammation. Hence, the amount of bound protein S increases, causing a decrease of free protein S concentration^[152]. It is known that the anticoagulant function of protein S is exerted by its free form. Thus, systemic inflammatory conditions may represent a risk factor for protein-S dependent thrombotic disorders^[152]. Finally, protein S exerts both APC-dependent and APC-independent anticoagulant effects. The APC-dependent mechanism, involving the cofactor function of protein S for the acceleration of APC-mediated degradation of factors VIII and V, is likely the physiologically dominant pathway. The APC-independent anticoagulant activity of protein S is attained by stimulating the inhibition of tissue factor (TF) by tissue factor pathway inhibitor (TFPI)^[153]. The latter blocks the intermediate complex of TF-FVIIa-FXa, thereby preventing substrate exchange of already activated FXa for new FX. Protein S enhances the inhibitory interaction of TFPI with the TF initiation complex, and thereby limits the extent of thrombin generation in plasma. This APC-independent anticoagulant activity of protein S is most pronounced at low TF levels. Due to the anticoagulant effects described above, severe protein S deficiency is

associated with severe thrombotic disorders.

The relevance of the PC system for the prevention of atherothrombotic diseases is further corroborated by studies in animals. Some interesting aspects of *in vivo* PC activation were unraveled by analyzing the role of EPCR in the response of mice to an inflammatory challenge with lipopolysaccharide (LPS)^[154]. In these studies, mice lacking EPCR showed substantially enhanced activation of coagulation, concomitantly with reduced APC formation attributable to the absence of EPCR. Yet, the plasma APC levels in LPS-challenged EPCR-deficient mice were almost identical to that measured in wild-type animals. The authors of this study then showed that in wild-type mice a large fraction (approximately 40%) of APC did not enter the systemic circulation but remained bound to endothelial cell-associated EPCR at its site of activation. It is this sequestered APC pool that is completely missing in EPCR deficient mice, and its absence apparently accounts for all the pro-coagulant and pro-inflammatory effects of EPCR deficiency in mice^[154].

Recent studies have shown that the anti-inflammatory effect of APC, at least in part, is mediated through the EPCR-dependent proteolysis of PAR-1 in endothelial cells^[155,156]. This finding seems paradoxical, as it is known that the cleavage of PAR-1 by thrombin elicits potent prothrombotic and pro-inflammatory responses^[157]. It is well known that thrombin is mainly responsible for the activation of PC in the presence of TM and that the enzyme also cleaves PAR-1 with a high catalytic efficiency, which is 3-4 orders of magnitude higher than that of APC. This finding raises the question as to how APC in the presence of thrombin is able to produce physiologically significant cleavage of PAR1 associated with protective signaling events^[158], as is presented in Figure 1. Notably, both APC and PC bind to EPCR with a similar equilibrium constant, so that it can be hypothesized that thrombin can increase the local concentrations of EPCR-bound APC^[159]. This phenomenon may induce channeling of the protease directly into the signaling pathway.

In a very elegant study, Rezaie and coworkers demonstrated that the critical receptors required for both protein C activation (TM and EPCR) and APC cellular signaling (EPCR and PAR-1) pathways colocalize in the membrane lipid rafts of endothelial cells. The co-localization of EPCR and PAR-1 in lipid rafts of endothelial cells is a fundamental requirement for the cellular signaling activity of APC, which leads to anti-inflammatory and anti-apoptotic cellular effects, such as phosphorylation of mitogen-activated protein kinase^[160] and suppression of NF κ B expression^[161].

TM co-localization with these receptors on the same membrane microdomain can also recruit thrombin to activate the EPCR-bound protein C, therefore eliciting PAR-1 signaling events that are involved in the APC protective pathways^[159] linked to dissociation of caveolin subunits (Figure 1). These findings explain how thrombin effectively channels endogenous APC to the protective signaling pathways, through cleaving the same receptor of thrombin.

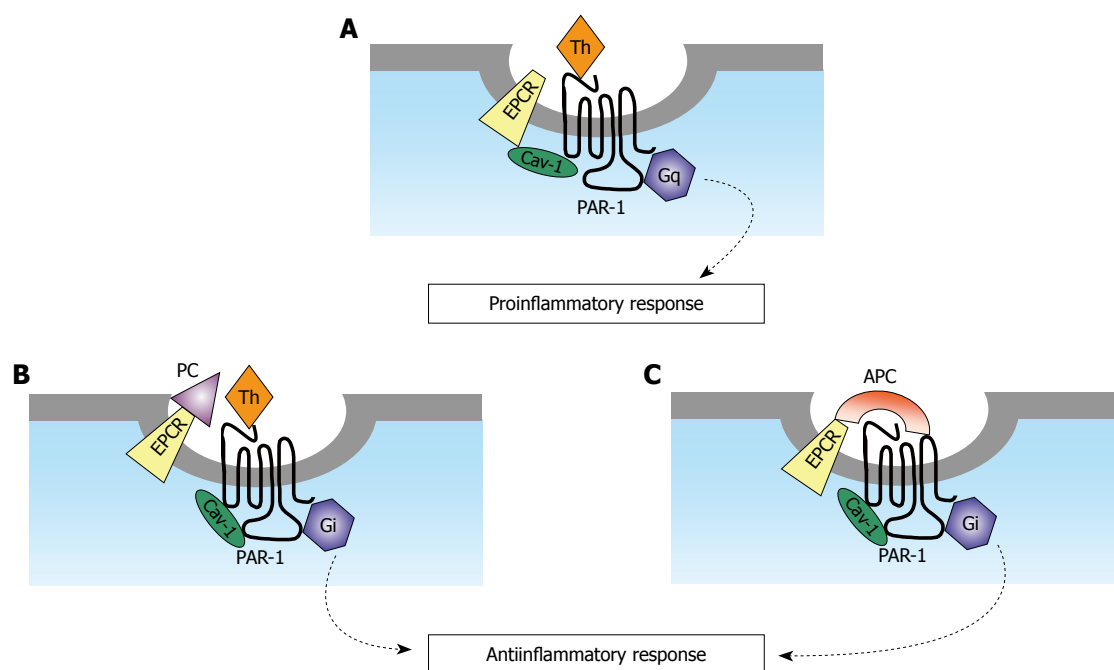


Figure 1 Models of protease-activated receptor-1 cleavage and activation by either activated protein C or thrombin when endothelial protein C receptor is occupied by its ligand protein C. A: The unoccupied endothelial protein C receptor (EPCR) is associated with caveolin-1 (Cav-1) within lipid rafts of endothelial cells. Upon thrombin cleavage of protease-activated receptor (PAR)-1, a pro-inflammatory signal is generated through G12/13 and Gq under these conditions; B: The occupancy of EPCR by protein C (PC) results in dissociation of EPCR from Cav-1. This process is linked with the coupling of PAR-1 to Gi. Thrombin cleavage of PAR-1 initiates an antiinflammatory response under these conditions; C: The same as (B) except that the EPCR and PAR-1 dependent protective signaling response is mediated by activated protein C (APC) (adapted from^[159]).

The APC pathway in IBD

TM and EPCR expression is diminished in the colonic mucosal microvasculature of IBD patients^[3,162-164], but is increased in their sera, suggesting increased shedding of TM and EPCR from cells. Inflammatory cytokines also down-regulate TM and EPCR by inhibiting transcription on cultured intestinal endothelial cells (HIMEC)^[162]. These changes in TM and EPCR expression would be expected to affect the conversion of protein C in its activated form, which, in addition to its anticoagulant properties, also has potent anti-inflammatory activity^[165], as described above.

Restoring the function of the PC pathway has anti-inflammatory effects on HIMEC, by decreasing pro-inflammatory cytokines secretion as well as adhesion molecules induced by TNF- α stimulation^[144,162,166]. Furthermore, restoration of APC by supplementation reduces stress-induced gastric mucosal injury in rats by inhibiting the decrease in gastric mucosal blood flow through attenuation of the activated neutrophil-induced endothelial cell injury *via* inhibition of TNF- α production^[167].

Overall, it can be concluded that a homeostatic balance exists between thrombin and APC in coagulation and inflammation. In particular, activated thrombin promotes the generation of APC and the two molecules influence the extent of both fibrin (clot) formation and the inflammatory response. This mechanism is mediated mainly by the cleavage of PAR-1 by either APC or thrombin on endothelial cells. Through binding to EPCR, APC would reverse the pro-inflammatory effects of thrombin on the same PAR^[127,168].

THERAPEUTIC PERSPECTIVES FOR COAGULATION ABNORMALITIES IN IBD

Based on the reported findings from several studies, one can conclude that the PC pathway is strategically located at the crossroads between coagulation and inflammation, where it exerts entirely unexpected roles in the damage that occurs in chronic inflammatory conditions^[169]. Unraveling the pathogenic role of the PC pathway offers a very promising tool in the therapeutic arsenal against IBD as well as many other chronic inflammatory diseases. Inflammation most likely mediates systemic hypercoagulability through various cytokines, which can affect the coagulation cascade at numerous points as well as platelet quantity and function. Unfortunately, perhaps due to this diversity of prothrombotic abnormalities that can exist in IBD patients and their likely multifactorial etiology, no specific therapy has ever been proposed in any clinical randomized trial to correct the cytokine-linked pro-inflammatory unbalance and pro-thrombotic phenomena occurring in IBD. Notably, unfractionated (UFH) and low-molecular-weight heparins (LMWHs), apart from their known anticoagulant/antithrombotic activities, display a broad spectrum of immune modulating and anti-inflammatory properties, such as modulation of cytokine production, T-lymphocyte cytotoxic activity^[170] and inhibition of leukocyte adhesion, activation and trafficking^[171]. Based on these features, these molecules have been proposed for the treatment of IBD. Some open studies suggested the efficacy of UFH^[172,173] and LMWHs^[174] for the treatment of active

UC. Conversely, large controlled studies using UFH and LMWHs did not show a clear efficacy^[175-178]. Moreover, a recent meta-analysis by Shen *et al.*^[179] indicated no significant additive benefit for heparins in the treatment of active UC. However, all studies included in the meta-analysis were very heterogeneous about their clinical, methodological and pharmacological features.

These studies, not only considered different definitions for response and remission, but also used different heparins, with theoretically very different anti-inflammatory activities^[179]. For these reasons, it is still difficult to set the real value of this therapeutic approach in IBD.

Recently, experimental data on animal models of IBD suggested efficacy of LMWHs, when selectively delivered in the site of disease, compared to the other route of administration. The multimatrix oral formulation MMX releasing parnaparin sodium at three different doses was evaluated in a clinical trial in patients with mild-to-moderate UC activity^[180]. This study, carried out on ten UC patients, showed no relevant side effects, including either interference with haemostatic parameters or increased bleeding. After treatment, seven patients were in clinical remission and only one achieved endoscopic healing. However, in a recent meta-analysis it was found that there is no evidence to support the use of UFH or LMWH for the treatment of active UC. In this study no further trials examining these drugs in patients with UC were warranted, except perhaps a trial of UFH in patients with mild disease^[181]. Furthermore, it has to be outlined that any benefit found using heparins in this clinical setting should be weighed against a possible increased risk of rectal bleeding, especially in patients with active UC.

In conclusion, a direct therapeutic approach for controlling inflammation-driven imbalances in the coagulation system in IBD patients is not yet available. The growing body of evidence concerning the molecular and cellular perturbations in this setting should be unraveled to promote a more efficacious, pathogenesis-oriented therapy for these disorders.

CONCLUSION

Overall, this paper was designed to underline the delicate and unstable equilibrium, at the mucosal level, between inflammation and coagulation. This complex equilibrium actively participates in the pathogenesis of several inflammatory disorders, in particular IBD.

Although the majority of available reports have looked at alterations in single coagulation components in IBD patients, no clear evidence of single alterations have been demonstrated to be crucial in IBD development. On the contrary, it is evident that several factors, with diverse relevance, are involved in maintaining chronic inflammation as well as a pro-coagulant profile in IBD. These factors include mainly classical coagulation components as well as inhibitors, and also cells, such as endothelium and platelets, which interact extensively at the mucosal level. Pathways that seem to play a major role in IBD pathogenesis are the APC, thrombin and Factor Xa pathways.

The delicate balance between these pathways, affecting different mucosal cell types, is responsible for controlling endothelium, leukocyte activation and trafficking, cytokines and chemokines secretion as well as the coagulation cascade. Despite a better understanding of the interaction between coagulation and inflammation, very few drugs targeting the coagulation pathways are available or under evaluation for clinical purposes. In conclusion, further studies are required to better characterize the relationship between coagulation and inflammation in different IBD patients and to identify good therapeutic targets.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP

Bacteriocinogeny in experimental pigs treated with indomethacin and *Escherichia coli* Nissle

Jan Bures, David Smajs, Jaroslav Kvetina, Miroslav Förstl, Jan Smarda, Darina Kohoutova, Martin Kunes, Jiri Cyrany, Ilja Tacheci, Stanislav Rejchrt, Jirina Lesna, Viktor Vorisek, Marcela Kopacova

Jan Bures, Darina Kohoutova, Jiri Cyrany, Ilja Tacheci, Stanislav Rejchrt, Marcela Kopacova, 2nd Department of Medicine, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, 500 05 Hradec Kralove, Czech Republic

David Smajs, Jan Smarda, Department of Biology, Masaryk University, Faculty of Medicine, 625 00 Brno, Czech Republic

Jaroslav Kvetina, Martin Kunes, Institute of Experimental Biopharmaceutics, Joint Research Centre of Czech Academy of Sciences and PRO.MED.CS Praha a.s., 500 02 Hradec Kralove, Czech Republic

Miroslav Förstl, Jirina Lesna, Institute of Clinical Microbiology, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, 500 05 Hradec Kralove, Czech Republic

Viktor Vorisek, Institute of Clinical Biochemistry and Diagnostics, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, 500 05 Hradec Kralove, Czech Republic

Author contributions: All authors contributed equally to this work.

Supported by Research project MZO 00179906 from the Ministry of Health of the Czech Republic, by institutional support from the Czech Republic (MSM0021622415) and by research grants GAČR 305/080535 and NS9665-4/2008 (Ministry of Health of the Czech Republic)

Correspondence to: Jan Bures, MD, PhD, Professor, 2nd Department of Medicine, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, 500 05 Hradec Kralove, Czech Republic. bures@lfhk.cuni.cz
 Telephone: +420-495-834240 Fax: +420-495-834785

Received: August 3, 2010 Revised: September 25, 2010

Accepted: October 2, 2010

Published online: February 7, 2011

Abstract

AIM: To evaluate bacteriocinogeny in short-term high-dose indomethacin administration with or without probiotic *Escherichia coli* Nissle 1917 (EcN) in experimental pigs.

METHODS: Twenty-four pigs entered the study: Group A (controls), Group B (probiotics alone), Group C (indomethacin alone) and Group D (probiotics and indomethacin). EcN (3.5×10^{10} bacteria/d for 14 d) and/or indomethacin (15 mg/kg per day for 10 d) were administered orally. Anal smears before and smears from the small and large intestine were taken from all animals. Bacteriocin production was determined with 6 different indicator strains; all strains were polymerase chain reaction tested for the presence of 29 individual bacteriocin-encoding determinants.

RESULTS: The general microbiota profile was rather uniform in all animals but there was a broad diversity in coliform bacteria (parallel genotypes A, B1, B2 and D found). In total, 637 bacterial strains were tested, mostly *Escherichia coli* (*E. coli*). There was a higher incidence of non-*E. coli* strains among samples taken from the jejunum and ileum compared to that of the colon and rectum indicating predominance of *E. coli* strains in the large intestine. Bacteriocinogeny was found in 24/77 (31%) before and in 155/560 (28%) isolated bacteria at the end of the study. Altogether, 13 individual bacteriocin types (out of 29 tested) were identified among investigated strains. Incidence of four *E. coli* genotypes was equally distributed in all groups of *E. coli* strains, with majority of genotype A (ranging from 81% to 88%). The following types of bacteriocins were most commonly revealed: colicins Ia/Ib (44%), microcin V (18%), colicin E1 (16%) and microcin H47 (6%). There was a difference in bacteriocinogeny between control group A (52/149, 35%) and groups with treatment at the end of the study: B: 31/122 (25%, $P = 0.120$); C: 43/155 (28%, $P = 0.222$); D: 29/134 (22%, $P = 0.020$). There was a significantly lower prevalence of colicin Ib, microcins H47 and V (probiotics group, $P < 0.001$), colicin E1 and microcin H47 (indomethacin group, $P < 0.001$) and microcins H47 and V (probiotics and indomethacin group, $P = 0.025$) compared to controls. *Escherichia fergusonii* (*E. fergusonii*) was identi-

fied in 6 animals (6/11 isolates from the rectum). One strain was non-colicinogenic, while all other strains of *E. fergusonii* solely produced colicin E1. All animals started and remained methanogenic despite the fact that EcN is a substantial hydrogen producer. There was an increase in breath methane (after the treatment) in 5/6 pigs from the indomethacin group (C).

CONCLUSION: EcN did not exert long-term liveability in the porcine intestine. All experimental pigs remained methanogenic. Indomethacin and EcN administered together might produce the worst impact on bacteriocinogeny.

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Key words: Bacteriocinogeny; *Escherichia coli* Nissle 1917; Experimental pigs; Indomethacin

Peer reviewer: Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

Bures J, Smajs D, Kvetina J, Förstl M, Smarda J, Kohoutova D, Kunes M, Cyrany J, Tacheci I, Rejchrt S, Lesna J, Vorisek V, Kopacova M. Bacteriocinogeny in experimental pigs treated with indomethacin and *Escherichia coli* Nissle. *World J Gastroenterol* 2011; 17(5): 609-617 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i5/609.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.609>

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) represent the group of most commonly used drugs worldwide. NSAIDs may cause severe injury to all parts of the gastrointestinal tract. The pathogenesis of NSAID-induced entero- and colopathy is more multifactorial and complex than formerly assumed but is not yet fully understood. A combination of local and systemic effects plays an important role in pathogenesis. NSAID-induced entero- and colopathy is a stepwise process involving direct mucosal toxicity, mitochondrial damage, breakdown of intercellular integrity, enterohepatic recirculation and neutrophil activation by luminal contents including bacteria. Unlike upper gastrointestinal toxicity, cyclo-oxygenase-mediated mechanisms are probably less important^[1-3]. Intestinal bacteria play a significant role in the pathogenesis of NSAID-induced entero- and colopathy. In experimental studies, NSAIDs cannot induce enteropathy in germ-free rats^[4].

Probiotic bacteria are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host^[5]. Probiotics likely function through enhancement of the barrier function of the gut, immunomodulation, and competitive adherence to the mucus and epithelium^[6]. Probiotic bacteria may exert a systemic anti-inflammatory effect^[7] and modulate apoptosis^[8]. Probiotics have been suggested for amelioration or prevention of

various diseases including antibiotic-associated diarrhoea, irritable bowel syndrome and inflammatory bowel disease. Further possible beneficial effects are being studied (including anti-cancer potential, lowering of serum cholesterol levels and blood pressure reduction, *etc.*)^[9-11]. It has been hypothesised that probiotic bacteria might reduce the adverse effects of NSAIDs on the small and large intestine. However, initial studies provided controversial results, both with ameliorating and deteriorating outcomes^[12-15]. NSAID-induced small intestinal injury is Toll-like receptor 4 dependent^[14]. Probiotic *Escherichia coli* Nissle 1917 (EcN) might ameliorate experimental colitis (induced by dextran sodium sulphate) *via* Toll-like receptor 2 and 4 pathways^[16,17].

Colicins and microcins, members of the bacteriocin family, are produced by bacteriocinogenic strains of *Escherichia coli* (*E. coli*) and some related species of *Enterobacteriaceae*. They are toxic to susceptible bacterial strains of the same family^[18-20]. However, some bacteriocins also exert an inhibitory effect on eukaryotic cells, including observed antineoplastic action *in vitro* and *in vivo*^[21-25]. Bacteriocins might induce apoptosis^[26] as some regulators of apoptosis (e.g. Bcl family with pro- and anti-apoptotic members) share similar structures with pore-forming colicins^[27]. The possible role of bacteriocins was also investigated in clinical studies on bacillary dysentery^[28], inflammatory bowel disease^[29] and colorectal cancer^[30]. Bacteriocins might have a dual role: they may act as both antibiotics and probiotics^[31]. One of the most commonly used probiotic bacterial strains, EcN, is a producer of microcins H47 and M^[32-34].

The aim of this study was to evaluate bacteriocinogeny in short-term high-dose indomethacin administration with or without probiotic bacteria EcN in an experimental porcine model. A small adult pig can be used in experiments as a representative of an omnivore due to its relatively similar gastrointestinal functions in comparison with man^[35-38].

MATERIALS AND METHODS

Ethics

The Project was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Record Number 1492006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes^[39].

Animals

Twenty-four healthy mature (4-5 mo old) female pigs (*Sus scrofa* f. *domestica*, hybrids of Czech White and Landrace breeds) weighing 33.0 ± 1.7 kg, were included in our study. The animals were divided into four groups: Group A (controls, 6 animals), Group B (probiotics alone, $n = 6$), Group C (indomethacin alone, $n = 6$) and Group D (probiotics and indomethacin, $n = 6$). All animals were fed twice a day (standard assorted food A1 of equal amounts).

Drug and probiotic bacteria administration

EcN (3.5×10^{10} live bacteria/d for 14 d) and/or indomethacin (Indomethacin suppositories, Berlin-Chemie, Germany; 15 mg/kg per day for 10 d) were administered as one-shot dietary bolus to hungry pigs.

Autopsy

Twenty-four hours after the last drug and/or probiotic bacteria administration (groups B to D) or after 14 d of stabling (Group A), the pigs were sacrificed (after 24 h of fasting) by means of pharmacological euthanasia (iv administration of embutramide, mebezonium iodide and tetracaine hydrochloride - T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 mL per kg) and exsanguinated. Immediate autopsy was performed and smears for bacterial cultures were taken.

Bacterial culture, isolation and identification

Before the experiment anal smears were taken from all animals. At autopsy, smears from mucosa of the jejunum, ileum, caecum, transverse colon and rectum were taken from each animal and immediately inserted into a transport liver-enriched broth. Standard primary cultures were inoculated on blood and MacConkey agars (24 h at 37°C), followed by standard clone isolation. Up to 9 different colonies of coliform bacteria were isolated from each sample (on blood, MacConkey and deoxycholate agars). Particular bacteria were precisely identified by the Vitek2 system (BioMérieux, Marcy l'Etoile, France). All bacterial strains were frozen in cryotube vials at minus 90°C until bacteriocin genotyping.

Analysis of bacteriocin production

The bacteriocin production of all strains was tested in parallel on 4 different agar plates containing (1) TY medium; (2) nutrient broth; (3) TY medium supplemented with mitomycin C; and (4) TY medium supplemented with trypsin. The TY medium consisted of yeast extract (Hi-Media, Mumbai, India) 5 g/L, tryptone (Hi-Media) 8 g/L, sodium chloride 5 g/L; the TY agar consisted of a base layer (1.5%, w/v, solid agar) and a top layer (0.7%, w/v, soft agar). A Difco™ nutrient broth (Difco Laboratories, Sparks, MD) 8 g/L, NaCl 5 g/L, was used for production of relatively unenriched 1.5% (w/v) agar plates. Mitomycin C (0.01%, w/v) and trypsin (0.1%, w/v) were used for induction of colicin production and for protease sensitivity tests, respectively. The previously described set of *E. coli* indicator strains including *E. coli* K12-Row, C6 (φ), B1, P400, and S40 was used to identify the producer strains together with *Shigella sonnei* 17 indicator^[40,41]. To test bacteriocin production, the agar plates were inoculated by needle stab and the plates were incubated at 37°C for 48 h. The tested macrocolonies were then killed with chloroform vapours and each plate was then overlaid with a thin layer of soft agar containing 10^7 cells/mL of an indicator strain and the plates were incubated at 37°C overnight. All investigated *E. coli* strains were tested on four parallel plates against 6 indicator strains stated.

Table 1 DNA primers used for polymerase chain reaction detection of colicin encoding genes

Bacteriocin type	Primer name	5'-sequence-3'	Length of PCR product (nt)
A	ColA-F	CGTGGGAAAAGTCATCATC	475
	ColA-R	GCTTIGCTCTTCCTGATGC	
B	colicinB-F	AAGAAAATGACGAGAAGACG	493
	colicinB-R	GAAAGACCAAAGGCTATAAGG	
D	ColD-F	CTGGACTGCTGCTGGTGATA	420
	ColD-R	GAAGGTGCGCCTACTACTGC	
E1	colicinE1-F	TGTGGCATCGGGCGAGAATA	650
	colicinE1-R	CTGCTTCGTGAAAAGCCTTTT	
E2	ColE2-F	TGATGCTGCTGCAAAAGAG	409
	ColE2-R	TTCAAAGCGTTCCTACCAC	
E3	ColE3-F	TAAGCAGGCTGCATTGTGATG	413
	ColE3-R	TCGGATCTGGACCTTTCAAC	
E4	ColE4-F	GAAGGCTGCATTGTGATGCT	409
	ColE4-R	CGGATCCGGACCTTTAATTT	
E5	ColE3-F	TAAGCAGGCTGCATTGTGATG	430
	ColE5-R	TTGAATTCTCGAATCGTCCA	
E6	ColE6-F	ACCGAACGTCCAGGTGTT	399
	ColE6-R	TTTAGCCTGTCGCTCCTGAT	
E7	ColE7-F	GCATCTCTGCCATCTGAAAT	431
	ColE7-R	CTTCTGCCCACCTTTCTTTTCG	
E8	ColE3-F	TAAGCAGGCTGCATTGTGATG	449
	ColE8-R	GACTGATTTGGCTTGTCGTGA	
E9	ColE3-F	TAAGCAGGCTGCATTGTGATG	418
	ColE9-R	GACTTTTCTCCCTCCGACCT	
Ia	ColIa-F	GCATGCAAATGACGCTCTTA	473
	ColIa-R	GAGGACGCCAGTCTCTGTC	
Ib	ColIb-F	AACGAGTGGGTCGATGATT	464
	ColIb-R	CCTTTTCTGCGCTCGTATTC	
Js	ColJs-F	TCAAAATGTTTGGGCTCCTC	254
	ColJs-R	TAATCTGCCCTGTCCCACTG	
K	ColK-F	CAGAGGTGCGTGAACATGAA	469
	ColK-R	TCCGCTAAATCCTGAGCAAT	
M	ColM-F	GCTTACCACTTCGCAAAACC	429
	ColM-R	GAGCGACTCTCCGATAATGC	
N	ColN-F	AGCTTGGCGAGTATCTTGGA	401
	ColN-R	CAACACAGCCCCGAATAAAC	
S4	ColS4-F	TATATGGCCCCAAGTCTGGT	456
	ColS4-R	CGTAAGGACGGACACCTGTT	
U	ColU-F	TGATTGCTGCGAGAAAAATG	485
	ColU-R	TCTGACAGCCTCTCCCTGTT	
Y	ColY-F	GCAGGCAGAAAAGAACAAGG	477
	ColY-R	CGGACGTTATTTCCTTCAT	
5	Col5-F	CATTGGCAAAAGCGAAATCT	443
	Col5-R	TGCAACTCTGGAAACAATCG	
10	Col10-F	GGTTACCGGATTTCTGGAT	448
	Col10-R	TTCTAGATGCTTGGCCCACT	

PCR: Polymerase chain reaction.

Identification of individual colicin types

All investigated strains were tested with colony polymerase chain reaction (PCR). A bacterial colony was resuspended in 100 µL of sterile water and 1 µL of this suspension was added to the PCR reaction. Individual colicin types (colicins A, D, E2-E9, Ia, Ib, Js, K, M, N, S4, U, Y, 5 and 10) were detected using PCR with primers designed using the Primer3 program^[42]. The list of primer pairs and the corresponding length of PCR products are listed in Table 1. Control bacterial producers stemmed from our stock and comprised *E. coli* BZB2101pColA - CA31, BZB2102 pColB - K260, BZB2103 pColD -

Table 2 Bacteriocinogeny of particular strains isolated at the end of experiment

Parameter	Small intestine			Colon and rectum		
	Bacteriocinogeny	Types of bacteriocin producers (No. of strains)	No. of unique bacteriocin producers	Bacteriocinogeny	Types of bacteriocin producers (No. of strains)	No. of unique bacteriocin producers
Group A	22/55 (40%)	E1 (1); E1, Ia, V (1); E1, V (2); Ia (2); Ia, B, K, M, H47 (2); Ia, H47, V (2); Ia, V (6); Ib (3); J25 (1); S4, U (1); S4, V (1)	11	30/94 (32%)	B (1); B, H47, Ib, K, M (2); B, M (1); C7, E1, Ib, V (1); E1 (1); E1, Ia (1); E1, Ia, V (1); E1, V (2); E7 (1); H47, Ia, V (1); H47, S4 (1); H47, V (2); Ia (6); Ia, V (4); Ib (3); M (1); S4, V (1)	17
Group B	11/43 (26%)	E1 (7); Ia (2); Ia, V, H47 (1); Ib (1)	4	20/79 (25%)	B, H47, K, M, Ia (1); B, M (1); E1 (4); E1, V (1); Ia (11); Ia, V (1); Ib (1)	7
Group C	17/58 (29%)	B, M, V (1); Ia (1); Ia, V (7); Ib (6); S4 (2)	5	26/97 (27%)	B, Ia, (1); E1, Ib (1); J25, Ia (1); Ia (5); Ia, E7, V (1); Ia, H47 (1); Ia, M (1); Ia, V (8); Ib (7)	9
Group D	9/45 (20%)	E1 (3); E1, Ia (1); E1, Ia, V (1); E1, Ib (1); Ia (1); Ia, V (1); Ib (1)	7	20/89 (22%)	E1 (4); E1, Ia (1); E1, Ia, V (1); E1, Ib (2); H47, V (1); Ia (7); Ia, V (1); Ib (2); Ib, V (1)	9

Small intestine: Bacterial strains isolated from mucosa of the jejunum and ileum; Colon and rectum: Bacterial strains isolated from mucosa of the caecum, transverse colon and rectum; Group A: Control animals with no treatment; Group B: Probiotics alone (see text for details); Group C: Indomethacin alone (see text for details); Group D: Probiotics and indomethacin (see text for details); Bacteriocinogeny: Number of bacteriocinogenic strains out of all tested; Types: Particular bacteriocin types found in single isolates; M: Colicin M, not for microcin M.

CA23, BZB2107 pColE4 - CT9, BZB2108 pColE5 - 099, BZB2150 pColE6 - CT14, BZB2120 pColE7 - K317, BZB2279 pColIa - CA53, BZB2202 ColIb - P9, BZB2116 pColK - K235, PAP1 pCol101M - BZBNC22, BZB2123 pColN - 284, *E. coli* 189BM pColE2 - P9, *E. coli* 385/80 pColE1, pColV, *E. coli* 185M4 pColE3 - CA38, *E. coli* W3110 pColE8, W3110 pColE9, *E. coli* K-12 pColS4, *Shigella boydii* M592 (serovar 8) pColU, *E. coli* K339 pColY, *Sh. sonnei* pColJs, *E. coli* pCol5, *E. coli* pCol10, *E. coli* 449/82 pColX (microcin B17), *E. coli* 313/66 pColG (microcin H47), *E. coli* 363/79 pColV (microcin V), *E. coli* TOP10F⁺ pDS601 (microcin C7), *E. coli* D55/1 (microcin J25), and *E. coli* B1239 (microcin L). Sequentially related colicin genes (colicins E2-E9, Ia-Ib, U-Y, and 5-10, respectively) often yielded PCR products with primer pairs of related colicin types and therefore all these PCR products were sequenced. The PCR detection primers for colicins B and E1 and for 6 microcin types including B17, C7, H47, J25, L, and V, were taken from Gordon *et al.*^[43]. The phylogenetic group of each *E. coli* strain was determined using the triplex PCR protocol according to Clermont *et al.*^[44]. Sequence analysis was performed using Lasergene software (DNASTAR, Inc., Madison, WI, USA).

Hydrogen and methane breath testing

Hydrogen and methane breath tests were performed before and the morning following completion of the treatment, carried out under general anaesthesia in spontaneously breathing animals. Alveolar air was aspirated by means of percutaneous puncture of the trachea. Immediate measurement of hydrogen and methane was accomplished in triplicate by means of gas chromatography (Microlyzer DP Plus Quintron, Milwaukee, WI, USA). Results were expressed as parts per million (ppm).

Statistical analysis

Data were statistically analysed with χ^2 with Yates cor-

rection and by Mann-Whitney rank sum test. Statistical software was used for this analysis (SigmaStat version 3.1, Jandel Co., Erkrath, Germany).

RESULTS

The general microbiota profile was rather uniform in all animals but there was a broad diversity in coliform bacteria (parallel genotypes A, B1, B2 and D found). In total, 637 bacterial strains were tested, mostly *E. coli*. The remaining isolates comprised *Salmonella enterica* ssp *Arizonae* (21 isolates), *Pasteurella aerogenes* (20), *Escherichia fergusonii* (*E. fergusonii*) (11), *Aeromonas hydrophila/caviae* (9), *Klebsiella pneumoniae* (8), *Enterobacter cloacae* (4), *Morganella morganii* (4), *Citrobacter braakii* (2), *Citrobacter youngae* (2), *Citrobacter freundii* (1), *Acinetobacter hvoffii* (1) and *Pseudomonas aeruginosa* (1). There was a higher incidence of non-*E. coli* strains among samples taken from the jejunum and ileum compared to that of the colon and rectum indicating predominance of *E. coli* strains in the large intestine (data not shown).

Bacteriocinogeny was found in 24/77 (31%) before and in 155/560 (28%) isolated bacteria at the end of the study. Altogether, 13 individual bacteriocin types (out of 29 tested) were identified among investigated strains. Incidence of four *E. coli* genotypes was equally distributed in all groups of *E. coli* strains, with majority of genotype A (ranging from 81% to 88%). The following types of bacteriocins were most commonly revealed: colicins Ia/Ib (44%), microcin V (18%), colicin E1 (16%) and microcin H47 (6%). There was a difference in bacteriocinogeny between control group A (52/149, 35%) and groups with treatment at the end of the study: B: 31/122 (25%, $P = 0.120$); C: 43/155 (28%, $P = 0.222$); D: 29/134 (22%, $P = 0.020$). See Table 2 for details. There was a significantly lower prevalence of colicin Ib, microcins H47 and V (probiotics group, $P < 0.001$), colicin E1 and microcin H47 (indomethacin group, $P < 0.001$) and microcins H47 and V (probiotics and indomethacin group, $P = 0.025$) com-

Table 3 Analysis of porcine alveolar breath for hydrogen and methane (in ppm - parts per million) before and after the treatment

Group	Hydrogen before	Hydrogen after	Statistical significance	Methane before	Methane after	Statistical significance
A	N/A	3.50 ± 2.81	N/A	N/A	69.33 ± 56.64	N/A
B	6.0 ± 2.82	2.0 ± 0	NS	106.50 ± 94.05	80.00 ± 48.02	NS
C	1.17 ± 0.41	5.0 ± 3.29	NS	34.67 ± 25.65	66.17 ± 38.83	NS
D	2.0 ± 1.16	6.0 ± 6.0	NS	60.75 ± 34.77	62.00 ± 27.71	NS

Group A (controls with no treatment, $n = 6$), Group B (probiotics alone, $n = 6$), Group C (indomethacin alone, $n = 6$) and Group D (probiotics plus indomethacin, $n = 6$). N/A: Not applicable; NS: Not significant.

pared to controls (Table 2). *E. fergusonii* was identified in 6 animals (6/11 isolates from the rectum). One strain was non-colicinogenic, while all other strains of *E. fergusonii* solely produced colicin E1.

Data on porcine alveolar breath analysis of hydrogen and methane are given in Table 3. All animals started and remained methanogenic. Differences between groups were not statistically significant. There was an increase in breath methane (after the treatment) in 5/6 pigs from the indomethacin group (C).

DISCUSSION

Probiotic bacteria might act in three different ways: they are able to modulate the host's defence mechanisms, they have a direct impact on other micro-organisms and finally probiotic effects may be based on actions affecting microbial products like toxins, host products (e.g. bile salts) and food ingredients^[45].

Our hypothesis for this study was that (1) indomethacin would suppress bacteriocin production of *Enterobacteriaceae*; (2) probiotic bacteria EcN would colonise the porcine gastrointestinal tract permanently; (3) they would protect intestinal microbiota from suppressive action of indomethacin; and (4) EcN would convert the starting methanogenic phenotype of pigs to a hydrogenic one. Surprisingly, most of our presumptions were not proved.

There is no simple way to explain this. The first question that should be addressed is a possible role of human probiotic bacteria in the porcine gastrointestinal tract. It is necessary to consider whether human probiotics can be also assumed to act as probiotic microbiota for domestic pigs. Criteria for probiotics of human origin were proposed^[46], however, potential probiotic bacteria isolated from porcine faeces are usually tested *in vitro* to be active against two or three common porcine pathogens only^[47-50].

Genotype B2 and production of microcin H47 were considered as markers of EcN in our study. None of our 637 isolates comprised these bacteria. Viability and sufficient amount of bacteria were ensured before their administration in our project. According to our results, it is unlikely that EcN could exert long-term viability in the porcine intestinal tract. Other swine studies by several authors^[51-54] were able to identify intestinal colonisation by EcN in pigs and piglets but not by all of them^[55]. There is no final proof of long-term colonisation of the gastroin-

testinal tract by EcN in healthy humans. In an interesting study by Schierack *et al.*^[56], probiotic *Enterococcus faecium* supplementation showed no significant effect on the numbers and diversity of *Enterobacteriaceae* species, or on the total counts, diversity and distribution of virulence gene-positive *E. coli* strains in healthy domestic pigs.

Aspirin and some NSAIDs, including indomethacin, influence intestinal bacteria^[57-60]. Indomethacin might exert some impact on intestinal microbiota in our study, as there was an increase in breath methane after the treatment in 5/6 pigs from the indomethacin group. Another interesting result from our current study showed a marked lower prevalence of colicin Ib, microcins H47 and V (probiotics group), colicin E1 and microcin H47 (indomethacin group) and microcins H47 and V (EcN and indomethacin group) compared to controls. We interpret this difference as a sign of adverse effects of probiotics and/or indomethacin on porcine microbiota. Bacteriocinogeny in controls (35%) was higher compared to the indomethacin (28%), probiotic (25%) and indomethacin and probiotic groups (22%). This evident trend did not reach statistical significance for the probiotic group (B) and indomethacin group (C). However, there was a statistically significant difference between controls and indomethacin and probiotic group (D). We can assume that indomethacin and EcN comprise the worst impact on bacteriocinogeny in the porcine gastrointestinal tract. This would be consistent with other studies showing that other probiotics might deteriorate NSAID-induced injury to the intestine^[13].

Composition of food, especially supplements with probiotics, might influence the probiotic effect of intestinal bacteria^[61,62]. This factor is unlikely to play an important role in our study. All animals received identical assorted food with cereals, animal fat, soya oil and a mix of supplements (lysine, threonine, methionine, lactic acid).

In our current study, the microbiota profile was rather uniform in all animals due to identical breed and feed. However, there was a broad diversity in coliform bacteria; the main four genotypes A, B1, B2 and D were identified in parallel. Similar diversity was also found in other porcine studies, with prevailing group A^[56,63]. Dixit *et al.*^[63] showed that differences among individual pigs accounted for 6% of the observed genetic diversity, whilst 27% of the genetic variation could be explained by clonal composition differences among gut regions (isolates obtained from the duodenum, ileum, colon and faeces of 8 pigs). Finally, the absence of virulence genes in these com-

mensals indicates that they may be suitable as a probiotic consortium, particularly if they also display increased adherence to enterocytes and antagonistic activity against pathogenic strains of *E. coli*^[63].

E. fergusonii was identified as a new species of *Enterobacteriaceae* in 1985^[64]. This is considered to be an opportunistic pathogen of farm animals including domestic pigs^[65]. We identified *E. fergusonii* in 6 animals, all pigs were healthy without any sign of infective disease. Interestingly 10/11 isolated bacteria solely produced colicin E1. Colicins produced by *E. fergusonii* strains closely resemble colicins encoded by *E. coli*^[66]. In a previous series of human isolates, only 6/50 (12%) strains were bacteriocinogenic, 3 of which produced colicin E1^[67].

In humans, all intestinal hydrogen and methane are produced by so called “hydrogenic and methanogenic” bacteria^[68-70]. However, most authors do not usually specify which particular bacteria constitute these producers. Hydrogen is produced by bacterial fermentation of saccharides in the intestinal lumen. Concurrently, hydrogen is consumed by other intestinal bacteria to synthesise methane, acetate and hydrogen sulphide. Methane is synthesised solely by bacteria in the intestine (four mols of hydrogen and one mol of carbon dioxide create one mol of methane and water). This reaction reduces the volume of gas that would otherwise be present in the colon^[71-76]. The question of intestinal methane producers has not been definitely solved yet. We hypothesised that common coliform bacteria could also synthesise methane^[77], however, this assumption was not proved by our further studies^[78,79]. McKay *et al.*^[80] found that several anaerobes (*Bacteroides*, *Clostridium* and others) produced hydrogen but rarely methane. Hydrogen is also produced by *Enterobacteriaceae*^[81]. In adult Caucasians, only 30%-50% of people produce methane while hydrogen is produced by 90%-98% of people^[69]. Kien *et al.*^[82] found low breath hydrogen and higher methane in piglets (even in a subgroup supplemented with lactulose). In our current study, all animals revealed a solely methanogenic phenotype (by the analysis of their alveolar breath). This fact could be explained as they came from an identical breed and received the same assorted food. All animals remained methanogenic despite the fact that EcN is a substantial hydrogen producer^[77]. This further supports our finding that EcN 1917 did not have major impact on porcine intestinal microbiota.

In conclusion, it is unlikely that probiotic EcN could exert long-term liveability in the porcine intestine. All experimental pigs remained methanogenic, despite the fact that EcN is a substantial hydrogen producer. The indomethacin and probiotic group had a significantly lower rate of bacteriocinogeny compared to controls with no treatment. These control pigs revealed higher bacteriocinogeny with simultaneous production of up to five different bacteriocins per single strain. Indomethacin and probiotics administered together might provide the worst impact on bacteriocinogeny in the porcine gastrointestinal tract.

COMMENTS

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) represent the group of most commonly used drugs worldwide. NSAIDs may cause severe injury to all parts of the gastrointestinal tract. The pathogenesis of NSAID-induced entero- and colopathy is more multifactorial and complex than formerly assumed but has still not been fully understood. A combination of local and systemic effects plays an important role in pathogenesis. NSAID-induced entero- and colopathy is a stepwise process involving direct mucosal toxicity, mitochondrial damage, breakdown of intercellular integrity, enterohepatic recirculation and neutrophil activation by luminal contents including bacteria. Unlike upper gastrointestinal toxicity, cyclo-oxygenase-mediated mechanisms are probably less important. Intestinal bacteria play a significant role in the pathogenesis of NSAID-induced entero- and colopathy. In experimental studies, NSAIDs cannot induce enteropathy in germ-free rats.

Research frontiers

Probiotic bacteria are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. Probiotics likely function through enhancement of the barrier function of the gut, immunomodulation, and competitive adherence to the mucus and epithelium. Probiotic bacteria might exert a systemic anti-inflammatory effect and modulate apoptosis. Probiotics have been suggested for amelioration or prevention of various diseases including antibiotic-associated diarrhoea, irritable bowel syndrome and inflammatory bowel disease. Further possible beneficial effects are being studied (including anti-cancer potential, lowering serum cholesterol levels and blood pressure reduction, *etc.*). It has been hypothesised that probiotic bacteria might reduce the adverse effects of NSAIDs on the small and large intestine. However, initial studies provided controversial results, both with ameliorating and deteriorating outcomes.

Innovations and breakthroughs

Based on the current study, it is unlikely that probiotic *Escherichia coli* Nissle 1917 (EcN) could exert long-term liveability in the porcine intestine. Genotype B2 and production of microcin H47 were considered as markers of EcN in the study. The authors did not find such bacteria among any of the 637 isolates. All experimental pigs remained methanogenic, despite the fact that EcN is a substantial hydrogen producer. The indomethacin and probiotic group had a significantly lower rate of bacteriocinogeny compared to controls with no treatment. These control pigs revealed higher bacteriocinogeny with simultaneous production of up to five different bacteriocins per single strain. Indomethacin and probiotics administered together might produce the worst impact on bacteriocinogeny in the porcine gastrointestinal tract.

Applications

Bacteriocins might induce apoptosis as some regulators of apoptosis (e.g. Bcl family with pro- and anti-apoptotic members) share similar structures with pore-forming colicins. Bacteriocins might have a dual role: they may act as both antibiotics and probiotics. One of the most commonly used probiotic bacterial strains, EcN, is a producer of microcins H47 and M.

Terminology

Colicins and microcins, members of the bacteriocin family, are produced by bacteriocinogenic strains of *Escherichia coli* and some related species of *Enterobacteriaceae*. They are toxic to susceptible bacterial strains of the same family. However, some bacteriocins also exert an inhibitory effect on eukaryotic cells, including observed antineoplastic action *in vitro* and *in vivo*.

Peer review

This is an innovative manuscript in basic research, which adequately addresses the ethics of the experiment. Its presentation is accurate but very complex in the reading and interpretation of many variables.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zheng XM

Analysis of the urinary peptidome associated with *Helicobacter pylori* infection

Di Xiao, Fan-Liang Meng, Li-Hua He, Yi-Xin Gu, Jian-Zhong Zhang

Di Xiao, Fan-Liang Meng, Li-Hua He, Yi-Xin Gu, Jian-Zhong Zhang, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

Author contributions: Zhang JZ and Xiao D designed the study, acquired the data and drafted the manuscript; Meng FL, He LH and Gu YX recruited volunteers, collected and analyzed urine samples, and performed 13C-urea breath test; all authors read and approved the final manuscript.

Supported by The National Science and Technology Pillar Program of the Ministry of Science and Technology of the People's Republic of China during the Eleventh Five-Year plan period, No. 2007BAID4B02

Correspondence to: Jian-Zhong Zhang, Professor, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping District, Beijing 102206,

China. zhangjianzhong@icdc.cn

Telephone: +86-10-58900754 Fax: +86-10-58900707

Received: August 14, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: February 7, 2011

DI-TOF MS. By optimizing the parameters of the model, the Genetic Algorithm model had good recognition capability (97%) and positive predictive value (94%). Based on the model, 2 markers with molecular masses of 6788 and 1912 Da were found that differentiated between *H. pylori* positive and negative volunteers. The m/z 1912 sequence was parsed as SKQFTSSTSYN-RGDSTF. The peptide was identified as isoform 1 of the fibrinogen α chain precursor, whose concentration in urine was markedly higher in *H. pylori* infected volunteers than in *H. pylori* non-infected ones.

CONCLUSION: The appearance of urinary fibrinogen degradation products is caused by an active *H. pylori*-induced process.

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Key words: Urinary peptidome profiling; MB-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *Helicobacter pylori*; Fibrinogen degradation products

Peer reviewer: David J McGee, PhD, Associate Professor, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States

Xiao D, Meng FL, He LH, Gu YX, Zhang JZ. Analysis of the urinary peptidome associated with *Helicobacter pylori* infection. *World J Gastroenterol* 2011; 17(5): 618-624 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/618.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.618>

Abstract

AIM: To investigate the relationship between urinary peptide changes and *Helicobacter pylori* (*H. pylori*) infection using urinary peptidome profiling.

METHODS: The study was performed in volunteers ($n = 137$) who gave informed consent. Urinary peptides were enriched by magnetic beads based weak cation exchange chromatography and spectrums acquired by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). ClinProTools bioinformatics software was used for statistical analysis and the recognition of peptide patterns. The marker peptides were identified by LTQ Orbitrap XL tandem MS.

RESULTS: Approximately 50 proteins or peptides which loaded onto the magnetic beads were detected by MAL-

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, micro-aerophilic bacterium adapted for survival in the human stomach, where it can cause chronic gastritis and peptic ulcer disease and is an important risk factor that may lead

to gastric cancer. Great progress has been made in understanding *H. pylori* pathogenicity since its discovery 25 years ago. *H. pylori* infection has been proposed as a risk factor not only for gastrointestinal diseases but also for cardiovascular diseases such as peripheral arterial disease^[1] and atherosclerosis^[2]. In addition, some studies have shown that *H. pylori* infection is associated with Henoch-Schönlein purpura^[3,4] and membranous nephropathy^[5,6]. Purpura nephritis is one of the serious complications of Henoch-Schönlein purpura^[7]. As a result of its long delitescence, rapid growth of drug resistance and the ease of infection, *H. pylori* infection has become a prominent chronic digestive system disease.

Recent progress in proteomic analysis and strategies for the identification of clinically useful biomarkers in biological fluids has shown that urine can be an excellent non-invasive reservoir^[8-10]. By virtue of its noninvasiveness and the availability of specimens, peptidome profiling of human urine is now becoming an important method for detecting novel disease-associated markers^[11,12]. Bruker Daltonics provides the mass spectrometry (MS)-based ClinProt™ system solution for preparation, measurement and visualization of peptides and proteins in body fluid^[13]. The Profiling Kit MB-WCX (Magnetic Beads based Weak Cation Exchange Chromatography) was developed for the enrichment of proteins and peptides from biological samples based on cation exchange chromatography prior to matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) analysis. Successful applications and reproducibility of the MB-WCX beads using serum, plasma and urine samples was demonstrated in various studies^[14-16]. Nanoliquid chromatography coupled to micro-electrospray ionization tandem MS (ESI-MS/MS) has become a powerful tool for identification and quantification in peptide analysis due to its higher sensitivity^[17,18]. In this study, we analyzed the urine peptidome profiles of *H. pylori* infected and non-infected volunteers by the ClinProt™ system, followed by MALDI-TOF MS, and we identified the biomarkers using Aquity nano-ultra-performance liquid chromatography coupled to a Thermo LTQ Orbitrap high resolution/high accuracy ultra-performance liquid chromatography (UPLC)-ESI-MS/MS.

MATERIALS AND METHODS

Protein/peptide marker discovery in urine

Urine specimen collection: Urine samples were collected from healthy volunteers ($n = 137$, 70 male, 67 female) who did not have cardiovascular diseases and had received a health checkup 3 mo prior to the study, and gave written informed consent before participation. The volunteers received ¹³C-urea breath tests to determine whether they were infected with *H. pylori*, and their midstream urine was collected the following morning^[19]. Urine samples were kept at a low temperature with ice and were transferred to the laboratory within 2 h, centrifuged at 3000 *g* for 20 min, aliquotted and stored at -80°C until use.

Urinary peptide enrichment

The urine samples were thawed at room temperature for 30 min, adjusted to pH 7, and centrifuged again. Urinary peptides were separated using MB-WCX kit (Bruker Daltonics, Bremen, Germany; particle size < 1 μm; mean pore size, 40 nm; specific surface area, 100 cm²/g). The magnetic beads were mixed thoroughly on a vortex device for 1 min, then a 30 μL urine sample was diluted in 60 μL MB-WCX binding solution, and 10 μL WCX beads were added. After thorough stirring, sample mixtures were incubated for 1 min at room temperature. The tube was placed into the magnetic separator and the beads at the wall of the tube were collected for 1 min. The supernatant was removed by using a pipette. Wash buffer (100 μL) was added to the tube, which was moved back and forth in the magnetic separator 10 times. The beads were collected at the tube wall for 1 min and the supernatant was removed carefully using a pipette. Elution buffer (5 μL) was added and the beads dissolved at the tube wall by pipetting up and down intensively 10 times. The beads were collected at the tube wall for 2 min and the clear supernatant was transferred into a fresh tube. Stabilization buffer (5 μL) was added to the eluate.

MALDI-TOF data acquisition

Sample solution (1 μL) was dropped onto an AnchorChip™ 600-μm target (Bruker Daltonics) and dried. Next, 1 μL of freshly prepared α-cyano-4-hydroxycinnamic acid [0.4 mg/mL matrix solution in ethanol/acetone (2:1, v/v)] was added onto the sample and crystallized. MALDI-TOF MS analysis of the peptidome profile was performed using an autoflex™ instrument (Bruker Daltonics), equipped with a N₂ laser ($\lambda = 377$ nm), with the ion source voltage as follows: source 1, 120 kV; ion source 2, 18.6 kV; lens 7.6 kV. The pulsed ion extraction delay was 320 ns and operated in positive ion linear mode (LP-ClinProt) with a total of 450 shots (30 shots at each of 15 different spot positions) per sample. All signals with a signal-to-noise ratio > 3 in a *m/z* range of 1000-10 000 Da were collected with the AutoXecute tool of the flexControl™ acquisition software (version 3.0; Bruker Daltonics). Mass calibration was performed with the standard calibration mixture of peptides and proteins (CPS, preparation method in the MB-WCX operation manual, MW range 1000-10 000 Da).

Statistical data analysis

The spectra were analyzed statistically using Clin-Prot™ (version 2.2 β; Bruker Daltonics) bioinformatics software. Parameters were as follows: peak definition: signal to noise ratio > 3; statistical analysis: Wilcoxon/Kruskal-Wallis; area normalization: against total ion count; integration: end point level; mass recalibration: maximal peak shift of 500 ppm; sort mode: *t*-test *P*-value/analysis of variance (ANOVA). The spectra from 90 samples (40 in the *H. pylori* infected group and 50 in *H. pylori* non-infected group) were used to build models and 47 samples (23 in the *H. pylori* infection group and 24 in the *H. pylori*

non-infected group) were used in model verification by the Genetic Algorithm (GA), Quick classifier, and Supervised Neural mathematical algorithms. The parameters k-nearest neighbor classification (KNN), maximal number of generations (MNG) were optimized and the best model was determined. The performance of the models was evaluated by recognition capability (RC) and positive predictive value (PPV): $RC = TP/n$ where TP is the number of true positives (correctly classified) in a data set and n is the number of samples in a data set and $PPV = TP/(TP + FP)$ where FP is the number of false positives (misclassified). The best model (RC and PPV values are a maximum one of 1) was implemented to determine the marker peptides. The *P*-value of the Anderson-Darling test (PAD) which can give information about the normal distribution: < 1 not normally distributed, > 1 normally distributed, the *P*-value of the *t*-test (2 classes) or ANOVA test (> 2 classes) (PTTA, preferable for normal distributed data) or the *P*-value of the Wilcoxon test (2 classes) or Kruskal-Wallis test (> 2 classes) (PWKW, preferable for abnormally distributed data) was used to confirm significant differences. If the PWKW or PTTA value was < 0.05 , the protein/peptide was confirmed to be significantly different.

Identification of significant peptides by nano UPLC-ESI-MS/MS

UPLC: The peptides from urine samples (the differential peptides are relatively abundant) were eluted from the magnetic beads and were analyzed by nano-UPLC-ESI-MS/MS using a nano Aquity UPLC (Waters Corporation, Milford, USA) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples of 5 μ L (the sample was diluted by 2 times) were loaded on a C18 precolumn (Symmetry[®]C18, 5 μ m, 180 μ m \times 20 mm, nanoAcquity[™]Column) at 15 μ L/min in 5% acetonitrile (Sigma-Aldrich, St Louis, MO, USA), 0.05% trifluoroacetic acid (Sigma-Aldrich) for 3 min. The precolumn was switched online with the analytical column (Symmetry[®]C18, 3.5 μ m, 75 μ m \times 150 mm, nanoAcquity[™]Column) equilibrated in 95% solvent A (5% acetonitrile, 0.1% formic acid; Sigma-Aldrich) and 5% solvent B (95% acetonitrile, 1.2% formic acid). Peptides were eluted using a 5% to 80% gradient of solvent B over 60 min at a flow rate of 400 nL/min.

UPLC-MS/MS and data analysis

The LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Full-scan survey MS spectra with 2 microscans (m/z 400–2000) were acquired with the Orbitrap with a mass resolution of 100 000 at m/z 400, followed by 10 sequential LTQ-MS/MS scans. Dynamic exclusion was used with 2 repeat counts, 10 s repeat duration and 60 s exclusion duration. For MS/MS, charge state 1 was rejected and precursor ions were activated using 25% normalized collision energy at the default activation q of 0.25. The mass spectra were searched against

Table 1 Comparison of results for the classification models

Model	Algorithms	KNN	MNG	Max. peaks	RC (%)	PPV (%)
1	GA	5	60	7	90.5	83.0
2	GA	3	60	10	91.3	85.1
3	GA	7	60	15	96.5	93.6
4	GA	3	60	20	93.3	91.5
5	GA	5	60	25	93.3	87.2
6	SNN			25	78.5	63.8
7	QC			25	78.8	66.0

Model 3 was the best. GA: Genetic Algorithm; QC: Quickclassifier; SNN: Supervised Neural Network; KNN: k-nearest neighbor classification; MNG: Maximal number of generations; RC: Recognition capability; PPV: Positive predictive value.

the human International Protein Index (IPI) database (IPI human v3.45 fasta with 71 983 entries) using Bioworks software (Version 3.3.1; Thermo Electron Co.) based on the SEQUEST algorithm. To reduce false positive identification results, a decoy database containing the reverse sequences was appended to the database. The parameters for the SEQUEST search were as follows: no enzyme, the variable modification was oxidation of methionine, peptide tolerance, 10 ppm, MS/MS tolerance, 1.0 Da. Positive protein identification was accepted for a peptide with Xcorr of greater than or equal to 3.20 for triply and 2.86 for doubly charged ions, and all with $\Delta Cn \geq 0.1$, peptide probability $\leq 2e-3$.

RESULTS

Urinary peptidome profiling

¹³C-urea breath tests showed that 74 volunteers were *H. pylori* negative and 63 volunteers were *H. pylori* positive (delta over baseline > 4). About 50 peaks with signal-to-noise ratios greater than 5 were detected between m/z 1000 and 10 000 in urine from the volunteers (Figure 1). The average intensities of peaks for the negative group and positive group are shown in Figure 2A, and the complete spectra from both the healthy group and the *H. pylori*-infected group are shown in Figure 2C.

Statistical data analysis and classification

When parameter KNN = 7, MNG = 60 and Max.peaks = 15, the GA model was the best fit: RC = 96.5%, PPV = 93.6% (Table 1). All the data PAD were < 1 , so the data were abnormally distributed and PWKW was used to confirm marker peptides. Two markers that differentiated between the *H. pylori* non-infected group and the *H. pylori* infected group (PWKW < 0.05) with molecular masses of 6788 and 1912 Da were found in urine (Table 2). The content of these peptides in urine was markedly higher in *H. pylori* infected volunteers than in non-infected subjects (Figure 2B and D).

Identification of peptides

The peptides from urine were separated using nano-UPLC. Product-ion-spectra of the doubly charged mol-

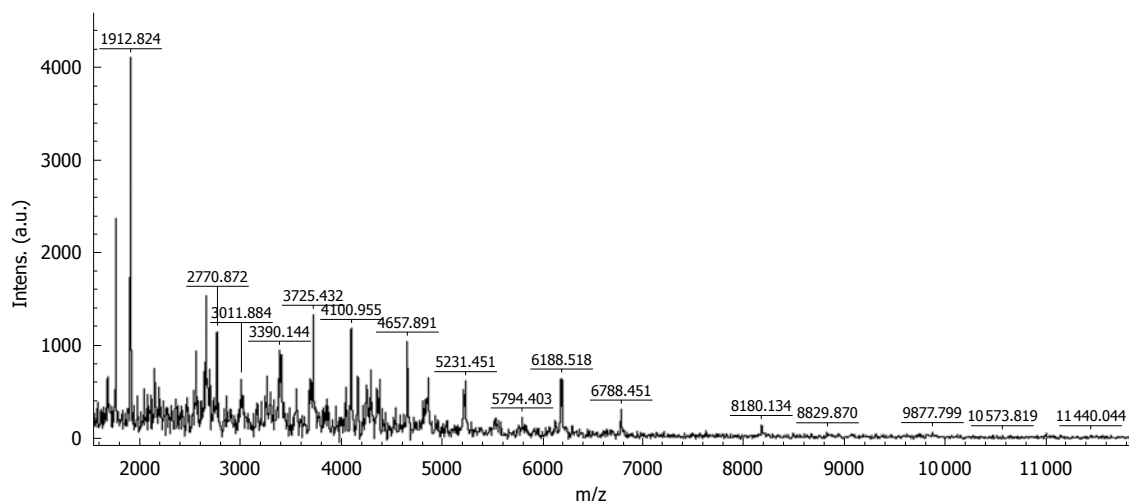


Figure 1 The mass spectrum of peptides in urine ranging between 1000 and 10000 Da.

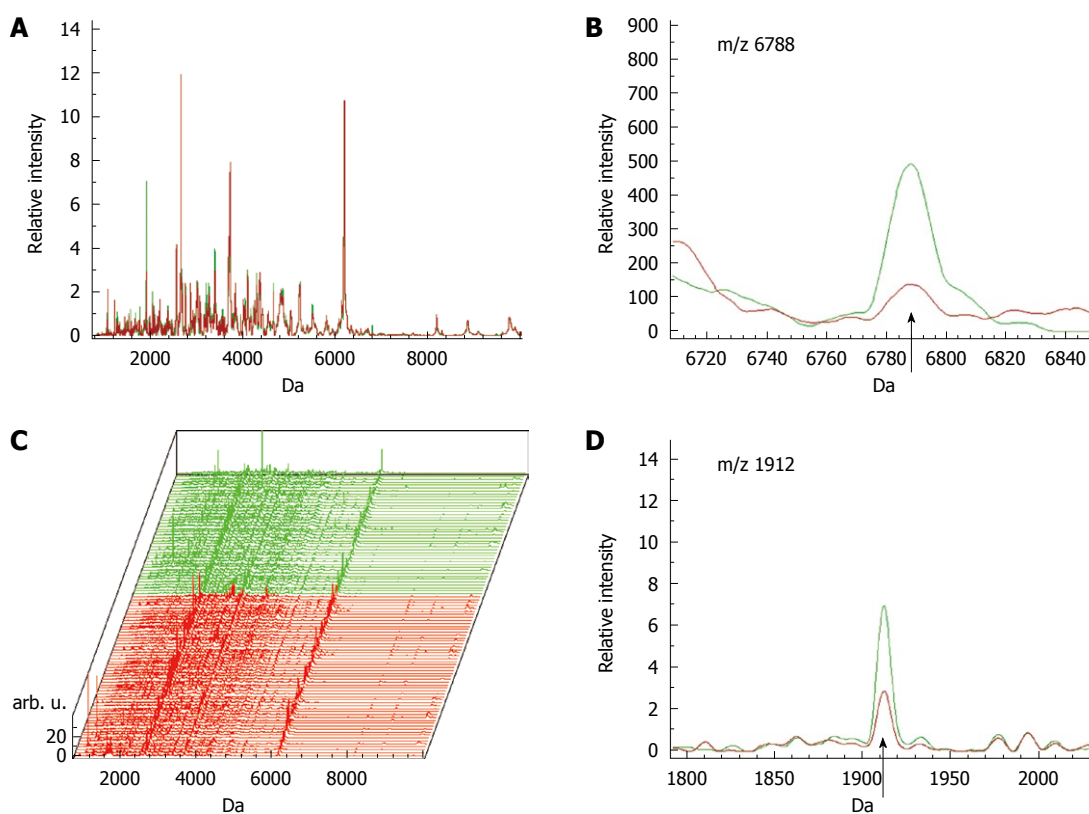


Figure 2 Differentially expressed low-mass peptides in human urine. A: The average intensities of matrix-assisted laser desorption/ionization time-of-flight peaks for the healthy group (red line), and the *H. pylori*-infected group (green line); B, D: The enlarged picture m/z 6788 and m/z 1912, respectively, the healthy group (red line) and the *H. pylori*-infected group (green line); C: The complete spectra from both the healthy group (red line) and the *H. pylori*-infected group (green line).

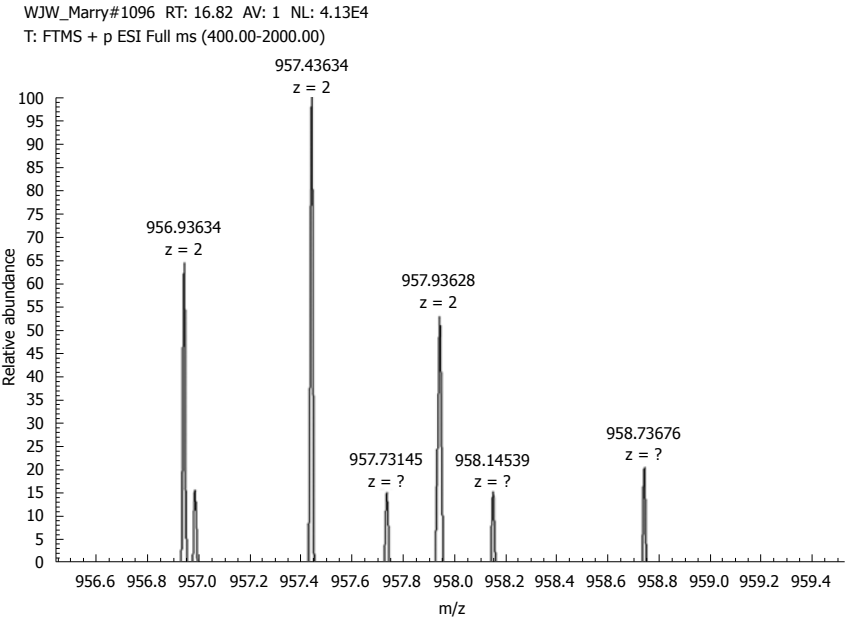
ecule m/z 957.436 for the 1912 Da peak was recorded with the linear ion trap (Figure 3A) and the sequence was parsed as SKQFTSSTSYNRGDSTF following MS/MS (Figure 3B). The sequence was identified as isoform 1 of fibrinogen α chain precursor (AC: IPI00021885) using the IPI database with Xcorr 3.201 (doubly charged ion), $\Delta C_n = 0.267$, $P = 1.10E-04$ and MS/MS tolerance 0.26 Da. Unfortunately, the m/z 6788 peak sequence was not identified. Because it was possible that the peptide m/z 1912

was from *H. pylori*, the sequence was searched against all the species in the NCBI nr. The fibrinogen was identified again as a fragment of human fibrinogen [gi|4503689|ref|NP_000499.1| fibrinogen, α polypeptide isoform α -E preproprotein (Homo sapiens)].

DISCUSSION

Urine is an especially attractive medium for biomarker

A



B

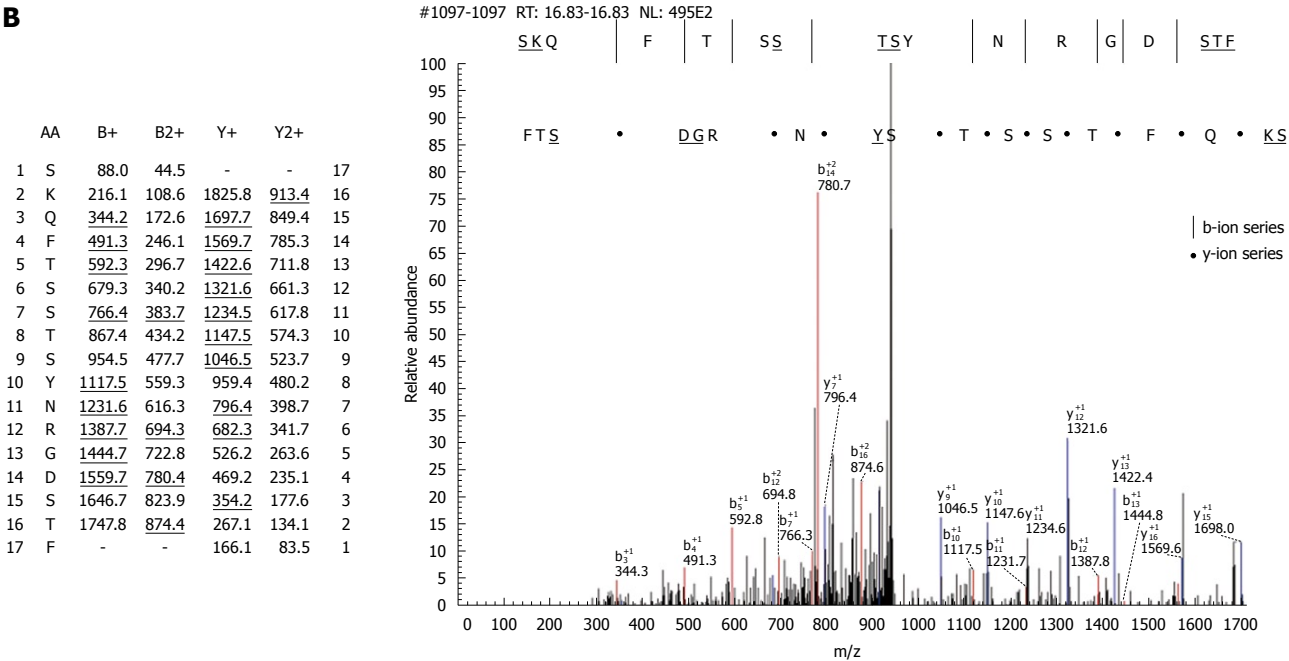


Figure 3 Protein identified by mass spectrometry/mass spectrometry. A: The enlarged picture of m/z 1912 (two charges 957.4); B: The b and y ions spectra used to identify the m/z 1912 as the fragment SKQFTSSTSYNRGDSTF. The underlined amino acids represent b or y ions of amino acids that cannot be found in the spectra. The underlined values represent the peaks where amino acids match with the b, y-ion peak.

Table 2 Statistical information for marker peptides of *Helicobacter Pylori* negative and positive groups

Index	Mass	DAve	PTTA	PWKW	PAD
56	6787.91	8.11	0.0000242	< 0.000001	0.00000418
9	1911.86	39.52	0.00847	0.00545	< 0.000001
23	3210.11	16.1	0.538	0.195	< 0.000001
27	3688.78	14.99	0.538	0.195	< 0.000001

DAve: Difference between the maximal and the minimal average peak area/intensity of all classes; PTTA: *P*-value of *t*-test (2 classes) or ANOVA test (> 2 classes); PWKW: *P*-value of Wilcoxon test (2 classes) or Kruskal-Wallis test (> 2 classes); PAD: *P*-value of Anderson-Darling test.

analysis, because urine can be obtained in large quantities using noninvasive procedures, and ample material is available for analysis and assessment of reproducibility. In addition, repeated sampling from the same individual is simple, facilitating longitudinal studies. Urine generally contains proteins and peptides of lower molecular mass (< 30 kDa) that are highly soluble. These features facilitate analysis of such polypeptides in their natural state, without any need for additional manipulation. Urinary polypeptides are stable and generally do not undergo significant proteolysis for several hours after collection^[20,21]. Urine has been known, or at least has been suspected, to

reflect pathological changes for centuries. Even early pathological changes are thought to be associated with disease-specific changes in the urinary proteome^[22]. In this study, we found 2 specific factors in human urine that were associated with *H. pylori* infection by urinary peptidome profiling. Urinary fibrinogen degradation products (FDP) increased with *H. pylori* infection.

Fibrinogen is a major plasma protein (340 kDa) that consists of pairs of 3 different polypeptide chains, α , β , and γ , joined by disulfide bonds to form a symmetric dimeric structure. The NH₂-terminal regions of all 6 chains form the central E-domain^[23]. Fibrinogen is directly involved in the clotting process as a clotting factor and is synthesized in hepatocytes^[24]. In addition, fibrinogen has a variety of other functions, such as a mediated platelet aggregation response^[25]. Many studies have found that an elevated level of plasma fibrinogen is an important risk factor for cardiovascular and cerebrovascular thrombotic diseases^[26,27] and renal failure^[28].

Fibrinogen can be digested either by plasmin or thrombin. When fibrinogen is cleaved by plasmin, it releases 2 D fragments (the COOH termini of the α , β , and γ chains), one E fragment (the NH₂ termini of the α , β , and γ chains), and several smaller fragments including a small peptide, β 1-42 (the NH₂ terminus of the β -chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH₂ termini of the α and β chains, respectively, while exposed polymerization sites form electrostatic bonds between the E-domain of one molecule and the D-domain of an adjacent one. Factor XIIIa, a transglutaminase, then introduces γ -glutamyl- ϵ -amino-lysine isopeptide cross-links between D domains of adjacent fibrin monomers, generating a stable polymer known as fibrin. Then, fibrin can be broken down by plasmin cleavage into the 3-stranded coils found between the D and E domains, yielding a D dimer, D fragment, and fibrin E fragment (which lacks the fibrinopeptides A and B) and smaller fragments^[29]. FDP, such as D-dimer, E-fragment and α , β -chain, have been widely studied in cardiovascular disease and cancer-related research fields^[30,31]. The m/z 1912 peptide is a fragment of an FDP (site 580-596). Our study shows that the peptide m/z 1912 in urine was significantly increased in patients with *H. pylori* infection.

The normal glomerular basement membrane has a filtration function, and the average pore size is 5.5 nm. Therefore, under normal circumstances, some small molecular weight proteins can filter through tiny pores in the glomerular membrane. Because of endocytosis, the major proteins are normally reabsorbed when they pass through the proximal tubule, so there is low protein content in urine, a random urinary protein of 0-80 mg/L. Although there are many kinds of fibrinogen degradation fragments, large fragments are retained by the glomerulus or are taken up by the renal tubule, therefore only small peptides are normally seen in the urine. In this study, the peptides or proteins below 10 kDa in the urine were captured by weak cation beads, so only the marker peptides 1912 and 6788 were detected, while the fragments of FDP that exceeded 10 kDa were not captured.

The reasons why *H. pylori* infection results in an FDP increase in urine are not clear. Our preliminary studies have shown that *H. pylori* will lead to human gastric adenocarcinoma epithelial cell calreticulin phosphorylation, and dephosphorylation of its calcium-binding protein (nucleobindin-2), which affects cell calcium ion channels^[32]. Fibrinogen achieves its biological functions by being degraded by plasmin or thrombin. The activities of plasmin and thrombin are regulated or progressively activated by calcium ions; therefore, the changes in the calcium ion channels will affect the fibrinolytic system. In short, the changes in FDP in urine are important for gaining a comprehensive understanding of the pathogenesis of *H. pylori*.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection has been proposed as a risk factor not only for gastrointestinal diseases but also for cardiovascular diseases and nephropathy. The pathogenic mechanisms of *H. pylori* are not yet clear since its discovery 25 years ago.

Research frontiers

The peptidome has been widely used in finding biomarkers with the development of mass spectrometry (MS). As it can be obtained in large quantities using noninvasive procedures, urine is an especially attractive medium for biomarker analysis. In this study, the authors analyzed the urine peptidome profiles of *H. pylori* infected and non-infected volunteers using the ClinProt™ system, followed by matrix-assisted laser desorption/ionization time-of-flight MS, and identified the marker peptides using liquid chromatography coupled to MS.

Innovations and breakthroughs

Cardiovascular diseases and nephropathy have been reported which associated with *H. pylori* infection. To date, the pathogenic mechanism is not clear. This study suggests that the appearance of urinary fibrinogen degradation products is caused by an active *H. pylori*-induced process. The results of this study are important to further the comprehensive understanding of the pathogenesis of *H. pylori*.

Applications

This study suggests that fibrinogen degradation products are associated with *H. pylori* infection. This result can help researchers in this field further understand the potential mechanism associated with *H. pylori* infection and cardiovascular diseases and nephropathy, and provide important information for prevention and control of *H. pylori*-related diseases.

Peer review

It would be of great interest in future experiments to investigate whether the urine fibrinogen peptide correlates with serum fibrinogen levels and *H. pylori* infection, as serum fibrinogen levels have been investigated in *H. pylori* infection in many studies.

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S- Editor Sun H L- Editor Cant MR E- Editor Zheng XM

Influence of CXCR4/SDF-1 axis on E-cadherin/ β -catenin complex expression in HT29 colon cancer cells

Lin Wang, Cui-Ling Li, Lei Wang, Wen-Bin Yu, Hai-Peng Yin, Guang-Yong Zhang, Li-Feng Zhang, Sheng Li, San-Yuan Hu

Lin Wang, Lei Wang, Wen-Bin Yu, Guang-Yong Zhang, Li-Feng Zhang, San-Yuan Hu, Department of General Surgery, Qilu Hospital, Shandong University, Jinan 250012, Shandong Province, China

Cui-Ling Li, Hai-Peng Yin, Key Laboratory for Modern Medicine and Technology of Shandong Province, Institute of Basic Medicines, Shandong Academy of Medical Sciences, Jinan 250062, Shandong Province, China

Sheng Li, Department of Hepatobiliary Surgery, Shandong Tumor Hospital, Jinan 250117, Shandong Province, China

Author contributions: Hu SY and Li S designed the study; Li CL, Yin HP, Zhang LF and Wang L performed the experiments; Wang L, Yu WB and Zhang GY analyzed the data; Wang L wrote the paper.

Supported by National Natural Science Foundation of China, No. 30571712 and 30810403081

Correspondence to: San-Yuan Hu, MD, Department of General Surgery, Qilu Hospital, Shandong University, Jinan 250012, Shandong Province, China. 33943219@qq.com

Telephone: +86-531-82169441 Fax: +86-531-82169441

Received: October 15, 2010 Revised: January 11, 2011

Accepted: January 18, 2011

Published online: February 7, 2011

Abstract

AIM: To study the influence of CXCR4/stromal cell-derived factor-1 (SDF-1) axis on E-cadherin/ β -catenin complex expression in HT29 colon cancer cells and its underlying mechanisms.

METHODS: Effect of SDF-1 on E-cadherin/ β -catenin expression was detected by immunocytochemistry. E-cadherin and β -catenin mRNA expression levels were measured by reverse transcriptase-polymerase chain reaction. SDF-1-induced phosphorylation of phosphatidylinositol 3-kinase (PI3K)/AKT and β -catenin was detected by Western blotting.

RESULTS: The E-cadherin and β -catenin mRNA ex-

pression levels in HT29 cells were lower 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL ($P < 0.05$). SDF-1-induced significant phosphorylation of PI3K/AKT and β -catenin. AMD3100 and LY294002 inhibited the phosphorylation of PI3K/AKT and β -catenin.

CONCLUSION: SDF-1 down-regulates the E-cadherin/ β -catenin complex expression in HT29 cells by decreasing mRNA synthesis and increasing β -catenin phosphorylation.

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Key words: CXCR4; Stromal cell-derived factor-1; E-cadherin; β -catenin; Phosphatidylinositol 3-kinase/AKT; Colon cancer

Peer reviewer: Julio Mayol, MD, PhD, Department of Digestive surgery, Hospital Clinico San Carlos, MARTIN-LAGOS S/n, Madrid, 28040, Spain

Wang L, Li CL, Wang L, Yu WB, Yin HP, Zhang GY, Zhang LF, Li S, Hu SY. Influence of CXCR4/SDF-1 axis on E-cadherin/ β -catenin complex expression in HT29 colon cancer cells. *World J Gastroenterol* 2011; 17(5): 625-632 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/625.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.625>

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers and the second leading cause of cancer-related death in the Western world. Death usually results from its uncontrolled metastasis. Although the 5-year survival rate approaches 90% for patients with local CRC, it has decreased to 19% for patients with distant metastasis^[1]. The metastatic process of CRC consists of a series of individ-

ual steps, which are required to establish the diagnosis of metastatic lesions^[2,3]. A number of molecules have been implicated in the metastatic process of CRC.

Chemokines are a group of chemoattractant cytokines that mediate several cellular functions. Stromal cell-derived factor-1 (SDF-1) is expressed in stromal cells, including fibroblasts and endothelial cells^[4,5], and interacts specifically with the seven-transmembrane, G protein-coupled receptor CXCR4^[6]. Recent studies showed that chemotaxis effect of CXCR4/SDF-1 axis is related with lymph node and liver metastasis of CRC^[7-10]. Although there is evidence that the CXCR4/SDF-1 signaling pathway is involved in the metastatic process of CRC, the precise molecular mechanism underlying SDF-1-induced chemotaxis effect has not been completely elucidated.

E-cadherin, a transmembrane glycoprotein located at the adheren junction, mediates calcium-dependent cell-cell adhesion^[11,12]. C terminus of E-cadherin is linked to α -catenin and actin cytoskeleton through the association with β -catenin. Strong cell-cell interactions result in a tight cell cluster as a community, and constrain cells from moving away. It has been shown that dysregulation of E-cadherin/ β -catenin complex expression is responsible for the invasion and metastasis of CRC^[13,14], indicating that the CXCR4/SDF-1 axis is correlated with E-cadherin/ β -catenin complex expression in invasion and metastasis of CRC.

This study was to observe whether SDF-1 can alter E-cadherin/ β -catenin expression in HT29 colon cancer cell line. In addition, the E-cadherin/ β -catenin mRNA expression level was measured and the phosphorylation of phosphatidylinositol 3-kinase (PI3K)/AKT and β -catenin was examined to provide insights into the mechanism underlying the change in E-cadherin/ β -catenin expression.

MATERIALS AND METHODS

Reagents

Antibodies against E-cadherin and β -catenin, and p- β -catenin antibody (Ser33/37) and p-AKT antibody (Ser473) were purchased from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). SDF-1 was bought from Pepro Tech Inc. (Rocky Hill, NJ, USA). AMD3100 was purchased from Sigma Aldrich, USA and LY29400 was bought from Beyotime, China.

Cell culture

Human colon cancer HT29 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Tumor cells were cultured in RPMI-1640 (Invitrogen product), supplemented with 10% newborn calf serum (Shanghai Mafa Corporation), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Cell proliferation assay

Exponentially growing HT29 cells were seeded in 96-well plates in RPMI-1640 containing 3% newborn calf serum at a density of 2×10^4 cells/well. After 24 h, either PBS or AMD3100 (100 ng/mL) was added and incubated for 2 h. SDF-1 was added into three wells daily at different concentrations (10, 20 and 40 ng/mL). MTT assay (Amersham Biosciences, USA) was performed after 24, 48 and 72 h. Absorbance was measured at 570 and 630 nm (630 nm as the reference wave length). The results were expressed as a mean of three wells in each group. Proliferation rate of HT29 cells was calculated by the absorbance of experimental groups divided by that of the control group. The results were expressed as a mean of three individual experiments.

Cell chemotaxis and migration assay

Migration of HT29 cells was assessed in a HTS transwell-24 system (Corning, Acton, MA, USA) with 8- μ m membrane pores. After rehydration for 2 h, RPMI-1640 and different SDF-1 concentrations (10, 20 and 40 ng/mL) were added into the lower chamber (0.5 mL per well) and 2×10^4 cells treated with PBS or AMD3100 (100 ng/mL) were added into the upper chamber 30 min before assay. After incubated at 37°C for 24 h, Matrigel and cells on the upper side of the membrane were wiped off with PBS-rinsed cotton swabs and invading cells migrated to the lower surface of the membrane were photographed and counted under an inverted light microscope at 100 \times magnification. Six random fields were counted for each well. Migration of HT29 cells was assayed in triplicate.

Immunocytochemistry

To observe the effect of SDF-1 on E-cadherin/ β -catenin complex expression, HT29 cells were seeded in 24-well plates with glass slides at a density of 2×10^5 cells/well in RPMI-1640 medium in the absence of serum. After incubated overnight, either PBS or CXCR4 antagonist AMD3100 (100 ng/mL) was added and incubated for 2 h. Then, SDF-1 was added at different concentrations (10, 20 and 40 ng/mL). Glass slides were collected after incubated for 24 and 48 h with SDF-1. Cells were washed thrice with PBS prior to fixation with 4% formaldehyde in PBS. Then, the cells were covered with 3% H₂O₂-methanol for 30 min at room temperature to inactivate the endogenous peroxidase. A methanol permeabilisation step was needed for β -catenin, during which cells were covered with ice-cold 100% methanol for 10 min in a freezer. After rinsed thrice with PBS, the cells were covered with 5% normal goat serum for 30 min at room temperature to block the non-specific binding. Primary rabbit anti-E-cadherin antibodies and mouse anti- β -catenin antibodies were applied to the slides and incubated overnight at 4°C. Slides were washed three times with PBS. Secondary antibodies were applied for 1 h at room temperature. Finally, the slides were stained with 0.025% diaminobenzidine tetrahydrochloride containing 4% H₂O₂ for 1-20 min, counterstained with hematoxylin

for an appropriate period of time, and analyzed under a light microscope. The level of nonspecific background staining was established for each measurement using control cells processed in the same way without exposure to primary antibodies.

Reverse transcriptase-polymerase chain reaction analysis

HT29 cells (1×10^6) treated as in immunocytochemistry were collected and washed three times with $1 \times$ PBS. Total RNA was extracted using trizol reagents and quantified by ND-1000 UV-visible spectrophotometry. cDNA was synthesized from 1 μ g of total RNA using Revert Aid™ M-MuLV reverse transcriptase. Reaction mixture contained 4 μ L of $5 \times$ reaction buffer, 2 μ L of 10 mmol/L dNTP mix, 1 μ g of Oligo(dT)18, 1 μ g of total RNA, 0.5 μ L of ribonuclease inhibitor, and 200 U Aid™ M-MuLV reverse transcriptase in a total volume of 20 μ L. PCR contained 2.5 μ L of $10 \times$ PCR buffer, 1.5 μ L of 25 mmol/L $MgCl_2$, 0.5 μ L of 10 mmol/L dNTP mix, 0.8 μ mol/L β -catenin primer, 0.04 μ mol/L GAPDH primer (for β -catenin) and 0.8 μ mol/L E-cadherin primer, 0.1 μ mol/L β -actin primer (for E-cadherin), 3 μ L of cDNA and 25 U *Taq* polymerase in a total volume of 25 μ L. The sequences of gene-specific primers are 5'-TTTGCCTGAGCAGGGTGC-3' (forward) and 5'-GCTGCATATGTCGCCACACC-3' (reverse) for β -catenin, 5'-CCACCCATGGCAAATTCATGGCA-3' (forward) and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (reverse) for GAPDH, 5'-GATTCTGCTGCTCTTGCTGT-3' (forward) and 5'-CCTGGTCTTTGTCTGACTCTG-3' (reverse) for E-cadherin, 5'-CCTTCCTGGGCATGGAGTCCT-3' (forward) and 5'-GGAGCAATGATCTTGATCTT-3' (reverse) for β -actin, respectively. The PCR conditions for β -catenin were as follows: denaturing at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, at 63°C for 30 s, at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR conditions for E-cadherin were as follows: annealing at 58°C for 5 min, followed by 35 cycles at 94°C for 30 s, at 63°C for 30 s, at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were separated on a 2% agarose gel in $1 \times$ TAE, visualized with ethidium bromide staining by BIO-RAD Gel Dos1000, and quantified using the Molecular Analyst software version 1.5 using GAPDH as an internal control for β -catenin, and β -actin as an internal control for E-cadherin. The hue value was calculated as the ratio of each group and the internal control group. Results were expressed as the mean value of three individual experiments.

Western blotting

To study the effect of SDF-1 on phosphorylation of various signaling proteins, HT29 cells were treated with 20 ng/mL SDF-1 for different periods of time (from 1 min to 2 d) or with different concentrations of SDF-1 (5-100 ng/mL) for 30 min after overnight starvation of

growth factors. Cell lysates were analyzed for the presence of phosphorylated AKT and β -catenin by phospho-specific antibodies to the specific phosphorylation sites of AKT (Ser473) and β -catenin (Ser33/37). For experiments using inhibitors, HT29 cells were pretreated with AMD3100 (100 ng/mL) or PI3K/AKT inhibitor LY294002 (20 μ mol/L) for 2 h, followed by stimulation with 20 ng/mL SDF-1. After each treatment, 4×10^6 HT29 cells were collected and washed three times with $1 \times$ PBS. Cytoplasm and membrane extracts were acquired according to the instruction datasheet of Pierce Biotechnology Corporation and quantified by Bradford protein assay. Different extraction proteins were separated by SDS-PAGE and transferred onto the PVDF membrane. Membranes were blocked with 5% BSA for 2 h at room temperature, incubated overnight at 4°C with primary antibodies (p- β -catenin 1:1000, p-AKT 1:1000 and β -actin 1:400) and washed three times prior to incubation with HRP-conjugated secondary antibodies (peroxidase conjugated goat anti rabbit IgG 1:10000) for 1 h at room temperature. Protein expression was visualized by chemiluminescence and quantified using the multigauge software. β -actin was used as a loading control. Data were shown as the ratio of phosphorylation and loading control. Western blotting assay was performed in triplicate.

Statistical analysis

All data were analyzed using the SPSS 16.0 and expressed as mean \pm SD. Statistical significance of differences was determined by Student's *t*-test in two groups and one-way ANOVA among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

SDF-1 enhanced viability of HT29 colon cancer cells

The viability of HT29 cells in any experiment groups was not different from that in control group 48 h after incubated with SDF-1. The cells grew much faster with a proliferation rate of 129% and 135%, respectively ($P < 0.05$) 72 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL. AMD3100 plus SDF-1 inhibited the cell growth. AMD3100 alone had no effect on cell proliferation.

SDF-1 promoted migration of HT29 colon cancer cells

MTT assay revealed no significant difference in viability of HT29 colon cancer cells in any experiment groups within 24 h after incubated with SDF-1. Therefore, the migration of HT29 colon cancer cells was assayed 24 h after incubated with SDF-1 to exclude the influence of cell viability. The migration ability of HT29 cells was significantly greater in experiment groups than in control group 24 h after incubated with SDF-1 at the concentration of 10 ng/mL (149 ± 13.3 vs 92.3 ± 12.4 , $P = 0.041$), 20 ng/mL (161 ± 13.5 vs 92.3 ± 12.4 , $P = 0.023$), and 40 g/mL (187.5 ± 14 vs 92.3 ± 12.4 , $P < 0.001$). AMD3100 plus SDF-1 inhibited

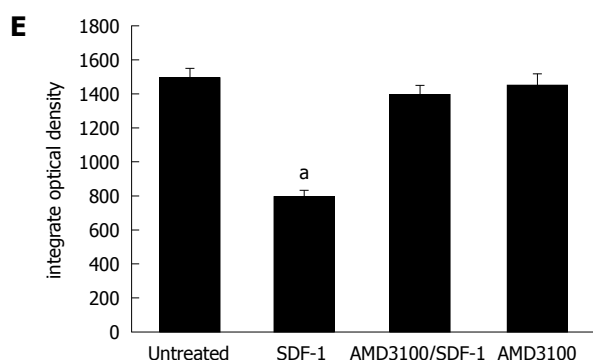
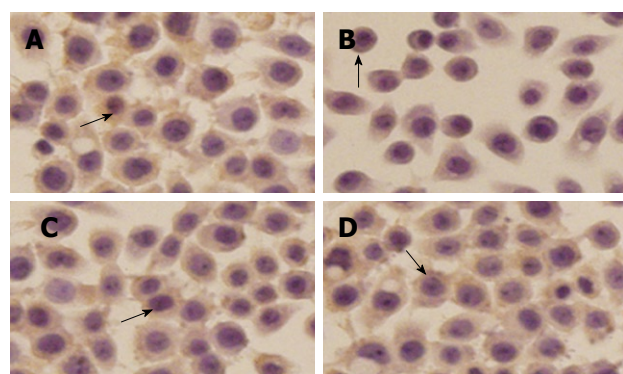


Figure 1 Effect of stromal cell-derived factor-1 (20 ng/mL) on E-cadherin expression ($\times 400$). A: E-cadherin expression in HT29 cells; B: Significantly lower E-cadherin expression level 48 h after incubated with stromal cell-derived factor-1 (SDF-1) ($P < 0.05$); C: AMD3100-inhibited E-cadherin expression; D: No effect of AMD3100 alone on E-cadherin expression. Arrows mean the expression of E-cadherin; Bars indicate mean \pm SD of six random fields. ^a $P < 0.05$.

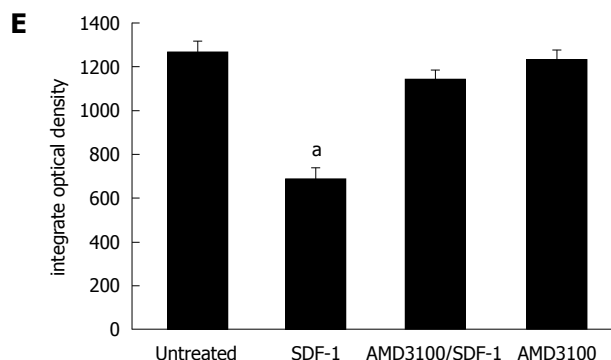
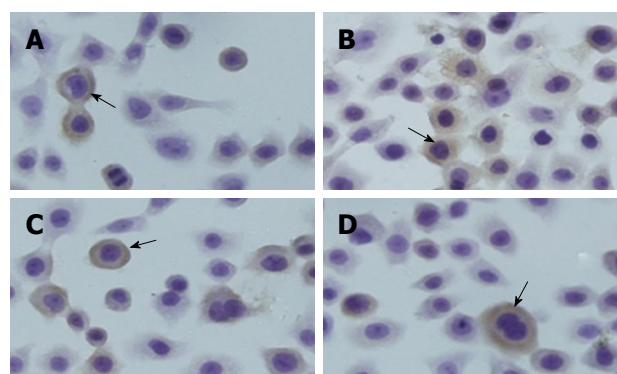


Figure 3 Effect of stromal cell-derived factor-1 (20 ng/mL) on β -catenin expression after 48 h ($\times 400$). A: β -catenin expression in HT29 cells; B: Significantly lower β -catenin expression level 48 h after incubated with stromal cell-derived factor-1 (SDF-1) ($P = 0.031$); C: AMD3100-inhibited β -catenin expression; D: No effect of AMD3100 alone on β -catenin expression. Arrows mean the expression of β -catenin; Bars indicate mean \pm SD of six random fields. ^a $P < 0.05$.

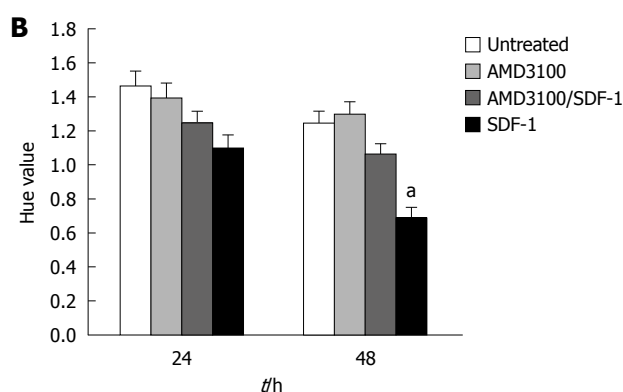
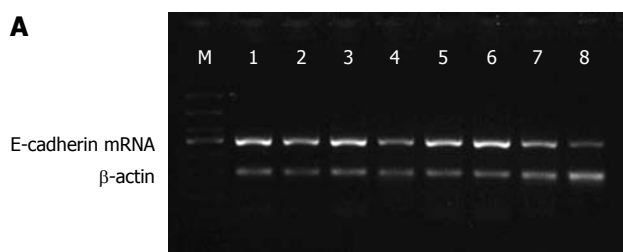


Figure 2 Effect of stromal cell-derived factor-1 (20 ng/mL) on E-cadherin mRNA expression after 24 h (A) and 48 h (B) in different groups. Bars represent mean \pm SD of three individual experiments. ^a $P < 0.05$. SDF-1: Stromal cell-derived factor-1.

the migration of HT29 cells. AMD3100 alone had no effect on cell migration.

SDF-1 down-regulated activation of CXCR4 and E-cadherin expression at protein and mRNA levels

Immunocytochemistry assay showed that E-cadherin was significantly expressed in HT29 cells (Figure 1A). No change was found in E-cadherin expression 24 h after incubated with SDF-1. The E-cadherin expression level was significantly lower 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL ($P < 0.05$, Figure 1B). HT29 cells treated with AMD3100 prior to administration of SDF-1 did not decrease the E-cadherin expression level. However, HT29 cells treated SDF-1 decreased the E-cadherin expression level (Figure 1C). AMD3100 alone had no effect on the E-cadherin expression (Figure 1D). The changes of E-cadherin expression in HT29 cells are demonstrated in Figure 1E.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that the E-cadherin mRNA expression level was lower 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL ($P < 0.05$). AMD3100 plus SDF-1 inhibited the E-cadherin mRNA expression. AMD3100 alone had no influence on E-cadherin mRNA expression (Figure 2A and B).

SDF-1 down-regulated β -catenin expression at protein and mRNA levels

β -catenin was strongly stained in HT29 cells not incubated with SDF-1 (Figure 3A). The β -catenin expression

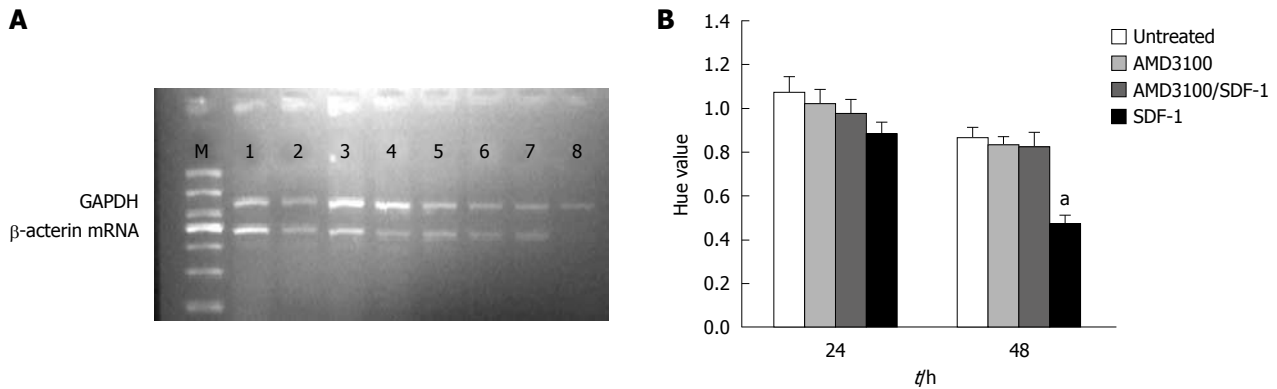


Figure 4 Effect of stromal cell-derived factor-1 (20 ng/mL) on β -catenin mRNA expression after 24 h (A) and 48 h (B) in different groups. Bars represent mean \pm SD of three individual experiments. ^a $P < 0.05$. SDF-1: Stromal cell-derived factor-1.

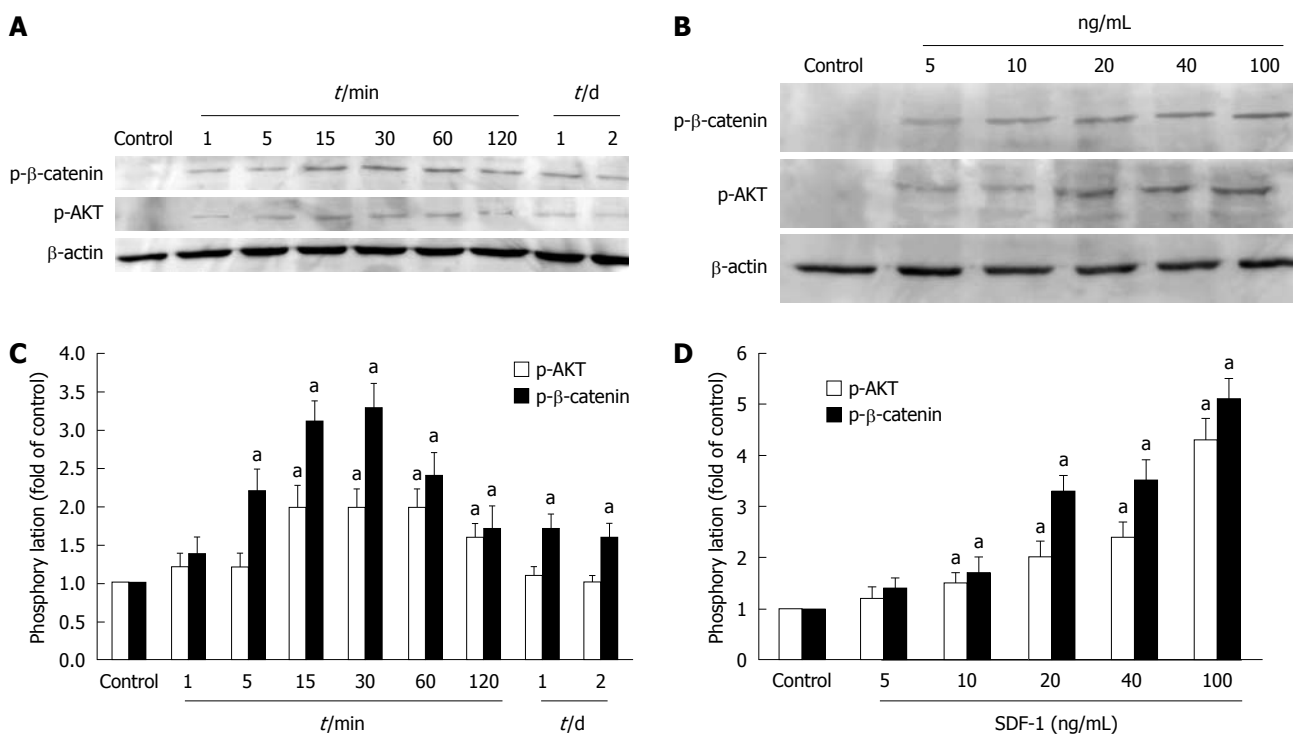


Figure 5 Effect of stromal cell-derived factor-1 on the phosphorylation of PI3K/AKT and β -catenin. A: Phosphorylation of PI3K/AKT and β -catenin after incubated with stromal cell-derived factor (SDF) at different periods of time (1, 5, 10, 15, 30, 60, 120 min and on days 1 and 2); B: Phosphorylation of PI3K/AKT and β -catenin after incubated with SDF-1 at different concentrations (5, 10, 20, 40 and 100 ng/mL) for 30 min; C and D: SDF-1 increases phosphorylation of PI3K/AKT and β -catenin in HT29 cells in a time- and dose-dependent manner. Bars represent mean \pm SD of triplicate experiments. ^a $P < 0.05$.

level was lower 24 and 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL ($P < 0.05$, Figure 3B). The β -catenin expression level was slightly lower in AMD3100- treated HT29 cells (Figure 3C). AMD3100 alone had no influence on β -catenin expression (Figure 3D). The changes of β -catenin expression in HT29 cells are demonstrated in Figure 3E.

RT-PCR analysis demonstrated that the β -catenin mRNA expression level was lower 24 and 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL ($P < 0.05$, Figure 4A and B). AMD3100 plus SDF-1 inhibited the β -catenin mRNA expression. AMD3100 alone did not influence the β -catenin mRNA expression.

Involvement of phosphorylation of PI3K/AKT and β -catenin in SDF-1-induced down-regulation of E-cadherin/ β -catenin expression

The phosphorylation of PI3K/AKT and β -catenin was detected to observe whether phosphorylation is involved in down-regulation of E-cadherin/ β -catenin complex expression, which demonstrated that SDF-1 increased the phosphorylation of AKT and β -catenin. The β -catenin was evidently activated at 5 min and the AKT was significantly phosphorylated at 15 min after incubated with SDF-1. The phosphorylation of β -catenin reached its peak 30 min after incubated with SDF-1. Then, β -catenin was evidently phosphorylated for 2 d (Figure 5A and C). The

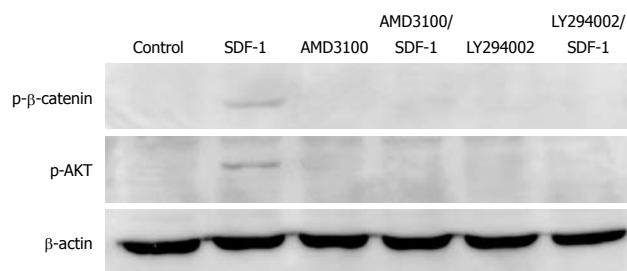


Figure 6 AMD3100 and LY294002 inhibit stromal cell-derived factor-1-induced phosphorylation of PI3K/AKT and β -catenin while AMD3100 or LY294002 alone has no effect on phosphorylation of PI3K/AKT and β -catenin. SDF-1: Stromal cell-derived factor-1.

phosphorylation of AKT reached its peak at 15–60 min after incubated with SDF-1, remained there for 2 h, and then slowly declined. To determine the dose-dependent effect of SDF-1 on the phosphorylation of PI3K/AKT and β -catenin, HT29 cells were treated with SDF-1 at different concentrations (0, 5, 10, 20, 40 and 100 ng/mL) for 30 min. Then, cell lysates were analyzed for the phosphorylation of PI3K/AKT and β -catenin. Administration of SDF-1 for 30 min increased the phosphorylation of PI3K/AKT and β -catenin in a dose-dependent manner. PI3K/AKT and β -catenin were phosphorylated after incubated with SDF-1 at the concentration of 5 ng/mL and reached its peak after incubated with SDF-1 at the concentration of 100 ng/mL (Figure 5B and D).

Inhibition of PI3K/AKT prevented phosphorylation of SDF-1-induced β -catenin

To investigate the relation between PI3K/AKT and β -catenin, AMD3100 and LY294002 were used to inhibit the effect of SDF-1 and the signal transmission through PI3K/AKT, which showed that AMD3100 inhibited the phosphorylation of PI3K/AKT and β -catenin (Figure 6). Further study demonstrated that administration of LY294002 prior to SDF-1 also prevented the phosphorylation of PI3K/AKT and β -catenin (Figure 6), suggesting that β -catenin is phosphorylated *via* the PI3K/AKT, and may be the downstream signaling molecule of PI3K/AKT.

DISCUSSION

Although the CXCR4/SDF-1 biological axis contributes to organ-selective metastasis of tumors^[15–17], the mechanism underlying the effect of chemotaxis remains unclear. In this study, the relation between CXCR4/SDF-1 axis and E-cadherin/ β -catenin complex expression was observed. Experiment on HT29 colon cancer cell line was performed because it shows a high expression level of CXCR4^[18]. SDF-1 promoted the proliferation of HT29 cells, thereby contributing to primary tumor formation and down-regulated the E-cadherin/ β -catenin expression by reducing the mRNA expression levels and decomposing their complex formation by phosphorylating the PI3K/AKT and β -catenin. AMD3100 and LY294002

blocked the two processes mediated by SDF-1, suggesting that they may be effective anti-metastatic agents, at least against CRC.

It was reported that CXCR4/SDF-1 axis-induced chemotaxis effect plays a role in the invasion of CRC cells^[16,17]. A number of molecules, such as vascular endothelial growth factor, matrix metalloproteinase (MMP)9, and MMP2, have been implicated in SDF-1-induced CRC invasion^[19]. E-cadherin is not only an adhesion molecule but also a tumor suppressor as well as the most important epithelial marker^[20]. In most cancers of epithelial origin, E-cadherin mediates cell-cell adhesion. Loss of E-cadherin-mediated cell-cell adhesion is implicated in tumor invasion and metastasis^[21,22]. Recent studies showed that Krüppel-like factor 4 inhibits epithelial to mesenchymal transition by regulating E-cadherin gene expression^[23]. In our study, the E-cadherin expression level at protein and mRNA levels was lower 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL, suggesting that E-cadherin is involved in SDF-1-induced chemotaxis effect. In this study, the molecular mechanism underlying SDF-1-induced chemotaxis effect was studied. The E-cadherin mRNA expression level was lower after incubated with SDF-1, which may account for the down-regulation of E-cadherin expression.

Tumor invasion is a complex process and loss of E-cadherin-mediated cell adhesion is not sufficient to confer an invasive phenotype to tumor cells. Cell migration requires precise control, which is altered or lost when tumors become invasive and metastatic. It has been shown that decreased β -catenin expression is often related with the absent or reduced E-cadherin, which contributes to the development of several cervical carcinoma cell lines^[24,25]. A recent study revealed that β -catenin membrane/cytosolic expression level is significantly lower in primary tumors than in corresponding matched metastases, suggesting that the low β -catenin expression level may be a prognostic factor for the occurrence of metastasis and a worse outcome^[26]. In this study, the β -catenin protein and mRNA expression levels were significantly lower 48 h after incubated with SDF-1 at the concentrations of 20 and 40 ng/mL, suggesting that β -catenin is involved in SDF-1-induced chemotaxis effect. Changes in β -catenin may also lead to the degradation of E-cadherin/ β -catenin complex and alter cytoskeleton, thus promoting cell migration.

Growth factors, such as epidermal growth factor, induce β -catenin and plakoglobin phosphorylation, resulting in breakage of E-cadherin binding to actin cytoskeleton and contact disassembly^[26]. In this study, the phosphorylation of β -catenin was increased after incubated with SDF-1, indicating that SDF-1 induces phosphorylation of β -catenin. Phosphorylation of β -catenin reduces β -catenin and may lead to decomposition of E-cadherin/ β -catenin complex. G protein-coupled receptor activation results in PI3K and downstream AKT activation^[27]. In this study, SDF-1-induced phosphorylation of PI3K/AKT and β -catenin in a time- and dose-dependent manner. Finally,

whether SDF-1 induces β -catenin phosphorylation *via* PI3K/AKT was also studied. Given that both AMD3100 and LY294002 inhibited the phosphorylation of PI3K/AKT and β -catenin, β -catenin may be the downstream signaling molecule of PI3K/AKT, indicating that the phosphorylation of β -catenin may account for the down-regulation of β -catenin, and the breakage of E-cadherin/ β -catenin complex. The phosphorylation of β -catenin may exert its effect *via* the PI3K/AKT pathway.

In conclusion, down-regulation of E-cadherin/ β -catenin complex expression is involved in SDF-1-induced chemotaxis. The decreased E-cadherin mRNA expression and the down-regulated β -catenin expression may account for the down-regulation of E-cadherin. CXCR4/SDF-1 axis stimulates the phosphorylation of PI3K/AKT and β -catenin. The down-regulation of β -catenin may be induced by the decreased β -catenin mRNA expression and the increased phosphorylation of PI3K/AKT. PI3K inhibitor LY294002 inhibits the SDF-1-induced phosphorylation of PI3K/AKT and β -catenin. β -catenin may be the downstream signaling molecule of PI3K/AKT.

ACKNOWLEDGMENTS

The authors thank Cui-Ling Li and Hai-Peng Yin for their excellent technical support in this study.

COMMENTS

Background

CXCR4/stromal cell-derived factor-1 (SDF-1) axis-mediated chemotaxis effect is crucial in organ-selective metastasis. E-cadherin/ β -catenin complex plays an important role in colorectal tumorigenesis. Relatively little is known about the role of E-cadherin/ β -catenin in CXCR4/SDF-1 axis-mediated chemotaxis effect.

Research frontiers

Recent studies demonstrated that the CXCR4/SDF-1 signaling pathway is involved in the metastatic process of colorectal cancer (CRC). In addition, inhibiting the interaction of SDF-1 and CXCR4 with CXCR4 antagonist AMD3100 prevents the chemotactic migration of CRC cells. However, the underlying molecular mechanism has not been elucidated.

Innovations and breakthroughs

Down-regulation of E-cadherin/ β -catenin complex is involved in SDF-1-induced chemotaxis effect in HT29 colon cancer cells. Moreover, decreased mRNA synthesis and increased β -catenin phosphorylation may down-regulate E-cadherin/ β -catenin. To the best of our knowledge, this is the first study that analyzes the relation between CXCR4/SDF-1 axis and E-cadherin/ β -catenin complex.

Applications

This study demonstrated the relation between E-cadherin/ β -catenin complex and SDF-1-induced chemotaxis effect in HT29 colon cancer cells, thus providing a possible molecular mechanism underlying the SDF-1-induced chemotaxis effect.

Terminology

SDF-1, also known as CXCL12, belongs to the CXC chemokine family and interacts specifically with the seven-transmembrane, G protein-coupled receptor CXCR4. E-cadherin is not only an adhesion molecule but also a tumor suppressor as well as the most important epithelial marker. β -catenin is a key component of adherens junctions, necessary for homophilic cell-cell adhesion. In addition to the membrane-associated pool, β -catenin plays a role in cell-signaling and gene transcription.

Peer review

This is a well designed study, which may explain the development of colorectal metastasis *in vitro*.

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S- Editor Sun H L- Editor Wang XL E- Editor Zheng XM

Natural history of cytomegalovirus infection in a series of patients diagnosed with moderate-severe ulcerative colitis

Valeria Criscuoli, Maria Rosa Rizzuto, Luigi Montalbano, Elena Gallo, Mario Cottone

Valeria Criscuoli, Mario Cottone, Dibimis (Biomedical Department of Internal and Specialist Medicine), "V.Cervello" Hospital Palermo, University of Palermo, Palermo 90143, Italy
 Maria Rosa Rizzuto, Elena Gallo, Institute of Pathology, "V.Cervello" Hospital Palermo, Palermo 90143, Italy

Luigi Montalbano, Division of Gastroenterology, "V.Cervello" Hospital Palermo, Palermo 90143, Italy

Author contributions: Criscuoli V and Cottone M planned, conducted the study and drafted the paper; Montalbano L performed proctoscopy and rectal biopsies; Rizzuto MR examined histologic specimens; Gallo E performed PCR assay; all authors approved the final version of the paper.

Correspondence to: Valeria Criscuoli, MD, PhD, Dibimis (Biomedical Department of Internal and Specialist Medicine), "V.Cervello" Hospital Palermo, University of Palermo, Via Imperatore Federico 70, Palermo 90143, Italy. vale.cic@libero.it
 Telephone: +39-91-540614 Fax: +39-91-7305218

Received: August 25, 2010 Revised: October 26, 2010

Accepted: November 2, 2010

Published online: February 7, 2011

Abstract

AIM: To evaluate the natural history of human cytomegalovirus (HCMV) infection in a series of 28 ulcerative colitis patients in whom the search for HCMV was positive.

METHODS: A series of 85 patients with moderate-severe ulcerative colitis flare-up were evaluated for a HCMV search by performing a haematoxylin and eosin stain, immunohistochemical assay and nested polymerase chain reaction on rectal biopsies. Among 85 screened patients (19 of whom were steroid resistant/dependant), 28 were positive for HCMV; after remission the patients were followed up clinically and histologically.

RESULTS: Among the 22 patients with complete follow-up, in 8 (36%) patients HCMV-DNA persisted in the intestinal specimens. Among the HCMV positive patients, 4 (50%) experienced at least one moderate-severe

flare-up of colitis without evidence of peripheral HCMV. Among the 14 HCMV negative patients, 3 with pouches developed pouchitis and 5 out of 11 (45%) experienced a colitis flare-up.

CONCLUSION: Our preliminary results suggest that HCMV may remain in the colon after an acute colitis flare-up despite remission; it seems that the virus is not responsible for the disease relapse.

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Key words: Ulcerative colitis; Cytomegalovirus; Natural history; Polymerase chain reaction; Outcome

Peer reviewer: Dr. Douglas K Rex, MD, FACP, FACG, FASGE, Department of Medicine, Indiana University School of Medicine, 550 N. University Blvd., Indianapolis, IN 46202, United States

Criscuoli V, Rizzuto MR, Montalbano L, Gallo E, Cottone M. Natural history of cytomegalovirus infection in a series of patients diagnosed with moderate-severe ulcerative colitis. *World J Gastroenterol* 2011; 17(5): 633-638 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/633.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.633>

INTRODUCTION

The etiology of inflammatory bowel disease (IBD) is still unknown. The most current hypothesis is that IBD derives from an unsuitable and exaggerated immune response to normal mucosal resident bacterial microflora, likely induced by an external agent, in genetically predisposed individuals. Among the external agents, environmental factors unquestionably play a major role in the pathogenesis of IBD. Evidence of associations of bacterial factors derives from both human and animal studies. The virus takes an unclear part in the pathogenesis and disease development, but is probably involved.

In recent years several papers have described the link between viral infection and IBD onset, reactivation or steroid resistance^[1-4].

No clear data is available on the natural history of HCMV infection superimposed on IBD. The following questions remain unanswered: (1) does the virus disappear after remission of acute colitis relapse? (2) does the persistence of the virus detected by sensitive assays increase the risk of relapse?

The aim of this study was an attempt to answer the questions raised above and to evaluate the natural history of human cytomegalovirus (HCMV) infection after a severe colitis exacerbation.

MATERIALS AND METHODS

From 1997 to 2007, a prospective study was conducted on 85 patients with ulcerative colitis who were admitted to the Medicine department of V. Cervello Hospital in Palermo due to a severe colitis attack (according to the Truelove-Witts criteria) (Figure 1). All patients were treated with conventional and standardized corticosteroid treatment (1 mg/kg per day) and were endoscopically evaluated with a different approach: from 1997 to 1999 sigmoidoscopy was performed only in steroid resistant patients (17 patients) whereas from 2000 to 2007 sigmoidoscopy was performed in all patients (68 patients) at ward admission, taking systematically rectal biopsies. Therefore a total of 85 patients (Table 1) was investigated for HCMV infection with 3 different techniques.

The rectal biopsies were immediately fixed with 10% buffered formalin for 2 h to obtain tissue fixation; afterwards the preparation was subjected to several processes (lavage, dehydration, clearing, paraffin impregnation and embedding) to prepare sections with a thickness of 3-4 μ m.

The histologic specimens were examined using the following techniques: (1) Light microscopy with hematoxylin and eosin (HE) stain in order to document the microscopic disease activity and allow the detection of cytomegalic cells, markers of infected viral cells. Cytomegalic cells, which are 2- or 4-fold larger (25-35 μ m) than surrounding cells, contain a basophilic intranuclear inclusion (8-10 μ m) eccentrically placed and sometimes surrounded by a clear halo giving it an "owl's eye" appearance, and thickened nuclear membrane, frequently associated with smaller granular intracytoplasmic inclusions. Intranuclear inclusions were observed in epithelial, endothelial, stromal and smooth muscle cells. A biopsy was regarded as positive by light microscopy for HCMV if a single cell showed intranuclear or cytoplasmic inclusions and cytomegalic characteristics (Figure 2); (2) Immunohistochemical (ICH) procedure for HCMV performed on a paraffin-embedded section with monoclonal mouse antibodies anti-Human CMV (clone BM204) and conjugated to a peroxidase-labeled amino acid polymer by peroxidase-antiperoxidase (PAP) method in order to detect viral proteins. Nuclear or cytoplasmic antigen was identified by the typical brown reaction product of the PAP method; and

Table 1 Clinical characteristics of the series *n* (%)

Patient characteristics	Total	HCMV+	HCMV-
Sex (M/F)	50/35	19/9	31/26
Age at ward admission (yr)			
≥ 50		17	40
< 50		11	17
Disease extension			
Left sided colitis	39	11 (39)	28 (49)
Subtotal and pancolitis	46	17 (61)	29 (51)
Previous azathioprine treatment	21	11 (39)	10 (17.5)
Previous biologic treatment	9	2 (7)	7 (12)
Active disease at sigmoidoscopy			
Severe	51	16 (57)	35 (61)
Mild/moderate	34	12 (43)	22 (39)
Onset of disease (new diagnosis)	5	4 (14)	1 (2)
Reactivation (established disease)	80	24 (86)	56 (98)

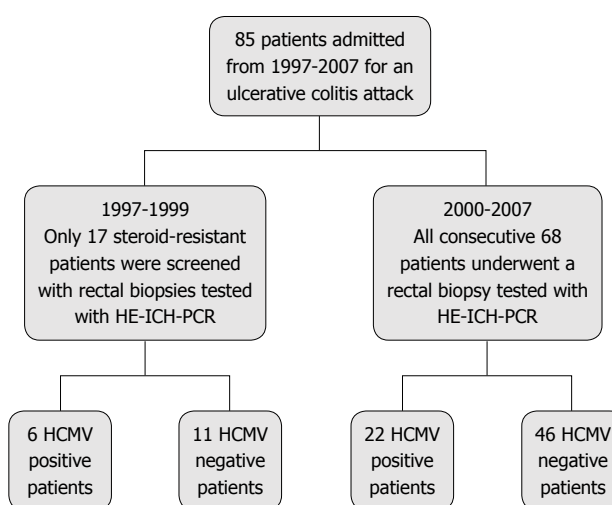


Figure 1 Series studied. HCMV: Human cytomegalovirus; ICH: Immunohistochemical; PCR: Polymerase chain reaction; HE: Hematoxylin and eosin.

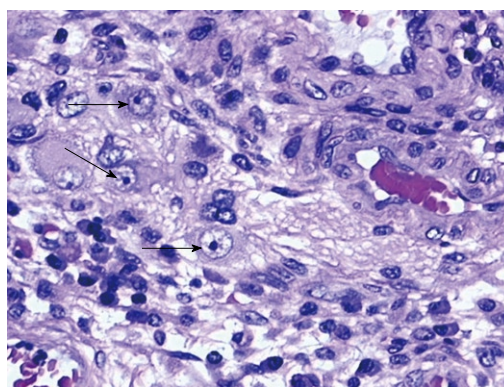


Figure 2 Original reproduction of epithelial cytomegalic cells (arrows) in rectal biopsy (hematoxylin and eosin stain, 40 \times).

(3) Nested polymerase chain reaction (nPCR): (a) DNA extraction: DNA was extracted from 10 mm sections of paraffin wax embedded tissues. Five sections were cut with a standard microtome from every paraffin wax block and transferred into a 1.5 mL microtube. To prevent cross

contamination between the samples, the microtome blade was washed with xylene and ethanol after sectioning of each block. DNA extraction was performed using a conventional method. The conventional method consisted of xylene/ethanol dewaxing followed by overnight proteinase K digestion in lysis buffer. The sample was heated at 95°C for 5 min to inactivate the proteinase K. We checked the quality of samples by PCR for the housekeeping gene β -globin (fragment of 268 bp); and (b) Detection of viral DNA: Nested PCR was used to detect the presence of viral DNA in colon tissues. Two pairs of primers annealed to the gB region of HCMV. Primers used for the first-round product and second-round PCRs are as follows (5' to 3'): first-round primer 1, GAGGACAACGAAATC-CTGTTGGGCA; first-round primer 2, GTCGACG-GTGGAGATACTGCTGAGG; second-round primer 3, ACCACCGCACTGAGGAATGTCAG, and second-round primer 4, TCAATCATGCGTTTGAAGAGGTA, to obtain a CMV fragment of 100 bp.

PCR reaction mixture contained 0.5 U *Taq* polymerase, 1 × PCR Buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl (pH 8.4), 0.2 mmol/L of each dNTPs, 1.5 mmol/L MgCl₂, 10 pmol of each primers and 100-200 ng of extracted DNA. The conditions for the first-round PCR were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by 5 min final extension at 72°C.

The conditions for second-round PCR were as follows: denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by 5 min final extension at 72°C.

The PCR amplification products were run on 2% agarose gel and stained with ethidium bromide and visualized under ultraviolet light.

HCMV-pp65 antigenemia

All patients were also tested for HCMV-pp65 antigenemia in peripheral leukocytes by CMV-CINAKit (Argene® Biosoft). The samples were considered positive for active HCMV infection using a cut-off value of 5 positive fluorescent nuclei/ 2×10^5 leukocytes^[5]; between 1 to 4 positive cells the result was considered questionable and the blood sample was repeated after 48 h to value a possible increase of cell nuclei positivity. If we detected histopathological examination positive we considered the patient HCMV-infected but not surely with active replication; if both tests (histology and pp65 antigenemia) were positive we judged the patients candidates for antiviral treatment due to a probable active replication. However the last decision about antiviral treatment was related to the disease activity without significant improvement of the disease course after treatment for underlying disease.

Sigmoidoscopy was performed in patients reaching clinical remission in order to control endoscopic activity and the presence of HCMV.

Table 2 Results and outcome of the series *n* (%)

	HCMV+ (HE-ICH/PCR)	HCMV- (HE-ICH/PCR)
Total patients	28	57
Steroid dependent/resistant (%)	68	19
Medical remission	22 (78)	51 (89)
Surgical remission	6 (21)	6 (10.5)
Death	3 (10)	0
Persistence of HCMV DNA+ at follow-up	8 (28.5)	0

HCMV: Human cytomegalovirus; ICH: Immunohistochemical; PCR: Polymerase chain reaction; HE: Hematoxylin and eosin.

The patients were followed clinically as outpatients quarterly to evaluate the remission time, the number of relapses, the immunosuppressive therapies and the need for surgery.

We define as remission a combination of clinical parameters (stool frequency ≤ 3 per day with no bleeding); the term relapse is used to define a flare of symptoms in a patient with established ulcerative colitis (UC) who is in clinical remission, either spontaneously or after medical or surgical treatment.

In the case of clinical relapse, sigmoidoscopy was performed with multiple biopsies and a search for HCMV in rectal biopsies by the 3 methods and peripheral pp65 antigenemia was conducted.

Moreover the asymptomatic patients were examined by colonoscopy every year in order to follow up the natural history of HCMV infection regarding histologic persistence, to understand more clearly the possible role of “bystander” or promoter to the disease relapse.

Oral and written informed consent was obtained from each patient before any procedure.

The rate of surgery and clinical relapse rates in the 2 groups of patients (HCMV positive and negative) were compared using χ^2 test.

RESULTS

The median clinical and endoscopic follow-up was 40 mo.

Among the 85 patients evaluated for HCMV infection from 1997 to 2007, in 28 (12 women and 16 men) the intestinal biopsy resulted positive by routine HE/ICH staining and α PCR assay according to the standard methods previously described. The overall prevalence data was 33% (Table 2). There was no association between HCMV and active immunosuppressive or biologic treatment. Ten patients among a total of 28 also displayed positive pp65 antigenemia (35%). Out of 10 positive patients for both histology and pp65 antigenemia, 7 received antiviral treatment (5 with ganciclovir and 2 with foscarnet) because of steroid resistance and clinical disease worsening, achieving remission in 5 patients. In these 5 treated patients HCMV disappeared at the first endoscopic control. Two patients were operated on because of a rapid clinical disease worsening: 1 patient died due to toxic megacolon. Among the 3 patients not treated with antiviral therapy for a contrain-

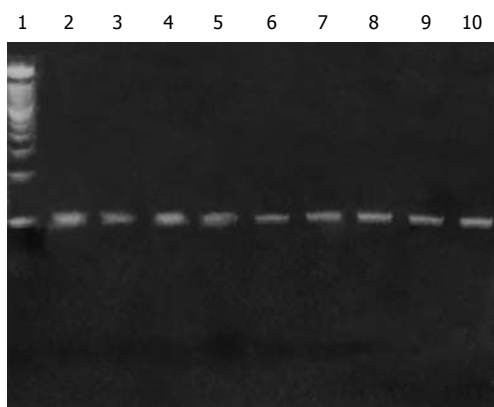


Figure 3 Ethidium bromide-stained 2% agarose gel demonstrating detectable second-round human cytomegalovirus products of nested polymerase chain reaction. Lane 1: Molecular size marker; Lanes 2-9: Positive patient samples; Lane 10: Positive control.

dication, 1 achieved remission with therapy for underlying disease, 2 patients were operated on for intractable disease and 1 of these 2 patients died due to post-operative complications.

Among the 18 patients who were histologically positive but pp65 antigenemia negative, 2 were operated on for intractable disease and 1 died; 16 improved with conventional therapy. Among the overall number of 28 patients who tested positive for HCMV, 6 underwent total colectomy (21%), 2 of them treated with antiviral treatment. Among the 57 patients who were negative for HCMV detection, 6 patients were operated on (10.5%).

Among the 25 surviving patients, 22 remained on follow-up (19 on medical remission and 3 with ileoanal pouch anastomosis) because 3 refused to undertake regular clinical and colonoscopic controls.

The 3 patients operated on underwent endoscopic and histological assessment of the ileoanal pouch during follow-up, showing a moderate grade of pouchitis according to PDAI 1 year after surgery, without detection of HCMV in the pouch.

Eighteen patients achieved clinical remission with medical treatment and 1 patient became steroid-dependent; all were followed up for an average period of 46 mo, all were clinically and endoscopically evaluated in accordance with the methods described above.

During the routine endoscopic and biopsy controls 11/19 patients were negative for HCMV detection both by traditional histology with HE and ICH in the intestinal biopsies and by α PCR assay, whereas 8 were positive for HCMV viral DNA detection by α PCR (Figure 3) and negative on light microscopy (HE and ICH) and on pp65 antigenemia.

Among the 8 patients in which positivity for HCMV-DNA persisted in the intestinal biopsies, 3 were treated with antiviral therapy at first detection of HCMV. Four experienced (50%) an early-moderate colitis flare up (within 3 mo) during the follow-up without detection of pp65 antigenemia in the peripheral leukocytes (Figure 4). A patient achieved remission with conventional steroid

Table 3 Clinical course and outcome of ulcerative colitis with persistent human cytomegalovirus at polymerase chain reaction

Patient No.	Age (yr)/sex	Immunomodulators at HCMV diagnosis	Disease relapse	Outcome
1	42/F	Aza	No	Remission with 5-asa
2	71/M	No	Yes	Remission with IFX
3	58/M	Aza	Yes	Procto-colectomy
4	74/M	No	Yes	Remission with IFX
5	65/F	No	No	Remission with 5-asa
6	65/M	No	Yes	Remission with steroid
7	45/M	Aza	No	Leukapheresis-IFX
8	28/M	No	No	Remission with Aza

HCMV: Human cytomegalovirus; Aza: Azathioprine; IFX: Infliximab.

treatment, 2 patients became steroid-dependent and were treated with anti-TNF therapy, both achieving remission. The fourth patient, who was steroid-dependent, intolerant to azathioprine and with a contraindication to anti-TNF therapy, underwent a total colectomy due to severity of disease. None were treated with antiviral treatment due to absence of positive antigenemia (Table 3). Among the 11 HCMV negative patients 5 (45%) experienced a reactivation of colitis and achieved remission with steroid treatment.

After remission, 8 patients (among 22) were treated with azathioprine (in 5 of them HCMV-DNA was present when the treatment was started) and 14 patients were treated with mesalazine (3 HCMV-DNA positive).

The χ^2 test comparing surgical intervention among positive HCMV (21%) and negative HCMV (10.5%) patients was not statistically significant ($P = 0.17$, odds ratio 2.32).

The χ^2 test comparing the relapse rate among HCMV positive (50%) and negative (45%) patients was not statistically significant ($P = 0.36$).

DISCUSSION

Our study was an attempt to answer two recurrent questions about the role of HCMV in IBD. The questions are about HCMV disappearance after remission of acute colitis relapse: we have demonstrated that HCMV persists in the colon after recovery of a colitis flare with a HCMV co-infection in a series of UC patients and remains detectable by sensitive methods such as α PCR without histological/ICH virus detection in a minority of patients. From this small series the persistence of virus in the colon of UC patients does not favour disease relapse because the virus persists in a latent state. The absence of pp65 antigenemia during the follow-up relapse and the response to the conventional corticosteroid treatment do not suggest a remarkable role of HCMV in the re-activation. Furthermore, it is unlikely that immunosuppressive treatment favours relapse of UC when HCMV persists in the colon.

To establish a connection between HCMV and UC

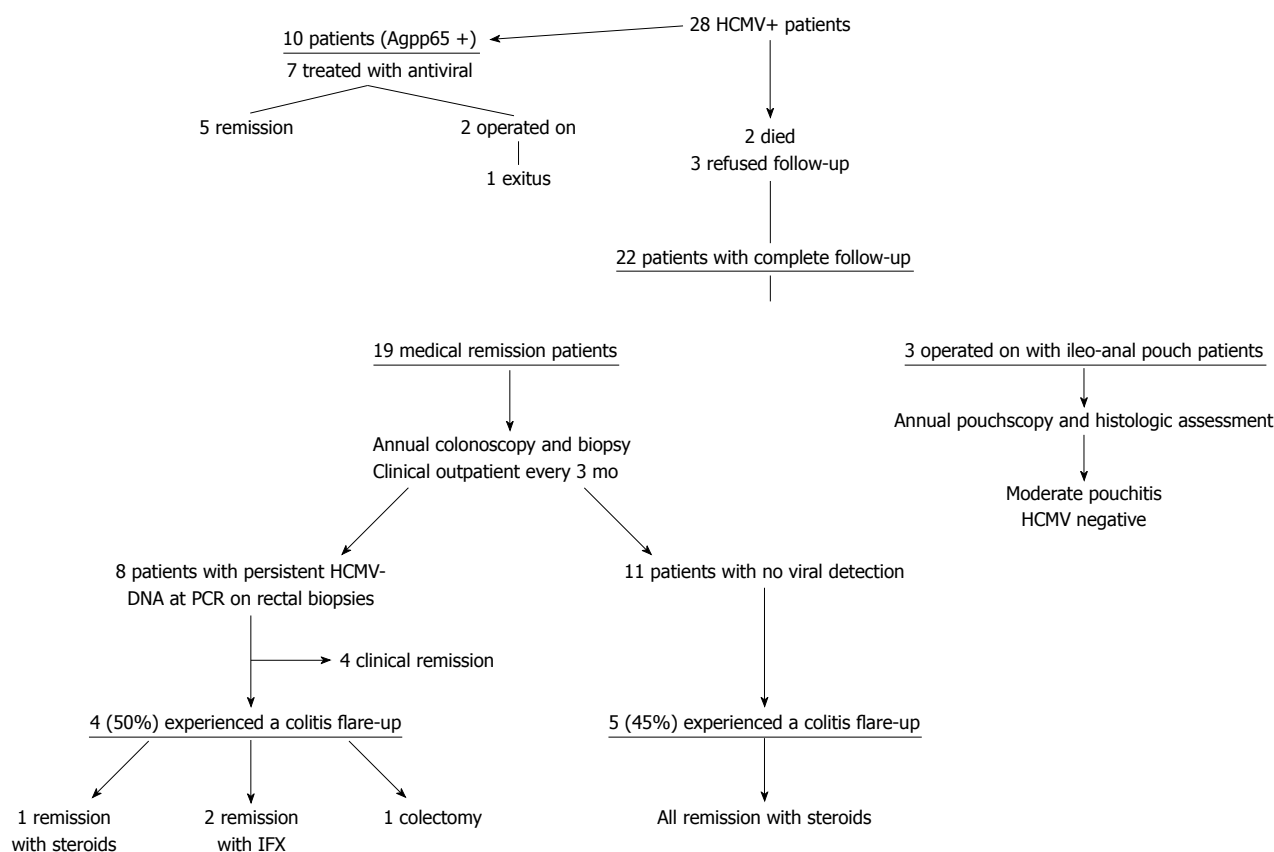


Figure 4 Follow-up human cytomegalovirus+ patients. IFX: Infliximab; HCMV: Human cytomegalovirus; PCR: Polymerase chain reaction.

more reports are needed. The interaction with the immune system plays a key role in the pathogenesis of HCMV disease, and the main determinant is an immunological impairment. As mentioned above, even most primary infections in humans are asymptomatic. No trace of the infection is observable except for seroconversion. In recent years, however, the disease has become more common probably due to the widespread use of immunosuppressants in oncology, in transplantation medicine and in chronic disease^[6].

What happens in intestinal tissue after persistence of HCMV-DNA is not known; maybe the colonic epithelial cells harbour the latent virus that became detectable using the highly sensitive PCR assay. The reactivation of virus from latency may depend on a complex interplay of biological factors with the host that, above all in patients with UC, are not clearly understood. Experimental studies suggest that latent CMV infection in the mouse may modulate mucosal immunity altering the susceptibility to gut microbiota without viral reactivation^[7].

Dimitroulia *et al*^[8] demonstrated that HCMV is frequently detected in IBD patients, showing that the virus genome was detected in intestinal tissue by polymerase chain reaction in 32.9% of the total IBD patients, while the HCMV genome in the blood was detected in 27.1% of these patients. Matsuoka *et al*^[9] showed that HCMV is frequently reactivated in a series of active UC seropositive patients, but that reactivation has little effect on the clinical

course and that most of the colitis reactivation with positive HCMV responds to conventional immunosuppressive therapies.

Kou *et al*^[10] demonstrated that the detected copy number of HCMV-DNA by PCR method is higher in the inflamed colonic tissue than in non inflamed colonic tissue in patients with UC refractory to immunosuppressive therapy. The author strongly supported the hypothesis that HCMV infection is involved in exacerbation of patients with IBD and the early detection of genome in intestinal tissue is important for an eventual change to the therapeutic approach.

We show that patients with HCMV infection were more frequently operated on than those without superimposed HCMV (even though not statistically significant) and this may suggest that the virus is a marker of risk of surgical treatment. This observation is an agreement with Cooper *et al*^[11] that showed in 1977 that HCMV infection might be responsible for acute toxic dilatation with increased colonic resection rate.

In patients who have undergone proctocolectomy we did not detect HCMV in the follow-up biopsies of the ileal pouch. Casadesus *et al*^[12] detected HCMV in a small series of patients with UC who underwent proctocolectomy and hypothesize that the virus may play an etiological role in pouchitis; other case reports^[13,14] demonstrate that HCMV is a rare but possible cause of refractory pouchitis to be considered for antiviral therapy.

In conclusion, our preliminary results suggest that HCMV may remain in the colon despite remission, probably activating a delicate balance with the host immune system in order to avoid its elimination, but the effects are not clear. The variability of genotypes may give an answer to the virulence and cell tropism so that we might be able to understand the different behaviour related to different host conditions.

A multicentre study including a more numerous population (almost 500 patients) with a longer follow-up is warranted to define whether the virus infection causes a disease complication or its presence does not alter the course of disease.

Limitations of the study

A limitation of study is that the assessment is based upon very few patients but it is very difficult to find a large population; possibly a multicentre study would solve this problem.

We performed the «PCR assay that detects only the presence of viral DNA in colon tissues. Maybe a quantitative assay that can distinguish between active and latent disease would give some useful information to correlate the replication of HCMV with bowel disease activity.

ACKNOWLEDGMENTS

The authors thank Professor Florian Mussnug for correcting the English text and Dr. Giuseppe Malizia for providing the photograph of nested PCR.

COMMENTS

Background

Cytomegalovirus (CMV) infection has been described as a cause of relapse of inflammatory bowel disease (IBD), in particular in ulcerative colitis patients, especially those receiving high-dose corticosteroid therapy. No clear data is available on the natural history of human CMV (HCMV) superimposed on IBD.

Research frontiers

The paper aimed to answer two questions concerning HCMV infection in ulcerative colitis patients.

Innovations and breakthroughs

This paper looks at the role of long-term HCMV persistence in patients with moderate to severe ulcerative colitis together with the likelihood this brings of relapse in patients that both cleared and did not clear the infection.

Applications

This is one of the first studies investigating the persistence of HCMV in colonic tissue of ulcerative patients after an acute flare up of disease.

Terminology

Nested polymerase chain reaction is a molecular assay that detects HCMV DNA using specific primers that amplify the gB region of HCMV. HCMV-pp65 antigenemia is an assay for polymorphonuclear leukocytes.

Peer review

I think there are some useful observations in the study, but some clarifications are needed.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zheng XM

Natural history of heartburn: A 10-year population-based study

Linda Bjork Olafsdottir, Hallgrimur Gudjonsson, Heidur Hrunn Jonsdottir, Bjarni Thjodleifsson

Linda Bjork Olafsdottir, Hallgrimur Gudjonsson, Bjarni Thjodleifsson, Department of Gastroenterology, Landspítali University Hospital, 101 Reykjavik, Iceland
Heidur Hrunn Jonsdottir, Department of Gastroenterology, The Social Science Research Institute, University of Iceland, 101 Reykjavik, Iceland

Bjarni Thjodleifsson, Department of Gastroenterology, Faculty of Medicine, University of Iceland, 101 Reykjavik, Iceland

Author contributions: Olafsdottir LB, Thjodleifsson B and Gudjonsson H designed research; Olafsdottir LB performed research; Jonsdottir HH analyzed data; Olafsdottir LB and Thjodleifsson B wrote the paper.

Supported by (in part) The Medical Research Fund of the National Hospital of Iceland; the Medical Research Fund of Wyeth, Iceland; AstraZeneca, Iceland; GlaxoSmithKline, Iceland; and the Icelandic College of Family Physicians

Correspondence to: Linda Bjork Olafsdottir, MSc Pharm, MBA, Department of Gastroenterology, Landspítali University Hospital, 101 Reykjavik, Iceland. linda04@ru.is

Telephone: +354-8966664 Fax: +354-5200801

Received: July 19, 2010 Revised: November 2, 2010

Accepted: November 9, 2010

Published online: February 7, 2011

Abstract

AIM: To study the natural history and prevalence of heartburn at a 10-year interval, and to study the effect of heartburn on various symptoms and activities.

METHODS: A population-based postal study was carried out. Questionnaires were mailed to the same age- and gender-stratified random sample of the Icelandic population (aged 18-75 years) in 1996 and again in 2006. Subjects were classified with heartburn if they reported heartburn in the preceding year and/or week, based on the definition of heartburn.

RESULTS: Heartburn in the preceding year was reported in 42.8% (1996) and 44.2% (2006) of subjects, with a strong relationship between those who experienced heartburn in both years. Heartburn in the preceding

week was diagnosed in 20.8%. There was a significant relationship between heartburn, dyspepsia and irritable bowel syndrome. Individuals with a body mass index (BMI) below or higher than normal weight were more likely to have heartburn. Heartburn caused by food or beverages was reported very often by 20.0% of subjects.

CONCLUSION: Heartburn is a common and chronic condition. Subjects with a BMI below or higher than normal weight are more likely to experience heartburn. Heartburn has a great impact on daily activities, sleep and quality of life.

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Key words: Heartburn; Follow-up; Questionnaire study; Epidemiology

Peer reviewer: Joachim Labenz, Associate Professor, Jung-Stilling Hospital, Wichernstr. 40, Siegen 57074, Germany

Olafsdottir LB, Gudjonsson H, Jonsdottir HH, Thjodleifsson B. Natural history of heartburn: A 10-year population-based study. *World J Gastroenterol* 2011; 17(5): 639-645 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/639.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.639>

INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most prevalent diseases worldwide^[1]. GERD is a chronic condition which usually manifests symptomatically, is a great burden for patients, and has significant socioeconomic implications^[2]. The prevalence of predominant gastroesophageal reflux symptoms appears to be stable over time^[3]. Heartburn is the typical GERD symptom and may be induced by various physiological and pathophysiological mechanisms^[4]. Heartburn, coupled with acid regurgitation and odynophagia, are considered to be highly specific for GERD^[1].

Functional heartburn is defined as episodic retrosternal burning in the absence of GERD, histopathology-based motility disorders or structural explanations^[5]. Heartburn alone has a prevalence of 17%-42% in Western populations^[2,3,5-7].

The prevalence of upper gastrointestinal symptoms in the general population is high and symptoms are associated with significant health-care utilization and diminished quality of life^[6]. In contrast, the natural history of heartburn has received limited attention and few epidemiological studies have focused on heartburn. Subjects with upper gastrointestinal symptoms are more likely to use prescription medication and are more likely to have seen a physician about symptoms than those with heartburn^[6]. There has been more focus on GERD than heartburn.

The aim of this present study was therefore to evaluate the natural history of heartburn in the Icelandic population prospectively over a 10-year period, as well as to evaluate different factors which are affected by heartburn both physically and sociodemographically. A parallel publication based on the same database, focusing on functional dyspepsia (FD), has been published^[8] as has another parallel publication regarding irritable bowel syndrome (IBS)^[9].

MATERIALS AND METHODS

Participants and setting

In 1996 an epidemiological study of gastrointestinal diseases was carried out in Iceland^[10], involving 2000 inhabitants in the range of 18-75 years of age. The individuals were randomly selected from the National Registry of Iceland. Equal distribution of sex and age was secured in each age group. In 2006 we attempted to contact all the same individuals as in 1996 as well as adding 300 new individuals in the 18-27 age group who were also randomly selected from the National Registry. A study questionnaire and explanatory letter were mailed to all eligible individuals at baseline. Reminder letters were mailed at 2, 4 and 7 wk, using the Total Method of Dillman^[11]. Individuals who indicated at any point that they did not want to participate in the study were not contacted further.

The questionnaire

The Bowel Disease Questionnaire (BDQ)^[12,13] was translated and modified for this study. The questionnaire was designed as a self-report instrument to measure symptoms experienced over the previous year and to collect the subject's past medical history^[14].

The Icelandic version of the BDQ questionnaire addresses 47 gastrointestinal symptoms and 32 items that measure past illness, health care use, items on sociodemographic and psychosomatic symptoms, together with a valid measure of non-gastrointestinal (non-GI) somatic complaints ascertained through the Somatic Symptom Checklist (SSC)^[15]. The SSC includes questions on 12 non-GI and 5 GI symptoms or illnesses. Individuals are instructed to indicate, on a 5-point scale, how often each symptom has appeared and how bothersome it has been. There were few changes to the later questionnaire (2006)

which addressed 51 gastrointestinal symptoms and 33 items that measure past illness, health care use, and sociodemographic and psychosomatic symptoms items. The 2006 Questionnaire furthermore addressed 17 items to identify heartburn and items related to heartburn.

Criteria for identifying heartburn

Subjects were classified with heartburn if they reported heartburn according to the following definition: Heartburn is a burning sensation in the retrosternal area (behind the breastbone). The pain often rises in the upper abdomen and may radiate to the chest.

Transition between disorders from initial and final surveys

A transition model used by Halder *et al.*^[14] was modified and applied for this study. The responses from the initial (1996) and final (2006) surveys were matched for each subject to examine the changes between disorders at an individual level for the 5 categories (FD, IBS, heartburn, frequent abdominal pain and no symptoms). A 5 × 5 table was used to model these multiple changes and collapsed into 6 groups, as illustrated in Table 1. Those with the most symptoms were prioritized higher. Those who developed more symptoms and those who reported fewer symptoms could be categorized into their respective groups. There were six patterns of symptoms, identified as follows: (1) symptom stability; (2) symptom increase; (3) symptom decrease; (4) symptom onset; (5) became asymptomatic; and (6) none of these symptoms.

Mortality data

For the 2006 survey we identified all deceased individuals with the assistance of the National Registry of Iceland (*Thjodskera*).

Statistical analysis

Tables were constructed to show frequency and percentage. Categorical data were analyzed using the χ^2 test. The type I error protection rate was set at 0.05. The exact *P* is listed in the tables and text. All the research data were imported into SPSS (Statistical Package of Social Science) software.

Ethics

The National Bioethics Committee of Iceland and The Icelandic Data Protection Authority (*Personuvernd*) gave their permission for the research.

RESULTS

Demographic data of involved individuals

In 1996 the response rate was 66.8% (1336/2000). Of the 1336 individuals who participated in 1996, 81 were deceased by 2006, five subjects were unable to answer, mainly because of old age, and 70 could not be traced to a current address. This left 1180 individuals, out of whom 799 responded. Therefore, the response rate in 2006 was 67.7% (799/1180). The mean age of the individuals in

Table 1 Transition among symptom subgroups between the initial and final surveys

FGID in 1996	Proportion of FGID in 2006 based on primary survey disorder (%)				
	FD	IBS	Heartburn	Frequent abdominal pain	No symptoms
FD (<i>n</i> = 111)	52.3 ¹	21.6 ³	14.4 ³	1.8 ³	9.9 ⁴
IBS (<i>n</i> = 152)	25.0 ²	30.3 ¹	19.7 ³	4.6 ³	20.4 ⁴
Heartburn (<i>n</i> = 173)	12.1 ²	12.1 ²	39.3 ¹	4.6 ³	31.8 ⁴
Frequent abdominal pain (<i>n</i> = 39)	12.8 ²	23.1 ²	17.9 ²	15.4 ¹	30.8 ⁴
No symptoms (<i>n</i> = 324)	3.4 ⁵	9.9 ⁵	17.3 ⁵	6.2 ⁵	63.3 ⁶

¹Stable; ²Increased symptoms; ³Decreased symptoms; ⁴Became asymptomatic; ⁵Developed symptoms; ⁶Remaining asymptomatic. FGID: Functional gastrointestinal disorder; FD: Functional dyspepsia; IBS: Irritable bowel syndrome.

Table 2 Study population: age and sex distribution

	Population 2006 (%)	Respondents 2006 (%)
Gender		
Male	50.3	42.2
Female	49.7	57.8
Age (yr)		
28-35	19.5	14.52
36-45	24.9	20.40
46-55	22.8	22.15
56-65	15.6	19.52
66-75	10.4	15.14
76-85	6.8	8.26
Total number	173859	799

1996 was 42 years, in 2006 it was 43 years, and 41 years for non-respondents in 2006. Women were more likely to respond than men in both years. A larger proportion of women than men responded again in 2006 (57.8%) than had responded in 1996, as is common in similar studies. The responders represented the population in all major factors concerning sex and age distribution. The response rate was also higher for older subjects than for younger ones. The age distribution and demographic details of the study cohort are presented in Tables 2 and 3.

Heartburn 10-year follow-up

At the 10-year follow-up, individuals were asked if they had experienced heartburn in the preceding year; 42.8% in 1996 and 44.2% in 2006 reported heartburn. There was a strong relationship between those who experienced heartburn in 2006 and those who reported heartburn in 1996. Two thirds of those who reported heartburn in 1996 also experienced heartburn in 2006. However, one third of those who reported heartburn in 2006 had not experienced it 10 years earlier.

Almost all who were on medication for heartburn reported relief with the medication. Individuals reported acid reflux once a month or more in 11% of cases in 1996 and 10% of cases in 2006.

There was a significant relationship between heartburn and dyspepsia and between heartburn and IBS, both in 1996 and in 2006.

Individuals of normal weight [body mass index (BMI) 18.5-24.9] were less likely to experience heartburn than individuals with a BMI below or higher than normal weight.

Individuals who smoked were not more likely to have heartburn than those who did not smoke. Individual alcohol consumption within the study group changed during the 10-year period of 1996 to 2006. Alcohol consumption was not associated with heartburn.

Transitions among symptom subgroups between the initial and final surveys

As described in the Methods section, the groups in this analysis were defined as mutually exclusive using a symptom hierarchy so that each subject appears in only one category for both the 1996 and 2006 surveys. There was a “no symptoms” category for those who did not meet any of the criteria applied for functional gastrointestinal disorders. Due to the hierarchical classification only a few participants occurred in some categories.

There was a substantial change in numbers in all the categories over time (Table 1). The group “no symptoms” was the most common (63.3%). Of the heartburn group 39.3% were stable and 31.8% reported “no symptoms”; 24.2% reported increased symptoms and 4.6% decreased symptoms. Of the FD group 52.3% remained stable and 9.9% reported “no symptoms” in 2006. Most of the subjects who were in the IBS group, or 30.3% of the total, were stable over the 10-year period; 20.4% reported “no symptoms” in 2006 and 25.0% showed an increase in symptoms over the 10 years. In 2006, 15.4% of the subjects reported stable frequent abdominal pain, 30.8% reported “no symptoms” and 53.8% reported increased symptoms.

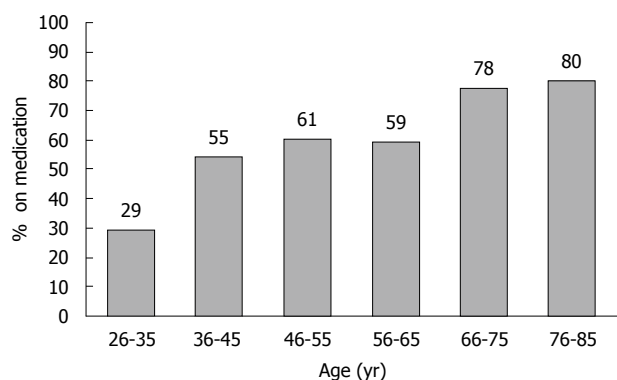
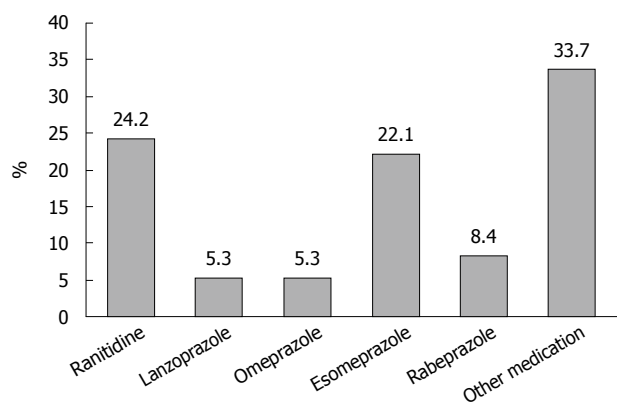
The distribution of the 6 transition groups was: 22.3% symptom stability, 12.6% symptom increase, 10.9% symptom decrease, 14.9% developed symptoms, 13.6% became asymptomatic, and 25.7% had no symptoms in either 1996 or 2006.

Heartburn in subjects in 2006

In the 2006 questionnaire individuals were asked additional questions regarding heartburn during the preceding week. Heartburn during the preceding week was reported by 20.8% of the subjects (19.0% male, 22.1% female). Of these, 60.5% reported taking medicine for heartburn. Increasing age was not a significant factor in prevalence of heartburn/reflux disease. Age was, however, a significant factor associated with the use of medication for heartburn (Figure 1). Most subjects took ranitidine or esomeprazole for their symptoms (Figure 2).

Table 3 Sociodemographic characteristics and the development and disappearance of heartburn

	<i>n</i>	Never HB (%)	Lost HB (%)	Retained HB (%)	Developed HB (%)	χ^2	<i>P</i> -value
Gender						1.687	0.640
Male	330	40.3	14.5	30.3	14.8		
Female	441	41.5	14.7	26.5	17.2		
Age group (yr)						15.542	< 0.05 ^a
66-85	170	54.8	10.6	27.1	10.6		
36-65	488	37.3	16.4	29.3	17.0		
28-35	113	40.7	13.3	24.8	21.2		
BMI (kg/m ²)						21.685	< 0.01 ^b
> 30	154	31.8	14.3	37.0	16.9		
> 25 and ≤ 30	314	37.3	14.3	31.5	16.9		
≤ 25	286	49.3	15.0	19.9	15.7		
Level of education						6.156	0.724
> 4 years' further education	225	39.6	12.9	28.9	18.7		
3-4 years' further education	279	41.9	17.6	25.1	15.4		
< 3 years' further education	92	39.1	13.0	33.7	14.1		
No further education	161	41.6	13.0	29.8	15.5		
Employment status						6.276	0.099
Employed	574	39.7	15.5	27.0	17.8		
No employment	189	44.4	12.2	31.7	11.6		
Alcohol						4.503	0.609
≥ 7 drinks per week	43	37.2	9.3	34.9	18.6		
1-6 drinks per week	404	39.1	14.6	28.2	18.1		
No alcohol	309	43.0	15.5	27.5	13.9		
Smoking						8.773	0.187
Smokers, > 15 cigarettes per day	63	34.9	20.6	25.4	19.0		
Smokers, ≤ 15 cigarettes per day	113	31.9	17.7	34.5	15.9		
No smoking	496	43.5	13.7	26.2	16.5		

^a*P* < 0.05, ^b*P* < 0.01. HB: Heartburn; BMI: Body mass index.**Figure 1** Age and use of medication.**Figure 2** Which medication do you take?**Table 4** Heartburn and relationship to medication, food/beverages and tiredness

Variable	<i>n</i>	% of heartburn prior week
On constant medication	30	27.3
Medication only when experiencing symptoms	77	85.6
Tiredness (lethargy)		
Frequent	20	13.2
Sometimes/seldom	73	48.0
Never	59	38.8
Heartburn caused by food and beverages		
Very often	32	20.0
Sometimes/seldom	118	73.8
Never	10	6.3
Increased heartburn caused by specific food		
Very often	35	22.7
Sometimes/seldom	92	59.7
Never	27	17.5

27.3% reported they were on constant medication. Most individuals (85.6%) reported taking medication only when they experienced symptoms (Table 4), although there was some overlap here between groups. Six subjects reported having had an operation for reflux disease.

Tiredness or lethargy was reported as occurring frequently by 13.2% of subjects, reported rarely or seldom by 48%, and reported as never having occurred by 38.8% (Table 4).

Heartburn caused by food or beverages was reported as occurring very often by 20%, 73.8% reported some or

Table 5 Symptoms or activities affected by heartburn (caused by heartburn)

Variable	<i>n</i>	% of heartburn prior week
Felt bad		
Frequent	21	13.1
Sometimes/seldom	119	74.4
Never	20	12.5
Less food and beverages consumption		
Frequent	9	5.9
Sometimes/seldom	77	50.3
Never	67	43.8
Less family activities		
Frequent	1	0.6
Sometimes/seldom	32	20.8
Never	121	78.6
Trouble with sleeping		
Frequent	9	5.8
Sometimes/seldom	70	45.2
Never	76	49.0
Felt hopeless, worried or impatient		
Frequent	9	5.8
Sometimes/seldom	42	27.3
Never	103	66.9
Felt worried or scared for their health		
Frequent	5	3.2
Sometimes/seldom	47	30.3
Never	103	66.5
Felt irritable		
Frequent	21	13.6
Sometimes/seldom	80	51.9
Never	53	34.4
Neglect specific food or alcohol		
Frequent	36	23.1
Seldom	66	42.3
Never	54	34.6
Affects their daily activities		
Frequent	3	1.9
Sometimes/seldom	32	20.5
Never	121	77.6
Unable to move (sports, hobbies and outside of home)		
Frequent	3	1.9
Sometimes/seldom	34	21.8
Never	119	73.6

minimal heartburn and 6.3% never. Increased heartburn caused by a specific food was reported as occurring very often by 22.7% and sometimes by 59.7%. A specific food significantly more often provoked considerable heartburn in women than in men (Table 4).

As can be seen in Table 5, heartburn can affect symptoms or activities in many cases. Three out of four heartburn subjects claimed that they felt badly sometimes or seldom. One out of three heartburn subjects felt hopeless, anxious or impatient. And one out of three also reported being worried or scared because of heartburn every week.

Only 1.9% of the subjects reported that heartburn frequently affected their daily activities, whereas one fifth claimed that their daily activities were only sometimes or seldom affected by heartburn. Three out of four subjects reported that heartburn made them irritable. And one out of four heartburn subjects reported that heartburn resulted in less family activities, affected their daily activities and meant they were unable to move in sports, hobbies and

outside of home. Half of the heartburn subjects reported trouble with sleeping because of heartburn.

Many heartburn subjects reported less food and beverage consumption and that they avoided specific food or alcohol because of the heartburn.

DISCUSSION

In this study our main focus was on the natural history of heartburn over a 10-year period in an Icelandic population. The only other long-term study, to our knowledge, that has focused on heartburn is a long-term community study in Sweden covering a maximum of 7 years^[3]. There are strengths and weaknesses in both studies, but taken together they give a reasonably accurate picture of the natural history of heartburn.

The strength of our study is the use of a stable, homogeneous and well-informed population. The sample was randomly selected from the National Registry of Iceland and represented the nation as a whole in selected age groups. The population of Iceland was around 300 thousand inhabitants at the time of the study and the sample was approximately 1% of the whole population from all around the country. The BDQ, the questionnaire used, assesses the whole range of gastrointestinal functional disorders.

The prevalence of heartburn is high in Iceland. More than two out of five subjects reported heartburn in the preceding year. Half of those reported heartburn in the preceding week. Heartburn was reported as still existing after 10 years for 2 out of 3 subjects in the study. The study by Agréus *et al*^[3] showed that the prevalence of predominant gastroesophageal reflux symptoms appears to be stable over time. Results from studies of patients suggest that GERD is a chronic disease in most cases^[3,16,17]. One third of subjects who did not report heartburn in 1996 had developed heartburn 10 years later. So even though the total prevalence of heartburn was almost the same in both 1996 and 2006, there was a change among over one third of subjects reporting heartburn.

Heartburn subjects with a BMI either lower than or higher than normal weight were more likely to experience heartburn than subjects with normal weight. A study by Aro *et al*^[18] found that reflux symptoms are linked to obesity and specifically that the presence of gastroesophageal reflux symptoms was linked to reflux esophagitis in the obese population. Festi *et al*^[19] concluded that it was likely that GERD and obesity are in some way linked and that it was possible to hypothesize that GERD may be a curable condition through the control of body weight. This may also be true for heartburn.

The transition analysis showed a substantial change in numbers in all the categories. The stability of each disease varied. FD subjects were the most stable throughout the 10 years (52.3%). Of the heartburn group 39.3% were stable, as were 30.3% of the IBS group and 15.4% of the frequent abdominal pain group. A quarter of the heartburn group had increased symptoms in 10 years, 4.6% decreased symptoms and one third developed no

symptoms in 10 years. There was a significant relationship between IBS and heartburn as well as FD and heartburn.

Half (45.1%) of the subjects who reported heartburn in the preceding year experienced heartburn in the previous week. Food and beverages play a large part in eliciting heartburn; very often in 20.0% of the cases and sometimes in 73.8% of the cases. Subjects also very often experienced increased heartburn caused by a specific food in 22.7% of the cases. Heartburn did not seem to be the cause for less food and beverage consumption, but one out of five heartburn subjects did avoid a specific food or alcohol because of heartburn. Festi *et al*^[19] report that no definitive data exist regarding the role of diet and specific foods or drinks in GERD clinical manifestations^[19].

Heartburn is associated with feeling tired (61.2%), feeling bad (87.5%) and with irritation (65.5%). One third felt worried or scared for their health because of heartburn symptoms and one third also felt that heartburn caused them to feel hopeless, worried or impatient (33.1%). Every fifth heartburn subject reported that heartburn affected activities such as daily and family activities, as well as that heartburn caused them to be unable to move normally and therefore affected their participation in sports, hobbies and outdoor activities. This effect of heartburn on normal life and activities may have affected the subjects in the manner of a chronic condition throughout the 10 years of the study, and therefore had a great impact on quality of life. This finding is in line with McDougall *et al*^[17] who showed in their study on reflux esophagitis and quality of life that it was not bodily pain and vitality that were impaired, but general health and social function.

Three out of five of all the heartburn subjects in 2006 reported taking medicine for heartburn. Almost all the subjects who were on medication for heartburn reported relief provided by the medication. Age was a significant factor for the use of medication for heartburn. Most subjects took ranitidine or esomeprazole for their symptoms.

Few studies have addressed the impact of nocturnal reflux symptoms in heartburn subjects. A study by Farup *et al*^[20] showed that the prevalence of nighttime heartburn in GERD patients under routine care was high, up to 49% for 1 of 3 years. A population-based survey in the United States claimed that the overall prevalence of nocturnal GERD symptoms was 10%, with 74% of subjects with GERD symptoms fitting the criteria for nocturnal GERD^[21]. In our study, sleep was frequently affected in 5.8% of cases and 45.2% of heartburn subjects were sometimes or seldom troubled with sleeping in the prior week. These numbers can be expected to be higher for the preceding year, since we asked specifically about the preceding week.

There are some limitations to our study. The subjects were not specifically interviewed or examined to evaluate the possibility of organic disease. However, a 10-year (postal) follow-up went some way towards making an organic cause of symptoms unlikely. Furthermore, since the response rate was 66.8% in 1996 and 67.7% in 2006, a dropout bias cannot be excluded.

In summary, heartburn is a common condition in the population of Iceland. The prevalence is slightly higher than reported elsewhere. Heartburn is a chronic condition, affecting every fifth person every week. Heartburn subjects with a BMI lower or higher than normal weight were more likely to experience heartburn than subjects of normal weight. Heartburn did not seem to result in less food and beverage consumption, but one out of five heartburn subjects did avoid a specific food or alcohol because of the heartburn. Heartburn had a great impact on daily activities and quality of life. Half of the heartburn subjects experienced sleep disturbances because of this condition.

COMMENTS

Background

Heartburn is a signature symptom of gastroesophageal reflux disease (GERD), which is a cluster of symptoms and signs associated with regurgitation of stomach acid up to the pharynx and mouth. Patient-based studies of GERD have shown high prevalence and chronicity, particularly in Western societies. GERD is associated with significant health-care utilization and diminished quality of life. Heartburn, coupled with acid regurgitation and painful swallowing are considered to be highly specific for GERD. Very few epidemiological studies have been performed with regard to heartburn, and only one has been population-based. The natural history of GERD or heartburn has received little attention. The pathophysiology of GERD and heartburn is basically unknown.

Research frontiers

The prevalence of upper gastrointestinal symptoms in the general population is high and symptoms are associated with significant socioeconomic consequences. The prevalence and natural history of heartburn is of importance as well as its association with functional dyspepsia and irritable bowel syndrome, and sociodemographic factors such as body mass index (BMI). The aim of the present study was therefore to evaluate the natural history of heartburn in the Icelandic population prospectively over a 10-year period, as well as to evaluate different factors which are associated with heartburn both physically and sociodemographically.

Innovations and breakthroughs

The prevalence of heartburn is high in Iceland. More than two out of five subjects reported heartburn in the preceding year. Half of those reported heartburn in the preceding week. Heartburn was reported as still existing after 10 years for 2 out of 3 subjects in the study. Heartburn subjects with a BMI either lower or higher than normal weight were more likely to experience heartburn than subjects with normal weight. There was an association between heartburn, functional dyspepsia and irritable bowel syndrome and patients floated over time between these categories. This suggests a common etiopathogenesis of these disorders. The quality of life was diminished due to a variety of factors such as worries, irritability, intolerance to specific foods and sleep disturbance.

Applications

The prevalence and natural history of heartburn and its risk factors are important for management and prognosis. Heartburn can be regarded as a reliable surrogate marker of GERD. This study creates a database for future studies and hopefully stimulates studies in other countries. Secular prevalence trends and international comparison can contribute towards understanding of the pathophysiology of the disease.

Terminology

A 10-year follow-up population-based, questionnaire study of the Icelandic population was performed. The primary aim was to study the prevalence and natural history of heartburn. Subjects were classified as having heartburn if they reported heartburn according to the following definition: Heartburn is a burning sensation in the retrosternal area (behind the breastbone). The pain often rises in the upper abdomen and may radiate to the chest.

Peer review

Heartburn alone has a prevalence of 17%-42% in Western populations and is associated with extensive health care expenses and diminished quality of life. Comparative international population-based studies are needed to document secular trends and to elucidate the reasons for the different prevalence in various countries.

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S- Editor Sun H L- Editor Logan S E- Editor Lin YP

Percutaneous aspiration and drainage with adjuvant medical therapy for treatment of hepatic hydatid cysts

Mohammed I Yasawy, Abdelrahman E Mohammed, Sammak Bassam, Mohammed A Karawi, Sohail Shariq

Mohammed I Yasawy, Department of Internal Medicine, Dammam University Hospital, Al-Khobar 31952, Saudi Arabia
 Abdelrahman E Mohammed, Consultant Gastroenterologist, Riyadh Military Hospital, Riyadh 11159, Saudi Arabia
 Sammak Bassam, Department of Radiology, Riyadh Military Hospital, Riyadh 11159, Saudi Arabia

Mohammed A Karawi, Department of Gastroenterology, Riyadh Military Hospital, Riyadh 11159, Saudi Arabia

Sohail Shariq, Department of Internal Medicine, King Fahd Military Medical Complex, Dhahran 31932, Saudi Arabia

Author contributions: Yasawy MI was in charge of adjuvant medical therapy and wrote the paper; Bassam S was the Senior Radiologist in charge of the radiological intervention; Mohammed AE and Karawi MA were consultants who offered and provided the cases; Shariq S was responsible for the literature search and collected the relevant references.

Correspondence to: Dr. Mohd I Yasawy, Consultant Internist/Gastroenterologist, Associate Professor, Department of Internal Medicine, Dammam University Hospital, PO Box 40143, Al-Khobar 31952, Saudi Arabia. yasawy@hotmail.com
 Telephone: +966-3-8966741 Fax: +966-3-8966741

Received: July 23, 2010 Revised: November 11, 2010

Accepted: November 18, 2010

Published online: February 7, 2011

Abstract

AIM: To determine the efficacy and success of percutaneous aspiration irrigation and reaspiration (PAIR) in the management of hepatic hydatidosis.

METHODS: Twenty-six patients with 32 hepatic hydatid cysts had PAIR. Twenty-two patients received at least 2 wk of drug therapy before the procedure was carried out to reduce the risk of recurrence from spillage during the procedure. The procedure was performed under local anesthesia with a 19-gauge 20 cm long needle, the cyst was punctured, cystic content (approximately 30 mL) was aspirated by a 12-14 F pigtail catheter and aspirated fluids were sent for analysis. Once the cyst was almost empty, two-thirds of the net amount of ma-

terial aspirated was replaced by hypertonic saline and left in the cavity for about 30 min, with the catheter left in place for reaspiration of most of the fluid. When the amount of fluid drained was less than 10 mL per 24 h, the drainage catheter was removed.

RESULTS: All 32 cysts showed evidence of immediate collapse after completion of the procedure, and before discharge from hospital, ultrasound examination showed fluid reaccumulation in all cysts. Serial follow-up showed a progressive decrease in the size and change in the appearance of cysts. To confirm the sterility of these cystic cavities, seven cysts were reaspirated on average 3 mo after the procedure. Investigations revealed no viable scolices.

CONCLUSION: PAIR using hypertonic saline is very effective and safe with proper precautions.

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Key words: Percutaneous aspiration irrigation and reaspiration; Hepatic hydatid cyst; Adjuvant medical therapy; Treatment outcome

Peer reviewer: Marco Vivarelli, MD, Assistant Professor, Department of Surgery and Transplantation, University of Bologna, S. Orsola Hospital, Bologna 40123, Italy

Yasawy MI, Mohammed AE, Bassam S, Karawi MA, Shariq S. Percutaneous aspiration and drainage with adjuvant medical therapy for treatment of hepatic hydatid cysts. *World J Gastroenterol* 2011; 17(5): 646-650 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/646.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.646>

INTRODUCTION

Until 1980, surgery was the only method of treatment for hepatic hydatidosis. Despite improved surgical techniques

and use of scolical compounds, a high incidence of hydatid cyst recurrence and dissemination is still a major problem. Spillage is known to occur at surgery^[1,2]. On the other hand, medical therapy is associated with side effects and it is effective only in some cases and need several courses to reach a response with albendazole alone^[3], but the outcome is better with combined therapy^[4]. In recent years, percutaneous drainage of hepatic hydatid cysts (HHC) has emerged as a minimally invasive, safe therapy, and a potential alternative to surgery. Different methods have been applied with variable success and healing rates^[5-7].

In this study, we prospectively assessed the value of percutaneous drainage with adjuvant medical therapy in 26 patients with confirmed 32 HHC over an average follow-up period of 10 years.

The Armed Forces Hospital in Riyadh is a well-known tertiary center in the region and is a 1000-bed hospital with facilities for hepatobiliary and liver transplant services. Percutaneous aspiration irrigation and reaspiration (PAIR) was introduced into the hospital in 1993. Between 1985 and 1992 albendazole was used alone in the management of hydatid cysts, and in 1993 praziquantel was added to albendazole as combination therapy.

Previously we reported our first patient with percutaneous drainage of a hydatid cyst of the liver in 1994^[8], and our first patient with a lung hydatid cyst with pleural effusion in 1991^[9].

MATERIALS AND METHODS

The study involved 26 patients (14 males and 12 females; age range 13-53 years) with 32 HHC (Table 1). Eleven patients were recently diagnosed with HHC and had no previous medical or surgical intervention. Seven patients had cyst recurrence following surgical excision. Eight patients had received long-term medical treatment with albendazole and praziquantel for an average of 24 mo with only a partial response of less than 30% reduction in size of the cysts. Nineteen patients had a solitary cyst and four others had multiple liver cysts. Three patients had extra hepatic disease in the lung ($n = 2$) and spleen ($n = 1$). Twenty-six cysts were located in the right lobe of the liver, five in the left, and one in the caudate lobe. All patients were complaining of right upper quadrant pain and/or pressure symptoms and had an abdominal ultrasound (US) examination. The average diameter was 10.2 cm (range, 5.5-18.5 cm). The diagnosis was confirmed by imaging modalities and positive serology (indirect hemagglutination titer $> 1:128$) in all patients. The cysts were classified by US according to the Gharbi *et al.*^[10] classification into Type I ($n = 4$), Type II ($n = 8$), Type III ($n = 2$) and Type IV ($n = 18$) (Table 2). Two of the Type IV cysts had a thin rim at their periphery. Four patients had abnormal liver function tests, 10 had elevated erythrocyte sedimentation rate, and six had high eosinophil count. The 26 patients and their results were evaluated by all relevant staff i.e. surgeons, gastroenterologist and senior

Table 1 Location and distribution of the cysts (n)

Type of patient	Site of cyst	Multiple liver cyst or extrahepatic
Prolonged medical therapy with albendazole (8)	Right liver lobe (8)	Lung cyst (1)
Recovered post surgery (7)	Left liver lobe (1)	
	Right liver lobe (4)	Multiple cysts (2)
	Left liver lobe (1)	
	Caudate lobe (1)	
New patients (11)	Right liver lobe (8)	Multiple cysts (4)
	Left liver lobe (3)	Lung cysts (1)
		Spleen cysts (1)

Table 2 Gharbi classification

Cyst type	No. of cysts
I	4
II	8
III	2
IV	18

radiologist. Management options i.e. a pharmacological approach, surgical intervention and PAIR were explained and all the above patients chose PAIR. Informed consent prior to the procedure was obtained from all patients. In 22 patients, albendazole 400 mg twice daily and praziquantel 50 mg/kg daily was given orally for at least 2 wk prior to and 4 wk after the procedure to all patients to reduce the risk of possible hydatid cyst fluid spillage and dissemination into the peritoneal cavity. The patient fasted overnight. The procedure was performed under heavy sedation (midazolam 5 mg iv and pethadine 50 mg im) with close monitoring to treat any potential complication including anaphylaxis. Under aseptic conditions, a Teflon sheath needle (19 gauge, 20 cm long; Meditech) was introduced percutaneously through the biopsy port of the 3.5 MHz probe into the cyst under US guidance (Aloka SSD 680). The puncture was made through thick normal liver tissue surrounding the cyst and whenever possible the right intercostal route was used to minimize the risk of hydatid fluid spillage into the peritoneum (Figure 1A). Once the cyst is punctured, a small amount of fluid (10-30 mL) was aspirated for cyst decompression followed by insertion of a 12F or 14F (van Sonnenberg sump drainage, Meditech) catheter, the use of such a large catheter was to prevent catheter clogging by membranes and daughter cysts during aspiration. Once the cyst was almost empty, injection of contrast medium under fluoroscopic control was performed to exclude cyst communication with the biliary system. Two-thirds of the aspirated material was replaced by hypertonic saline (23.4%) and left in the cavity for 20-30 min. The fluid was then reaspirated as much as possible and the catheter was left in place to drain by gravity. Immediately after aspiration, examination of fluid to identify scolices, hooklets, pieces of laminated membranes or daughter cysts were performed in the parasitology laboratory. After the procedure, the patients were closely observed for possible com-

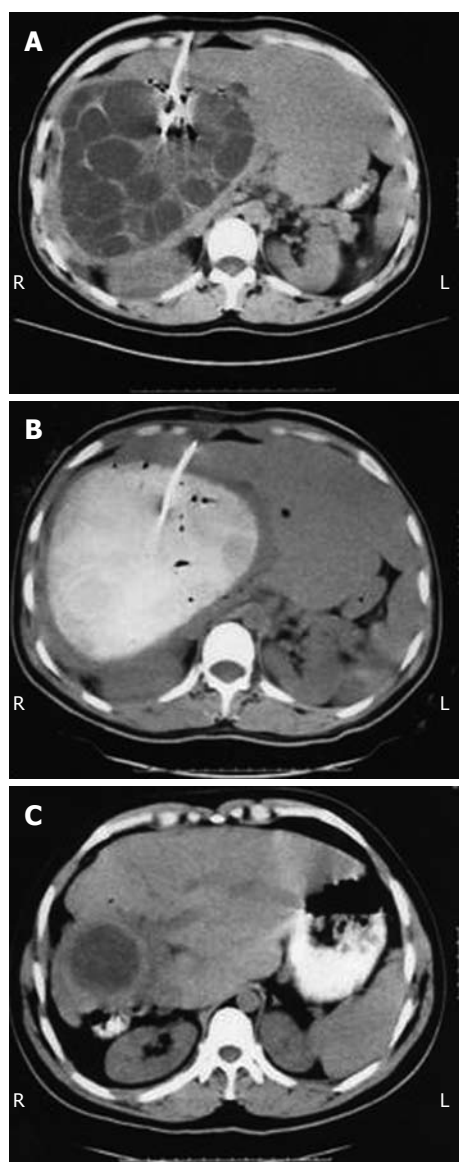


Figure 1 Ultrasound examination. A: Puncture of the hydatid cyst and insertion of a catheter; B: End of percutaneous aspiration irrigation and reaspiration procedure; C: Six months later, aspiration showed only granulation and necrotic tissue without any evidence of hydatidosis.

plications for 48 h. The drainage catheter was left in place for an average period of 3 d (range, 2-6 d) (Figure 1B) and removed when the amount of fluid drained was less than 10 mL/24 h. Follow-up examination in the form of clinical assessment, blood tests, serology and imaging examination with US and/or computed tomography (CT), were performed at 1, 2 and 3 mo after drainage for an average period of 6 mo (range, 2-11 mo), followed by 6-monthly blood tests and yearly imaging for an average period of 10 years.

RESULTS

All 32 cysts were successfully treated by PAIR, with relief of symptoms in all 26 patients with an average hospital stay of 6 d. There was collapse of all cysts immediately

Table 3 Complications post aspiration

Complications	No. of patients
Anaphylactic reaction	1
Urticarial rash	2
Hypernatremia	2
Fever	10
Pleural effusion	6
Total	21

after completion of percutaneous drainage and removal of the drainage catheter. Before discharge from hospital, US examination showed fluid reaccumulation in all cysts within an average of 2 d after catheter withdrawal reaching an average size of 59% (range, 48%-74%) compared to the size of the cysts before drainage. Serial follow-up CT and US examination showed a progressive decrease in the size and change in the appearance of the cysts. Two patterns of healing were observed, the first was a predominantly cystic cavity with detached membranes. All the cystic cavities lost their rounded contour appearance suggestive of being under less tension. The second pattern was a predominantly solid mass.

Asymptomatic fluid reaccumulation following drainage and catheter removal happened in nearly all cysts with an average size of 59% (range, 48%-74%) compared to the predrainage cyst size. However, on regular follow-up examinations, a progressive decrease in the residual cavity with two distinctive healing patterns was observed. A cystic residual cavity with internal membranes was predominantly seen in patients with Type I - II cysts, and a solid mass was predominantly seen in patients with Type IV cysts. The complex large Type IV HHC with a predominantly solid component showed better results following drainage, with an overall reduction in size of 51.5% compared to 29% in patients with Type I and II cysts in whom the cysts had a predominantly fluid component.

To confirm the sterility of the residual cystic cavities, seven out of 32 cysts were reaspirated, three at an average of 3 mo after drainage, and four at an average of 6 mo after drainage. All reaspirated cyst cultures for microorganisms were negative, and microscopy revealed debris of hydatid membranes and hooklets in some cases but no viable scolices (Figure 1C). Serial follow-up serological examination showed a 2-fold elevation in the indirect hemagglutination titer following drainage in 18 patients compared to the titer level before drainage, and it remained elevated at an average follow-up period of 16 mo. No major complications developed during or after the procedure except for a mild anaphylactic reaction which responded very well to immediate treatment (Table 3). Two patients developed urticarial reactions 8 h following drainage, but responded well to antihistamines and steroids. Fever occurred in 10 patients but was mild and transient, and cultures of fluid from the drainage catheters were negative. Minimal right pleural effusion occurred in six patients. The liver cysts in these six patients were rela-

tively large and reached the right hemidiaphragm. However, the pleural effusion was small and resolved completely before the patients were discharged. Two patients developed transient hypernatremia and one patient showed an anaphylactic reaction during the procedure but responded to immediate management. No radiological evidence of reactivation of aspirated cysts was seen during the average of follow-up of 10 years.

DISCUSSION

Surgery is considered as the standard treatment for HHC. However, surgery is not without risks and there is a high incidence of dissemination, infection and recurrence of 2% to 25%, with morbidity of 0.5% to 4%^[11-16]. Furthermore, surgery is not advisable in elderly patients with cardiac or pulmonary disease, nor in recurrent cases. Medical treatment alone in the form of mebendazole, and recently albendazole and praziquantel, have been used as an alternative therapy to surgery, but the success rate in terms of a reduction in size of HHC and the change in echotexture has been variable^[17-19]. Another prospective randomized study compared albendazole, percutaneous drainage and both modalities combined. These studies showed that cyst size reduction was best achieved by the combined therapy when compared to albendazole or percutaneous drainage alone^[20,21]. Percutaneous drainage of HHC was started by Mueller *et al.*^[22], and since then several series of percutaneous drainage have been published with no single fatality related to the procedure has been reported^[23,24]. Reversible anaphylactic shocks, mild to severe allergic reactions, and pleural effusions have been reported in the recent literature^[25,26], and any other complications were minor and infrequent. The reason for the pleural effusion is probably due to diaphragmatic irritation by the sudden collapse of the cyst following drainage and/or catheter manipulation during the procedure. However, pleural effusion was discovered incidentally during follow-up and in US examination, and was small and resolved completely before the patients were discharged. Fever was also a common complication, occurring in 10 patients, but was mild and transient, and cultures of fluid from the draining catheter were negative. Two patients developed an urticarial reaction hours following drainage but the patients responded well to antihistamines and steroid therapy. Only one patient developed an anaphylactic reaction which required immediate intubation and management, but there was a full recovery.

Drainage of complex Type IV cysts have been attempted before. Eighteen cysts in our series belonged to this group, including two patients with a partially calcified wall with multiple daughter cysts, in whom active disease was confirmed by serology and clinical assessment prior to the procedure and microscopy following drainage. It should be remembered that a calcified cyst does not mean always mean an inactive cyst. In our study, a 12F or 14F catheter was used to drain all types of HHC. Such large caliber catheters have not been used before in percutaneous drainage. We used a large catheter in order to

minimize clogging of the catheters by membranes and daughter cysts, and to ensure that all the cyst cavities were completely evacuated, though finer catheters might be safer, despite frequent clogging. Future studies will clarify this and many other issues. Follow-up indirect hemagglutination tests were performed in all patients. There was slight elevation of the indirect hemagglutination titer in 18 patients after the procedure and it remained elevated in comparison to the predrainage value during an average follow-up period of 16 mo. This observation has been reported by others^[27,28], and we believe that a longer follow-up period is needed for the indirect hemagglutination titer to start decreasing.

Our results have shown that percutaneous drainage of all types of HHC with adjuvant medical therapy is minimally invasive, safe and effective therapy with proper precautions. It can be used as an alternative to surgery, and in some cases is superior to surgery. Further evaluation by means of organized multicenter studies and long-term evaluation will answer questions regarding the use of a larger caliber or fine catheter, types of sedation or anesthesia, duration and requirement of adjuvant medical therapy, possible recurrence and many other unanswered questions.

COMMENTS

Background

Human hydatid disease (Echinococcosis) was recognized by Hippocrates over 2000 years ago. Al Razi and Avicenna made references in 900 AD and 11200 respectively and was described as liver cysts filled with water. However, it is still seen all over the world and is endemic and common in many countries i.e. Africa, central Asia, the Mediterranean, South America and Middle East and remains a problem for the World Health Organization. It is a slow growing cyst and may produce no symptoms for up to 10 years. In the most common form of the disease (Echinococcosis granulosis) dogs are the definitive host. Humans and sheep are the intermediate victims. Therefore, human hygiene and dogs' sanitation (removing the tapeworm from the dog) are essential issues in the prevention of this disease.

Research frontiers

Any organ and any part of the body could be affected but the most common sites are the liver and lungs. Over the recent decades substantial improvement has been made in the diagnosis and management of hydatid disease, through diagnostic tools such as imaging procedures including ultrasound (US), computed tomography (CT), magnetic resonance imaging and endoscopic retrograde cholangiopancreatography.

Innovations and breakthroughs

Concerning treatment, until recently the only definitive treatment for hydatid disease had been surgery. Different surgical techniques and procedures have been carried out and even in some cases, a liver transplant has been required. Advances in drug therapy has been influenced by the introduction of albendazole and accelerated by addition of praziquantel, but this requires a long period of treatment i.e. up to a year or more, and is not effective for everyone.

Applications

Percutaneous aspiration irrigation and reaspiration (PAIR) under direct US or CT guidance is a real achievement in the management of hydatid disease. The procedure was associated with reversible complications, no mortality, very short hospitalization and minimal cost. All 32 cysts showed evidence of immediate collapse after completion of the procedure. Serial follow-up showed progressive decrease in the size and change in the appearance of the cysts. At 10 years follow-up, the longest follow-up in the literature, there was no evidence of recurrence. Therefore, the authors confirm and believe that PAIR using hypertonic saline with adjuvant medical therapy has encouraging results and, with appropriate precautions, is very safe.

Peer review

The authors retrospectively analyzed a series of 26 patients whose hydatid liver cysts were treated with percutaneous aspiration and hypertonic solution injection. Albendazole was given prophylactically and after the procedure. The study might provide some confirmation of the efficacy of a non-surgical approach to the treatment of liver hydatidosis.

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S- Editor Sun H L- Editor Cant MR E- Editor Zheng XM

A population-based case-crossover study of polyethylene glycol use and acute renal failure risk in the elderly

Nam-Kyong Choi, Yoosoo Chang, Sun-Young Jung, Yu-Kyong Choi, Joongyub Lee, Jin-Ho Lee, Ju-Young Kim, Byung-Joo Park

Nam-Kyong Choi, Yoosoo Chang, Ju-Young Kim, Byung-Joo Park, Department of Preventive Medicine, Seoul National University College of Medicine, Seoul 110-799, South Korea
Nam-Kyong Choi, Byung-Joo Park, Medical Research Center, Seoul National University, Seoul 110-799, South Korea
Yoosoo Chang, Department of Occupational Medicine, Health Screening Center, Kangbuk Samsung Hospital, Sungkyunkwan University College of Medicine, Seoul 110-746, South Korea
Sun-Young Jung, National Evidence-Based Healthcare Collaborating Agency, Seoul 110-450, South Korea
Yu-Kyong Choi, Department of Internal Medicine, Gumi Hospital, Gumi 730-909, South Korea
Joongyub Lee, Byung-Joo Park, Medical Research Collaborating Center, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-799, South Korea
Jin-Ho Lee, Department of Internal Medicine, Dongguk University Ilsan Hospital, School of Medicine, Dongguk University, Goyang 410-733, South Korea
Ju-Young Kim, Department of Family Medicine, Health Promotion Center, Seoul National University Bundang Hospital, Bundang 463-707, South Korea

Author contributions: Park BJ suggested the study; Choi NK, Chang Y and Jung SY planned and designed the study; Choi NK analyzed the data; Choi NK, Chang Y, Jung SY, Choi YK, Lee J, Lee JH, Kim JY and Park BJ interpreted the results; Choi NK drafted the manuscript; Chang Y, Jung SY, Choi YK, Lee J, Lee JH and Kim JY revised the manuscript; all authors contributed to modifying the manuscript and the final editing of the paper; Park BJ is the guarantor.

Correspondence to: Byung-Joo Park, MD, MPH, PhD, Professor and Chairperson, Department of Preventive Medicine, Seoul National University College of Medicine, 103 Daehakno, Chongno-Gu, Seoul 110-799, South Korea. bjpark@snu.ac.kr
Telephone: +82-2-7408325 Fax: +82-2-7474830

Received: October 5, 2010 Revised: November 3, 2010

Accepted: November 10, 2010

Published online: February 7, 2011

tween polyethylene glycol (PEG) and acute renal failure (ARF) in elderly patients using a health insurance claims database.

METHODS: We conducted a population-based case-crossover study using information obtained from Korean Health Insurance Review and Assessment Service (HIRA) claims from January 1, 2005 to December 31, 2005 (Seoul, Korea). The study population consisted of elderly patients who received PEG prior to experiencing their first ARF-related hospitalization from April 1, 2005 to December 31, 2005. For each patient, one case and two control periods were matched. PEG use in a 2- or 4-wk window period prior to hospitalization for ARF was compared with PEG use in two earlier 2- or 4-wk control window periods. Conditional logistic regression analysis was used to estimate odds ratios (ORs) and 95% CI, adjusting for concomitant uses of diuretics, angiotensin converting enzyme inhibitors, non-steroidal anti-inflammatory drugs, antibiotics, anti-cancer drugs, and contrast media.

RESULTS: Within the HIRA database which contained 1093262 elderly patients, 1156 hospitalized ARF cases were identified. Among these cases, PEG was prescribed to 17 (1.5%) patients before hospitalization. The adjusted ORs when applying the 2- and 4-wk window periods were 0.4 (95% CI: 0.03-5.24) and 2.1 (95% CI: 0.16-27.78), respectively.

CONCLUSION: No increased risk of ARF was found in elderly PEG users. However, based on the limited number of study subjects, further analysis should be performed to confirm these results.

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Key words: Polyethylene glycol; Acute renal failure; Adverse drug reaction; Health insurance claims database; Case-crossover

Abstract

AIM: To evaluate the possibility of an association be-

Peer reviewer: Venkatesh Shanmugam, MBBS, MS (Gen. Surg.), Dip.NB (Gen. Surg.), FRCS (Glasg.), MD, Specialist Registrar (Trent Deanery), Royal Derby Hospital, Uttoxeter Road, Derby, DE22 3NE, United Kingdom

Choi NK, Chang Y, Jung SY, Choi YK, Lee J, Lee JH, Kim JY, Park BJ. A population-based case-crossover study of polyethylene glycol use and acute renal failure risk in the elderly. *World J Gastroenterol* 2011; 17(5): 651-656 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/651.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.651>

INTRODUCTION

Colonoscopy is a common diagnostic or therapeutic procedure. As early detection of colorectal cancer can significantly decrease mortality^[1], regular screening is recommended for those aged 50 years or over^[2]. The success and accuracy of colonoscopy are largely dependent on appropriate cleansing of the colon^[3]. Ideally, a colon preparation would provide safe and rapid cleansing with little or no discomfort to patients^[4]. Currently, polyethylene glycol (PEG) bowel preparation and oral sodium phosphate (NaP) are predominantly used as bowel cleansing agents before colonoscopy based on the fact that they are effective and generally well tolerated^[5-7]. PEG is a non-digestible, non-absorbable, osmotically-balanced laxative lavage solution that does not cause physiologic changes and can even be administered to patients in poor general condition^[8-12].

However, several recent studies have raised concerns about the safety of oral NaP preparations due to their reported association with an increased risk of serious electrolyte disturbances and renal failure^[13]. Conversely, although a large volume of PEG produces discomfort to the examinee, it is considered to be a relatively safe agent. Therefore, there has been a tendency to prescribe PEG more than NaP for renally impaired or elderly patients^[14]. Recently, several studies have shown that the risk of renal impairment is similar between PEG and NaP users^[15,16]. In addition, a case report has raised a possible association between the use of PEG and acute renal failure (ARF)^[17]. The patient in that case was a 55-year-old male without pre-existing renal disease who visited the emergency room with severe abdominal pain and frequent diarrhea after ingesting PEG 2 h earlier as pre-treatment for a follow-up colonoscopy. He was diagnosed as having prerenal ARF and improved after intensive fluid administration. Also, a recent cohort study revealed that following colonoscopy, those over 65 years of age without preexisting renal disease were at risk for impaired renal function^[16]. Unfortunately, most of the evidence for PEG risk to date has been based on a limited number of hospital patients or the case report mentioned.

There have been no quantitative epidemiological studies analyzing a relationship between PEG preparation and the development of ARF using a national health insur-

ance database. Therefore, this case-crossover study was performed to evaluate the risk of ARF following the use of PEG among elderly patients using information gathered from a Korean national health insurance database.

MATERIALS AND METHODS

Data source

We used the Korean Health Insurance Review and Assessment Service (HIRA) database that contains information on all claims including prescribed medications for approximately all 50 million Koreans^[18]. We obtained claims data for elderly patients (age 65 years or older) that had been submitted by healthcare providers based in Seoul between January 1, 2005 and December 31, 2005. Seoul is the capital and largest city of South Korea. A megacity with a population of over 12 million, it is one of the largest cities in the world. The study database contained information on 1 093 262 elderly patients with 11 842 586 prescriptions^[19]. This study was exempted from review by the Institutional Review Board of the Seoul National University College of Medicine/Seoul National University Hospital because researchers only accessed a de-identified database which included age, gender, diagnosis, and a list of prescribed drugs.

Study design

We employed a case-crossover approach, using cases at previous time points as their own controls, thereby eliminating time-invariant confounders between subjects through within-subject difference comparisons^[20]. In this design, only patients experiencing an event of interest were included and their exposures were measured during case- and control-time windows. Accordingly, the number having medication available in the case period (which is the period immediately before the event of interest) is compared with the number having medication available in the control period (which is a period prior to but of the same length as the case period)^[21]. Thus this design eliminates the effects of many potential confounders by keeping characteristics such as age, gender, socioeconomic status, and comorbidity fixed^[21,22].

Study subjects

The study population consisted of patients 65 years of age or older who received PEG prior to their first ARF-related hospitalization (ICD-10 code: N17) from April 1, 2005 to December 31, 2005. The index date was defined as the first hospital admission date for ARF. To identify initial ARF admission patients, we excluded those with pre-existing ARF during the preceding 3 mo, from January 1, 2005 to March 30, 2005.

Case and control periods

For each patient, one case and two control periods were matched to increase the study power and improve the precision of the estimates^[23]. The time windows, of 2 and 4 wk, were used to determine the periods over which as-

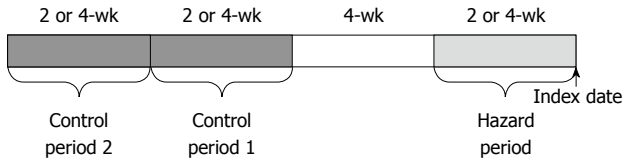


Figure 1 Definition of hazard and control periods in this case-crossover study. Hazard period was defined as the 2- or 4-wk window before the index date. A 4-wk washout period was chosen between the end of the hazard period and the start of the control period. Two consecutive control periods were defined as 2- or 4-wk windows.

assessment of drug exposure occurred. We defined the case period as a 2- or 4-wk window prior to the index date. A 4-wk washout period was chosen between the end of the case period and the start of the control period. Two consecutive control periods were also defined as 2- and 4-wk windows (Figure 1). Accordingly, for each patient, PEG prescription in each window period prior to hospitalization for ARF was compared with PEG prescription in two earlier control-window periods.

Statistical analyses

Descriptive statistics were used to illustrate the characteristics of the first ARF-hospitalized patients by age and gender. For the study population, the distribution of diagnoses on the day of PEG prescription was analyzed. Diagnoses were constructed from records made at the time of PEG prescription and grouped as colorectal cancer (ICD-10 codes: C18-C21, D12), gastric or duodenal ulcer, gastritis or duodenitis, intestinal disorders (K25-K59), renal disease (N03-N20), fibrosis and cirrhosis of the liver (K74), liver cancer (C22), and pancreatic cancer (C25). Conditional logistic regression analysis was used to estimate odds ratios (ORs) and 95% CI. The date of PEG exposure was regarded as the date of PEG prescription in the database. Use of concomitant medications that could induce ARF^[24,25] was included and evaluated in the model. Concomitant drugs included diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), non-steroidal anti-inflammatory drugs (NSAIDs), aminoglycosides, β -lactams, sulfonamides, anti-viral agents, antimycotics, anti-cancer drugs, and contrast media. We assessed PEG prescriptions and uses of concomitant drugs included in the case- or control-window periods. Exposure to PEG and other concomitant drugs was considered as a dichotomous variable in the model (exposed at least once during each specific time window: yes or no). Statistical analysis was performed using the SAS statistical application program (Release 9.1, SAS Institute Inc., Cary, NC, USA).

RESULTS

The total number of elderly patients who had at least one claim for any healthcare service in Seoul between January 1, 2005 and December 31, 2005 was 1 093 262. Their mean age was 71.0 ± 6.1 years and 59.9% were female.

Table 1 Characteristics of elderly patients who had at least one claim for any healthcare service in Seoul between January 1, 2005 and December 31, 2005 and those who were hospitalized for acute renal failure among the population *n* (%)

	Total of elderly patients	Patients hospitalized for ARF
Age (yr)		
mean \pm SD	72.0 \pm 6.1	75.9 \pm 7.3
65-69	472 614 (43.2)	270 (23.4)
70-74	297 348 (27.2)	260 (22.5)
75-79	179 562 (16.4)	272 (23.5)
80-84	96 131 (8.8)	197 (17)
≥ 85	47 607 (4.4)	157 (13.6)
Sex		
Male	438 795 (40.1)	587 (50.8)
Female	654 497 (59.9)	569 (49.2)
Total	1 093 262 (100)	1156 (100)

ARF: Acute renal failure.

Table 2 Characteristics of elderly patients with polyethylene glycol prescription prior to hospitalization for acute renal failure

	<i>n</i> (%)
Age (yr)	
mean \pm SD	70.6 \pm 4.6
65-69	8 (47.1)
70-74	5 (29.4)
75-79	3 (17.6)
80-84	1 (5.9)
Sex	
Male	14 (82.4)
Female	3 (17.6)
Diagnoses on the day of PEG prescription	
Colorectal cancer	6 (35.3)
Gastric or duodenal ulcer, gastritis or duodenitis, intestinal disorders	6 (35.3)
Renal disease	3 (17.6)
Fibrosis and cirrhosis of liver	2 (11.8)
Liver cancer	1 (5.9)
Pancreatic cancer	1 (5.9)
Total	17 (100)

PEG: Polyethylene glycol.

Among them, we identified 1156 patients hospitalized for ARF with a mean age of 75.9 ± 7.3 years. Patient sex was split relatively equally with males accounting for 50.8% of cases (Table 1). Among the cases of ARF, 17 (1.5%) had received PEG prior to their hospitalization. Their mean (SD) age was 70.6 (4.6) years and 82.4% (14 cases) of them were male. The most frequent diagnoses on the day of PEG prescription were colorectal cancer (35.3%), gastric or duodenal ulcer, gastritis or duodenitis, or intestinal disorders (35.3%) (Table 2). Using the 2- and 4-wk windows, the crude ORs for ARF were 0.7 (95% CI: 0.07-6.41) and 1.3 (95% CI: 0.22-7.99), respectively. After adjusting for the use of concomitant drugs the adjusted ORs applying the 2- and 4-wk windows were 0.4 (95% CI: 0.03-5.24) and 2.1 (95% CI: 0.16-27.78), respectively (Table 3).

Table 3 Association between polyethylene glycol and the risk of acute renal failure with respect to time-window periods by matched ratio of case and control period

Time-window period	Case period (<i>n</i> = 17)	Control period (<i>n</i> = 34)	Crude OR (95% CI) ¹	Adjusted OR (95% CI) ²
2 wk				
PEG non-users	16	31	1	1
PEG users	1	3	0.7 (0.07-6.41)	0.4 (0.03-5.24)
4 wk				
PEG non-users	15	31	1	1
PEG users	2	3	1.3 (0.22-7.99)	2.1 (0.16-27.78)

¹Calculated by conditional logistic regression; ²Calculated by conditional logistic regression adjusted for use of nephrotoxic drugs (diuretics, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, non-steroidal anti-inflammatory drugs, aminoglycoside, β -lactams, antiviral agents, antimycotics, anti-cancer drugs, and contrast media). PEG: Polyethylene glycol; OR: Odds ratio.

DISCUSSION

This population-based case-crossover study showed that PEG did not increase the risk of ARF in elderly patients. When several different time-window periods were applied, the use of PEG was not associated with ARF risk. This study supports findings of previous reports which have suggested that PEG does not increase the risk of ARF^[12,26-29]. Known PEG-associated common adverse effects include volume-related symptoms of abdominal fullness, nausea, and bloating, with minimal discomfort^[26]. Also, previous results of a prospective, randomized, multicenter, controlled trial comparing PEG plus ascorbic acid to NaP solution in 352 patients had shown that PEG use was associated with fewer adverse events and no clinically relevant changes in laboratory values^[27]. In a study addressing age, 557 patients were stratified into two groups, > 60 years and \leq 60 years of age, all of whom received either PEG or a cathartic preparation for colonoscopy, barium enema, or elective colon surgery^[28]. Patients in the older age group reported significantly fewer cramps ($P < 0.05$) and no differences in overall discomfort compared to their younger PEG counterparts, confirming the generally accepted understanding that PEG is safe and tolerable^[12,29].

In our study, we excluded patients admitted with a diagnosis of ARF 3 mo before the study starting date; therefore we could infer that PEG did not increase the risk of ARF among patients without recent worsening of renal function. However, because decreased renal function is extremely common in elderly persons^[30], the study population might have asymptotically decreased renal function. Further studies should be performed to examine the possibility that PEG could worsen existing renal impairment and hasten its progression to ARF. Moreover, although the study results did not show a statistically significant risk for ARF in PEG users, it may be desirable to ensure adequate hydration before, during, and after PEG bowel preparation and provide renal function monitoring before and after colonoscopy in high risk patients.

We applied a case-crossover design optimal for evaluating short-term effects after transient exposures, particularly by removing time-invariant between-subject confounding factors^[31]. Results of clinical trials are sometimes difficult to generalize to clinical practice and rarely detect adverse event incidents because they include only small numbers of highly selected patients. Also, the estimates of adverse drug effects derived from observational studies are vulnerable to unmeasured or unknown confounding factors, associated with both the exposure and the outcome^[20]. Actually, a previous cohort study which aimed to compare the risk of renal dysfunction related to the use of PEG and NaP mentioned that its results could be affected by potential selection bias^[11]. The cohort study was conducted using clinical records of patients undergoing colonoscopy in one hospital. Accordingly, the baseline patient characteristics might have affected which drugs were prescribed and the two groups were not comparable^[32,33]. In the present study, using the case-crossover technique, only cases with incident renal failure were considered and their PEG exposures were compared during two different time-windows. Since inherent confounders remain invariant over time, the case-crossover design which is optimal for transient exposures with short-term effects has an advantage in that it can minimize between-subject confounding and assure an optimal sample size^[31].

This study has several strengths. Firstly, we evaluated patients from an entire target population of over one million elderly derived from the national health insurance claims database in Seoul, South Korea, rather than use a sample population. Therefore, our results reflect unbiased real world conditions. Nevertheless, we identified only 17 cases of ARF following PEG use. This means that there is little possibility the PEG would increase the risk of ARF. Secondly, this study included elderly patients who are not usually involved in clinical trials or safety studies, but are at high risk of renal failure related to bowel preparations. Thirdly, although we controlled unmeasured confounders which were stable over time by using a case-crossover design, we further adjusted for other medication use which could affect the development of ARF such as diuretics, ACE inhibitors, ARBs, β -blockers, NSAIDs, aminoglycosides, β -lactams, anti-viral agents, antimycotics, anti-cancer drugs, and contrast media^[24,25].

However, our results should also be interpreted with caution. Although ARF is generally defined as an abrupt and sustained decline in the glomerular filtration rate (GFR)^[34], we defined incident cases of ARF as hospitalization with diagnosis of ARF in the HIRA database. Since the database did not contain laboratory test results such as GFR, a validation study was used to compare the diagnosis derived from the HIRA database with the actual diagnosis in the patients' medical records. The overall positive predictive value of the diagnoses was 81.8% in cases of hospitalized patients^[35]. Also, ARF as defined in this study only included symptomatic and serious events requiring hospitalization. We defined the date of PEG exposure as the prescription date of PEG; however, there could be a

difference of several days or more between the date of prescription and actual administration. Nonetheless, the date of PEG administration followed the prescription, so the period from the actual PEG exposure date to ARF hospitalization might in fact be shorter than calculated.

In this study, PEG was found not to be associated with an increased risk of ARF in elderly patients. However, further studies should be conducted to confirm an association or lack thereof.

COMMENTS

Background

Polyethylene glycol (PEG), a commonly used solution for colonoscopy bowel preparation, is regarded as effective and tolerable. Recent reports have cited an increased risk of acute renal failure (ARF) in the elderly. Until now there have been no quantitative population-based epidemiological studies analyzing a possible relationship between PEG and ARF.

Research frontiers

Colonoscopy is a common diagnostic or therapeutic procedure. The success and accuracy of colonoscopy are largely dependent on appropriate cleansing of the colon. Ideally, a colon preparation would provide safe and rapid cleansing with little or no discomfort to patients. Currently, PEG bowel preparation and oral sodium phosphate (NaP) are predominantly used as bowel cleansing agents before colonoscopy based on the fact that they are effective and generally well tolerated.

Innovations and breakthroughs

Several recent studies have raised concerns about the safety of oral NaP preparations due to their reported association with an increased risk of serious electrolyte disturbances and renal failure. There have been no quantitative epidemiological studies analyzing a relationship between PEG preparation and the development of ARF using a national health insurance database. Therefore, this case-crossover study was performed to evaluate the risk of ARF following the use of PEG among elderly patients using information gathered from the Korean national health insurance database.

Applications

No increased risk of ARF was found in elderly PEG users. However, based on the number of study subjects, further analysis should be performed to confirm an association or lack thereof.

Peer review

This is very well constructed paper regarding the possibility of an association between PEG and ARF in elderly patients.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

Effects of intestinal mucosal blood flow and motility on intestinal mucosa

Yan-Bin Wang, Jing Liu, Zhao-Xu Yang

Yan-Bin Wang, Zhao-Xu Yang, Department of Gastroenterology, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China

Jing Liu, Department of Neurology, Beijing Dianli Hospital, Beijing 100073, China

Author contributions: Wang YB and Yang ZX designed the research; Liu J performed the research; Wang YB wrote the paper. Correspondence to: Jing Liu, Associate Chief, Department of Neurology, Beijing Dianli Hospital, Beijing 100073, China. maogou0806@sina.com

Telephone: +86-10-67096580 Fax: +86-10-67096567

Received: July 7, 2010 Revised: September 30, 2010

Accepted: October 7, 2010

Published online: February 7, 2011

Abstract

AIM: To investigate the role of intestinal mucosal blood flow (IMBF) and motility in the damage of intestinal mucosal barrier in rats with traumatic brain injury.

METHODS: Sixty-four healthy male Wistar rats were divided randomly into two groups: traumatic brain injury (TBI) group ($n = 32$), rats with traumatic brain injury; and control group ($n = 32$), rats with sham-operation. Each group was divided into four subgroups ($n = 8$) as 6, 12, 24 and 48 h after operation. Intestinal motility was measured by the propulsion ratio of a semi-solid colored marker (carbon-ink). IMBF was measured with the laser-Doppler technique. Endotoxin and D-xylose levels in plasma were measured to evaluate the change of intestinal mucosal barrier function following TBI.

RESULTS: The level of endotoxin was significantly higher in TBI group than in the control group at each time point (0.382 ± 0.014 EU/mL vs 0.102 ± 0.007 EU/mL, 0.466 ± 0.018 EU/mL vs 0.114 ± 0.021 EU/mL, 0.478 ± 0.029 EU/mL vs 0.112 ± 0.018 EU/mL and 0.412 ± 0.036 EU/mL vs 0.108 ± 0.011 EU/mL, $P < 0.05$). D-xylose concentrations in plasma in TBI group were significantly higher than in the control group (6.68 ± 2.37 mmol/L vs $3.66 \pm$

1.07 mmol/L, 8.51 ± 2.69 mmol/L vs 3.15 ± 0.95 mmol/L, 11.68 ± 3.24 mmol/L vs 3.78 ± 1.12 mmol/L and 10.23 ± 2.83 mmol/L vs 3.34 ± 1.23 mmol/L, $P < 0.05$). The IMBF in TBI group was significantly lower than that in the control group (38.5 ± 2.8 PU vs 45.6 ± 4.6 PU, 25.2 ± 3.1 PU vs 48.2 ± 5.3 PU, 21.5 ± 2.7 PU vs 44.9 ± 2.8 PU, 29.4 ± 3.8 PU vs 46.7 ± 3.2 PU) ($P < 0.05$). Significant decelerations of intestinal propulsion ratio in TBI groups were found compared with the control group ($0.48\% \pm 0.06\%$ vs $0.62\% \pm 0.03\%$, $0.37\% \pm 0.05\%$ vs $0.64\% \pm 0.01\%$, $0.39\% \pm 0.07\%$ vs $0.63\% \pm 0.05\%$ and $0.46\% \pm 0.03\%$ vs $0.65\% \pm 0.02\%$) ($P < 0.05$).

CONCLUSION: The intestinal mucosal permeability is increased obviously in TBI rats. Decrease of intestinal motility and IMBF occur early in TBI, both are important pathogenic factors for stress-related damage of the intestinal mucosal barrier in TBI.

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Key words: Traumatic brain injury; Intestinal mucosa barrier; Stress; Intestinal mucosa blood flow; Intestinal motility

Peer reviewer: Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad - 500 082, India

Wang YB, Liu J, Yang ZX. Effects of intestinal mucosal blood flow and motility on intestinal mucosa. *World J Gastroenterol* 2011; 17(5): 657-661 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/657.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.657>

INTRODUCTION

Multiple system organ dysfunction syndrome (MODS) often occurs following the stress of severe trauma, burn and acute necrotic pancreatitis^[1-4]. However, its exact mecha-

nism remains unclear. The gut origin hypothesis suggests that damage of intestinal mucosal barriers as a result of these stress permits bacterial and endotoxin translocation, which triggers systemic immunoinflammatory response to release cytokines and inflammatory mediators. All of these might exacerbate systemic inflammatory response syndrome (SIRS) and MODS. Many patients with severe traumatic brain injury (TBI) often die of MODS^[5], but not of the injury itself. So to prevent SIRS and MODS in TBI patients is one of the important factors that affect the prognosis and sequela.

Our previous studies have found the damage of intestinal mucosal morphology and barrier function following TBI^[6]. Although very common, the pathophysiology of this stress-related change is far from understood.

Fortunately, researches over the past decades have provided insight into the potential mechanisms responsible for the pathogenesis of stress-induced gastrointestinal dysfunction. The stressful situation is a multi-factorial disorder involving dysregulation within the brain-gut axis. Upon activation of the brain-gut axis by stress, the release of brain-gut peptides can profoundly affect gastrointestinal physiology and it is frequently associated with gastrointestinal motor, gastrointestinal mucosal blood flow (IMBF), enteric and central nervous system irregularities, and neuroimmune dysregulation^[7].

The aim of this study was to further elucidate the effects of TBI on intestinal motility and IMBF, and to explore the putative mechanism of this stress-induced change in the TBI process.

MATERIALS AND METHODS

Animal model of TBI

Sixty-four healthy male Wistar rats, weighing 200-250 g (provided by Experimental Animal Center of Genetics and Developmental Biology Institute, Chinese Academy of Sciences), were randomly assigned to TBI model group ($n = 32$) and control group ($n = 32$). Each group was divided into four subgroups as 6, 12, 24 and 48 h after operation ($n = 8$). Experimental procedures complied with the ethical requirements for animal care.

Establishment of animal models

TBI group ($n = 32$): RATS with traumatic brain injury by free falling body method^[8]. Rats were deprived of food for 12 h prior to experiment, and then was anesthetized with injection of 10% chloral hydrate (0.4 mL/100 g) and fixed on a stereotaxic apparatus. Scalp was cut along the median line and exposed the skull under sterilized conditions. At the point of 2.0 mm rearward from the coronal suture and 2.0 mm left to the sagittal suture, open a 3.5 mm diameter bone window and maintain the integrity of the duramater. Then 20 g metal bar was released and fallen freely from 50 cm height to strike the meninges to cause the brain injury.

Control group ($n = 32$): rats with sham-operation with skull open operation alone and no brain injury.

Determination of endotoxin

One mL blood was collected from portal vein and placed into an apyrogenic tube (containing heparin) immediately. The levels of endotoxin were measured by chromogenic limulus amebocyte lysate test. The test kit was purchased from Shanghai Yihua Clinical Technology Company (Shanghai, China).

Measurement of D-xylose concentrations in plasma

Intestinal permeability was quantified by D-xylose concentrations in plasma. The 5% D-xylose solution of 1.5 mL was administered into the stomach by gastric tube feeding, and blood samples were collected into chilled tubes containing 100 U heparin 1 h later. The blood was centrifuged at 3000 r/min at 4°C for 10 min. The plasma was stored at -70°C until assayed. Levels of D-xylose in plasma were measured with D-xylose kit.

Measurement of IMBF

IMBF was measured with Laser Doppler Flowmetry (LDF) equipment (PeriFlux System 5000, Perimed, Sweden). The laser probe was inserted through a small enterotomy at the point that 20 cm from pylorus of the jejunal sac and held in a fixed position in the chamber solution at a distance of 1-2 mm above the mucosa. The measurement was taken as the average flow over a 10-min period following an initial 20-min period of stabilization.

Measurement of intestinal transit

Rats were fasted for 24 h prior to experiment, and 0.5 mL carbon-ink was administered into the stomach by gastric tube feeding. Twenty min later, the rats were killed at each time point, their intestines were removed from the pylorus through the ileocecal junction. The distance of carbon-ink from the pylorus to the most distal point of stain was expressed as migration distance. Results were expressed as propulsion ratio (%) of the migration distance to the total length of the small intestine (the distance between the pylorus and the ileocecal junction).

Statistical analysis

Software SPSS 11.0 was used for the statistical analysis. The data were expressed as mean \pm SD. Experimental results were analyzed by unpaired *t* test and $P < 0.05$ was considered as significant difference.

RESULTS

Serum endotoxin levels

There were significant differences of endotoxin levels between the TBI group and control group at each time point (0.382 ± 0.014 EU/mL *vs* 0.102 ± 0.007 EU/mL, 0.466 ± 0.018 EU/mL *vs* 0.114 ± 0.021 EU/mL, 0.478 ± 0.029 EU/mL *vs* 0.112 ± 0.018 EU/mL and 0.412 ± 0.036 EU/mL *vs* 0.108 ± 0.011 EU/mL, $P < 0.05$, respectively). As shown in Table 1, the endotoxin was significantly increased 6 h after TBI, and reached the peak at 24 h, and then declined at 48 h, but was still higher than that of the control group.

Table 1 Changes of endotoxin in plasma (mean \pm SD) (EU/mL)

Groups	6 h	12 h	24 h	48 h
Control	0.102 \pm 0.007	0.114 \pm 0.021	0.112 \pm 0.018	0.108 \pm 0.011
TBI	0.382 \pm 0.014 ^a	0.466 \pm 0.018 ^a	0.478 \pm 0.029 ^a	0.412 \pm 0.036 ^a

^a*P* < 0.05 *vs* control. TBI: Traumatic brain injury.Table 2 Changes of D-xylose in plasma (mean \pm SD) (mmol/L)

Groups	6 h	12 h	24 h	48 h
Control	3.66 \pm 1.07	3.15 \pm 0.95	3.78 \pm 1.12	3.34 \pm 1.23
TBI	6.68 \pm 2.37 ^a	8.51 \pm 2.69 ^a	11.68 \pm 3.24 ^a	10.23 \pm 2.83 ^a

^a*P* < 0.05 *vs* control. TBI: Traumatic brain injury.

D-xylose concentrations in plasma

D-xylose concentrations in plasma in TBI rats were significantly higher than in the control group (6.68 \pm 2.37 mmol/L *vs* 3.66 \pm 1.07 mmol/L, 8.51 \pm 2.69 mmol/L *vs* 3.15 \pm 0.95 mmol/L, 11.68 \pm 3.24 mmol/L *vs* 3.78 \pm 1.12 mmol/L and 10.23 \pm 2.83 mmol/L *vs* 3.34 \pm 1.23 mmol/L, *P* < 0.01, respectively), indicating that the intestinal mucosal barrier was damaged (Table 2).

Changes of IMBF

As shown in Table 3, IMBF was significantly lower in TBI group than that in the control group (38.5 \pm 2.8 PU *vs* 45.6 \pm 4.6 PU, 25.2 \pm 3.1 PU *vs* 48.2 \pm 5.3 PU, 21.5 \pm 2.7 PU *vs* 44.9 \pm 2.8 PU, 29.4 \pm 3.8 PU *vs* 46.7 \pm 3.2 PU) (*P* < 0.05). It began to decrease at 6 h, reached the lowest at 24 h, and did not reach the baseline by 48 h.

Changes of intestinal transit

The overall mean ratio of intestinal propulsion under TBI stress was lower than that of the control group (0.48% \pm 0.06% *vs* 0.62% \pm 0.03%, 0.37% \pm 0.05% *vs* 0.64% \pm 0.01%, 0.39% \pm 0.07% *vs* 0.63% \pm 0.05% and 0.46% \pm 0.03% *vs* 0.65% \pm 0.02%) (*P* < 0.05), indicating that TBI stress could inhibit small intestinal motility (Table 4).

DISCUSSION

Gastrointestinal dysfunction is a common complication of stress. Damage of the gastrointestinal function, especially of the gastrointestinal barrier function, permits translocation of enterogenic bacteria and endotoxins, triggers systemic immunoinflammatory response to release cytokines and inflammatory mediators, which is an important initiator as well as a stimulator for occurrence of SIRS, sepsis and MODS following major stress^[9]. The stress including severe trauma, hemorrhagic shock, severe pancreatitis and burn^[10,11]. So the gastrointestinal barrier function is one of the important factors that affect the prognosis and sequelae.

Intestinal mucosal barrier function could be evaluated by measuring the permeability of saccharide mo-

Table 3 Changes of intestinal mucosal blood flow (mean \pm SD) (PU)

Groups	6 h	12 h	24 h	48 h
Control	45.6 \pm 4.6	48.2 \pm 5.3	44.9 \pm 2.8	46.7 \pm 3.2
TBI	38.5 \pm 2.8	25.2 \pm 3.1 ^a	21.5 \pm 2.7 ^a	29.4 \pm 3.8 ^a

^a*P* < 0.05 *vs* control. TBI: Traumatic brain injury.Table 4 Ratio of intestinal propulsion (mean \pm SD) (%)

Groups	6 h	12 h	24 h	48 h
Control	0.62 \pm 0.03	0.64 \pm 0.01	0.63 \pm 0.05	0.65 \pm 0.02
TBI	0.48 \pm 0.06 ^a	0.37 \pm 0.05 ^a	0.39 \pm 0.07 ^a	0.46 \pm 0.03 ^a

^a*P* < 0.05 *vs* control. TBI: Traumatic brain injury.

lecular probe. Lactulose/mannitol and D-xylose have previously been used to assess intestinal mucosal permeability^[12-15]. Shi *et al.*^[16], reported that chronic restraint stress could cause damage of the intestinal barrier function and increased intestinal permeability to D-xylose.

In this study, we used endotoxin and plasma D-xylose to evaluate the intestinal mucosa barrier function. We found that the endotoxin and plasma D-xylose levels in the TBI group were significantly higher than in the control group at 6 h following TBI, and reached its peak at 24 h, and then declined at 48 h, but still markedly higher than that in the control group. All of these demonstrated that TBI stress could be an initiating factor to increase the permeability of intestinal mucosa, suggesting that the intestinal mucosal barrier dysfunction initiated at the early stage of TBI.

At present, the specific pathogenesis and progress of the intestinal mucosal barrier damage still remain unclear. Stress is known to alter ingestive behaviors and associated physiological events such as gastric acid secretion and gastrointestinal motility. Mast cells translate the stress signal that has been transmitted through brain-gut axis to release a wide range of neurotransmitters and proinflammatory mediators, some of them are brain-gut peptides, such as 5-HT, SP, CGRP, CRP, CCK, NO, NE and VIP. Evidences implicated that the brain-gut peptides are involved in these physiological effects which can change the intestinal motility, modulate tight junction proteins and increase the intestinal permeability^[7,17]. Animal studies suggest that cholecystokinin (CCK) acts *via* a vagal afferent pathway to decrease gastrointestinal motility^[18] and substance P can stimulate a contractile function of smooth muscle^[19]. Studies in animal models showed that burn injury and cardiopulmonary bypass markedly down-regulated the expression of occludin and tight junction associated protein ZO-1 in intestinal mucosa of rats. The close correlation between expression of tight junctions and plasma levels of diamine oxidase or *D*-lactate supports the hypothesis that intestinal permeability increases during and after stress events because of decreases in the expression of tight junctions^[20,21].

IMBF plays a vital role in intestinal mucosal defense system. Sufficient IMBF brings oxygen and nutrients to the mucosal cells, maintains the normal structure and function of intestinal mucosa and is closely associated with the pathogenesis and healing of intestinal mucosal lesions^[22]. Our results revealed that IMBF decreased significantly at the early stage of TBI, and the intestinal mucosal permeability increase occurred at the same time. As intestinal mucosa is very sensitive to the shortage of blood and oxygen, ischemia/reperfusion (I/R) is the main pathogenesis of intestinal mucosal damage. The physiopathology of intestinal mucosal damage by I/R is not fully understood. But, it is believed that cytotoxic substances such as free radicals, nitric oxide, pro-inflammatory cytokines, leukotrienes, serotonin and other related products, play important roles^[23,24]. I/R not only damages the intestinal mucosal barrier function but also alters the gastrointestinal motility^[25].

It is widely believed that delayed intestinal motility could cause small intestinal bacterial overgrowth (SIBO). Gangarosa^[26] demonstrated that intestinal motility served as a normal cleansing mechanism of the intestine. Leveau *et al.*^[27] noticed a delay in intestinal transit time, appearing as an early event in acute pancreatitis, preceding SIBO, and suggested that impairment in intestinal motility may play a role in the development of SIBO. Tsukada *et al.*^[28-30] demonstrated that the small intestinal transit was significantly inhibited by restraint stress. Our results revealed that, at the early stage of TBI, the intestinal propulsion ratio decreased significantly as compared with control group ($P < 0.05$). Damage of intestinal mucosal barrier function occurred at the same time, indicating that the inhibition of intestinal motility might be another vital factor of gastrointestinal barrier dysfunction.

The mechanism may be explained by the fact that the prolonged small intestinal transit makes it possible that the small intestinal content remains in the intestinal tract for a long time, preceding SIBO, increasing the chance of bacterial and endotoxin translocation and producing a great deal of gas. The defect of intestinal barrier and the above factors of small intestinal dysfunction may enhance each other.

In summary, the damage of intestinal mucosal barrier function following TBI is caused by multiple factors, the close correlation between decrease of intestinal blood flow and motility and increase of intestinal permeability supports the hypothesis that both of them might play a very important role in the regulation of intestinal epithelial barrier dysfunction during and after TBI. Therefore, maintaining intestinal barrier function is a systematic engineering project. Further research that more precisely characterizes the role of intestinal mucosal blood flow and intestinal motility in these diseases could put new insights into the new therapies for stress-induced injury of intestinal mucosal barrier function.

COMMENTS

Background

Multiple system organ dysfunction syndrome (MODS) often occurs following

the stress of traumatic brain injury (TBI). Although being very common, the pathophysiology of this stress-related change is far from understood. The gut origin hypothesis suggests that damage of intestinal mucosa barriers as a result of these stress permits bacterial and endotoxin translocation, which triggers systemic immunoinflammatory response to release cytokines and inflammatory mediators. All of these might exacerbate the systemic inflammatory response syndrome (SIRS) and MODS.

Research frontiers

Gastrointestinal dysfunction is a common complication of stress. Damage of gastrointestinal function, especially of the gastrointestinal barrier function is an important initiator as well as a stimulator for occurrence of SIRS, sepsis and MODS following major stress, including severe trauma, hemorrhagic shock, severe pancreatitis and burn. Studies in animal models showed that brain-gut axis/ brain-gut peptides are involved in these physiological effects which can change the intestinal motility, modulate tight junction proteins and increase intestinal permeability.

Innovations and breakthroughs

The specific pathogenesis and progress of stress-induced damage of intestinal barrier function remain unclear. But, disruption of the intestinal mucosal protection is certainly involved. Intestinal blood flow plays a vital role in intestinal mucosal defense system and intestinal motility served as a normal cleansing mechanism of the intestine. This study revealed that the intestinal blood flow and motility decreased significantly at the early stage of TBI, and the intestinal mucosal permeability increase occurred at the same time. The results suggested that both might be important pathogenic factors for intestinal barrier function damage during and after TBI.

Applications

Many patients with severe TBI often die of MODS, but not of the injury itself. So to protect the mucosal barrier function at the early stage of TBI will be of significance for reducing the stress-related SIRS and MODS.

Terminology

Brain-gut axis is composed of main regulatory cores in the central nervous system that are connected to peripheral (enteric and autonomic) nervous systems through a series of networks of afferent and efferent nerves. Brain-gut peptide is named because of its distribution in both nervous system and gastrointestinal tract. Intestinal mucosal barrier function include mechanical barrier, chemical barrier, immunologic barrier and biological barrier, any damage of these barriers will damage the intestinal mucosal barrier function.

Peer review

This is a well conducted randomized controlled trial on animal models. The authors presented the results of their study that decreased intestinal blood flow and motility occur early in TBI, which supports the hypothesis that both are important pathogenic factors for increasing the intestinal permeability. So resuming the intestinal blood flow and motility might be a useful method for maintaining intestinal barrier function during and after TBI.

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S- Editor Sun H L- Editor Ma JY E- Editor Ma WH

Suspended moxibustion relieves chronic visceral hyperalgesia and decreases hypothalamic corticotropin-releasing hormone levels

En-Hua Zhou, Xiao-Mei Wang, Guang-Hong Ding, Huan-Gan Wu, Li Qi, Hui-Rong Liu, Shu-Jing Zhang

En-Hua Zhou, Guang-Hong Ding, Department of Mechanics and Engineering Science, Fudan University, Shanghai 200433, China

En-Hua Zhou, Xiao-Mei Wang, Huan-Gan Wu, Li Qi, Hui-Rong Liu, Shu-Jing Zhang, Key Laboratory of Acupuncture-Moxibustion and Immunological Effects, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Guang-Hong Ding, Shanghai Research Center of Acupuncture and Meridians, Shanghai 200433, China

Author contributions: Zhou EH and Ding GH performed the majority of experiments; Wu HG, Wang XM and Qi L provided vital reagents and analytical tools and were also involved in editing the manuscript; Liu HR and Zhang SJ analyzed the data; Zhou EH and Wu HG designed the study and wrote the manuscript.

Supported by The National Basic Research Program of China (973 program), No. 2009CB522900; the Shanghai Leading Academic Discipline Project, No. S30304; and Shanghai Foundation for Development of Science and Technology, China, No. 08DZ1973000

Correspondence to: Dr. Huan-Gan Wu, Key Laboratory of Acupuncture-Moxibustion and Immunological Effects, Shanghai University of Traditional Chinese Medicine, 650 South WanPing Road, XuHui district, Shanghai 200030, China. wuhuagan@126.com

Telephone: +86-21-64644238 Fax: +86-21-64644238

Received: August 9, 2010 Revised: September 14, 2010

Accepted: September 21, 2010

Published online: February 7, 2011

SM. Each treatment lasted for 30 min. Rats undergoing treatment with SM were not anesthetized. Untreated CVH rats and normal rats were used as controls. The abdominal withdrawal reflex was determined 30-90 min after the seven treatments. The hypothalamic corticotropin-releasing hormone (CRH) mRNA level was measured using real-time quantitative reverse transcription-polymerase chain reaction.

RESULTS: We found that SM treatment significantly decreased visceral sensitivity to colorectal distention in this rat model. In treated animals, SM also decreased the relative hypothalamic CRH mRNA expression level to control levels.

CONCLUSION: Lower hypothalamic CRH levels may mediate the beneficial effects of SM in this rat irritable bowel syndrome model.

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Key words: Chronic visceral hypersensitivity; Corticotropin-releasing hormone; Irritable bowel syndrome; Rat; Suspended moxibustion

Peer reviewer: Ricardo Alberto Cruciani, MD, PhD, Department of Pain Medicine and Palliative Care, Beth Israel Medical Center, First Ave. and 16th Street, New York, NY 10003, United States

Zhou EH, Wang XM, Ding GH, Wu HG, Qi L, Liu HR, Zhang SJ. Suspended moxibustion relieves chronic visceral hyperalgesia and decreases hypothalamic corticotropin-releasing hormone levels. *World J Gastroenterol* 2011; 17(5): 662-665 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/662.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.662>

Abstract

AIM: To evaluate the effect of suspended moxibustion (SM) on rectal sensory thresholds and to analyze the possible mechanisms involved in SM treatment of chronic visceral hypersensitivity (CVH) in rats.

METHODS: SM was administered once daily to 37-d-old CVH rats for 7 d. The two pairs of acupoints (ST25 and ST37, bilateral) were simultaneously treated with

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional disorder

characterized by chronic recurring abdominal pain or discomfort and altered bowel habits^[3]. Several recent studies have demonstrated that electro-acupuncture (EA) can decrease chronic visceral hypersensitivity (CVH) in a rat IBS model induced by mechanical colorectal irritation in the postnatal period^[2-6]. Previously, we reported that EA in this model can decrease hypothalamic corticotropin-releasing hormone (CRH) levels^[7]. CRH, a 41 amino acid peptide produced mainly in the paraventricular nucleus of the hypothalamus, is regarded as a major mediator of the stress response^[8]. IBS patients are reported to be hypersensitive to routine stress^[9], and stressful life events are known to contribute to the clinical course of IBS^[10].

Moxibustion is an alternative or complementary therapy that is also used to treat IBS. Methods of moxibustion include suspended moxibustion (SM, also known as warming moxibustion), scarring moxibustion and herb-partition moxibustion. Previously, we reported that both SM^[11] and herb-partition moxibustion^[12] could decrease CVH in a rat IBS model induced by mechanical colorectal irritation in the postnatal period. Moreover, we found that rats were relaxed or asleep during SM, indicating that the procedure was not stressful to the animals^[11]. Therefore, in the present study, we focused on whether SM could decrease hypothalamic CRH levels in rats.

MATERIALS AND METHODS

Animals

We used a rat model of CVH^[13] induced by mechanical colorectal irritation during postnatal development. Neonatal male Sprague-Dawley rats (5 d old) were obtained from the Experiment Animal Center, Shanghai University of Traditional Chinese Medicine. The animals were maintained in a plastic cage containing corn chip bedding at a controlled temperature (21°C) in a light-dark cycle (12 h:12 h). The maximum number of rats per cage was five. All rats were used strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals.

Neonatal rats were subjected to daily mechanical colorectal distention (CRD) from the age of 8 d to the age of 21 d. Neonatal rats received CRD twice daily, at 30-min intervals using a procedure modified from previous studies^[6,13]. A balloon constructed from a condom (length 20.0 mm and diameter 3.0 mm) was inserted rectally into the descending colon. The balloon was distended with 0.5 mL air for 1 min. It was then deflated and withdrawn. The rats were reared until they reached adulthood (at least 6 wk old), and behavioral responses to visceral pain induced by acute CRD were then examined. SM was administered to CVH rats ($n = 8$) for 7 d. CVH rats without SM ($n = 8$) and normal rats ($n = 8$) were used as controls. After seven treatments, the abdominal withdrawal reflex (AWR) was monitored over a period of 30-90 min. The animals were then sacrificed by intraperitoneal anesthesia using sodium pentobarbital (80 mg/kg), and the hypothalamus was isolated immediately and frozen in liquid nitrogen.

SM treatment

In the CVH + SM group, one ignited moxa stick was suspended perpendicularly 2 cm above Tianshu (ST25)^[11] and Shangjuxu (ST37) (5 mm lateral to the anterior tubercle of the tibia and 20 mm below the knee joint). ST25 and ST37 were the two key acupoints chosen in this study based on our clinical treatment of patients with IBS since the 1980s. The two pairs of acupoints (ST25 and ST37) were simultaneously treated with SM. Each treatment consisted of 30 min of moxibustion (15 min for each pair of acupoints). SM was administered once daily to CVH rats for 7 d. The animals were not anesthetized before SM, but were held in the supine position in one gloved hand. Rats from both the normal group and CVH group were also held in one gloved hand in the supine position, but not treated with SM^[11], these were used as controls.

Colon stimulation and testing of the AWR

Behavioral responses to CRD in young adult rats were assessed by recording AWR scores, using a procedure modified from previous studies^[5,13]. After anesthesia with diethyl ether, a balloon (3 cm in length, made using one finger of a latex glove) was inserted into the descending colon. The rats were then housed in small lucite cubicles (20 cm × 8 cm × 8 cm) on a platform and allowed to wake up and adapt for 20 min. CRD was induced by rapidly inflating the balloon at pressures of 20, 40, 60 and 80 mmHg for a duration of 10 s. AWR scores were observed by two blinded observers using the scale developed by Al-Chaer *et al.*^[13].

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from the hypothalamus using TRIZOL Reagent (TAKARA Biotechnology Co., Ltd., China), and quantified using a UV-3000 spectrophotometer (UNICO, USA). First-strand cDNA was synthesized using oligo(dT)15 primer, Moloney murine leukemia virus reverse transcriptase (TAKARA Biotechnology Co., Ltd., China), and 4 µL RNA. CRH and GAPDH (housekeeping gene) primers were designed in different exons to amplify cDNA using ABI Prism 7500 SDS Software (Applied Biosystems Co., Ltd., USA). CRH primers were: sense 5'-TGGCCTGCAGTGCAATGC-3' and antisense 5'-CCTGGCACTCAGAATAATTACAC-3'. Real-time quantitative polymerase chain reaction (qPCR) was performed with 5 µL of first-strand cDNA reaction in the presence of 0.5 µL dNTP, 10 µL specific buffer, 1 µL *Taq* polymerase, SYBR green fluorescent dye and the appropriate sense and antisense primers (0.5 µL) in a final volume of 50 µL (qPCR™ Core Kit, Shanghai DaWei'K Biology Technology Co., Ltd., China). PCR was carried out using the 7500 Sequence Detection System (Applied Biosystems). The reaction conditions were as follow: initial denaturation for 5 min at 95°C followed by 40 cycles with denaturation at 95°C for 15 s and annealing and extension at 60°C for 45 s. Three SYBR cycle threshold values were

Table 1 Abdominal withdrawal reflex scores in response to graded colorectal distention at 20, 40, 60 and 80 mmHg

Group	n	AWR score			
		20 mmHg	40 mmHg	60 mmHg	80 mmHg
Normal	8	0.13 ± 0.13	0.38 ± 0.18	0.75 ± 0.25	1.50 ± 0.33
CVH	8	1.38 ± 0.18 ^{b,d}	1.80 ± 0.25 ^{b,d}	2.75 ± 0.25 ^{b,d}	3.50 ± 0.27 ^{b,d}
CVH + SM	8	0.25 ± 0.16	0.50 ± 0.19	1.13 ± 0.30	1.63 ± 0.18

AWR: Abdominal withdrawal reflex; SM: Suspended moxibustion; CVH: Chronic visceral hypersensitivity. ^b*P* < 0.01 *vs* normal group; ^d*P* < 0.01 *vs* CVH + SM group.

averaged for each sample, and the RNA input for the target gene was calculated from the standard curve.

Statistical analysis

All values are expressed as mean ± SE. Statistical analyses were performed using one-way ANOVA followed by Fisher's PLSD procedure using SPSS 10.0 (SPSS Inc., USA). Dunnett's T3 test was used if variances were unequal. *P* < 0.05 was considered to be significant.

RESULTS

AWR scores in response to graded CRD at 20, 40, 60 and 80 mmHg

As shown in Table 1, the AWR scores in response to graded CRD (20, 40, 60, and 80 mmHg) in the normal control group were lower than in the CVH group (*P* < 0.01). SM treatment significantly reduced AWR scores in the CVH rats in response to CRD (20, 40, 60 and 80 mmHg).

Relative hypothalamic CRH mRNA expression

The relative CRH mRNA expression level was significantly higher in the CVH group than in the normal control group (*P* < 0.01). However, the relative CRH mRNA expression level was markedly lower in the CVH + SM group than in the normal control group (*P* < 0.01) (Table 2).

DISCUSSION

In our previous study, we found that SM depressed AWR scores following CRD stimulation at 20 mmHg. However, in this study SM depressed AWR scores following CRD stimulation at 20, 40, 60 and 80 mmHg, which may be due to the increased number of treatments (1 treatment *vs* 7 treatments), and acupoints (1 pair of acupoints *vs* 2 pairs of acupoints). Overall, the results of this experiment demonstrated the efficacy of SM in decreasing CVH in a rat IBS model induced by mechanical colorectal irritation in the postnatal period.

We previously reported that EA can decrease the hypothalamic CRH levels in a rat IBS model^[7]. Moreover, the rats were relaxed or asleep during SM, indicating that the procedure was not stressful to the animals^[11]. Stress is known to lead to central CRH release, and we have confirmed that hypothalamic CRH levels are elevated in IBS rats. In the present study, we focused on whether SM

Table 2 Relative hypothalamic corticotropin-releasing hormone mRNA expression

Group	n	CRH mRNA (relative expression)
Normal	8	0.29 ± 0.03
CVH	8	3.62 ± 0.23 ^{b,d}
CVH + SM	8	0.47 ± 0.06

CRH: Corticotropin-releasing hormone; SM: Suspended moxibustion; CVH: Chronic visceral hypersensitivity. ^b*P* < 0.01 *vs* normal group; ^d*P* < 0.01 *vs* CVH + SM group.

could decrease hypothalamic CRH expression level in rats. Our results showed that the relative hypothalamic CRH mRNA expression level also decreased in IBS rats, suggesting that the modulation of hypothalamic CRH may mediate the decreased visceral sensitivity arising from SM.

The effects of CRH on different tissues are mediated *via* CRH receptors on the cell membrane^[14]. CRH receptors are expressed in different brain regions^[15,16] and in several peripheral organs^[17]. Both central CRH receptor 1 (CRH-R1) and peripheral CRH-R1 are believed to be responsible for colorectal distension-induced sensitization^[18]. Moreover, the activation of CRH-R2 reduces visceral sensitivity induced by colorectal distension in conscious rats.

We hope that this study will pave the way for further studies on the relationship between CRH, CRH receptors, and SM. It will be necessary to determine whether SM can regulate the expression or activity of CRH receptors in IBS rats. Further experiments with CRH receptor antagonists would shed light on the functional relationship between SM and changes in CRH levels.

In conclusion, SM increased pain thresholds in a rat model of IBS and decreased relative hypothalamic CRH mRNA expression level. We suggest that reduced hypothalamic CRH levels may mediate the beneficial effects of SM in a rat IBS model induced by mechanical colorectal irritation in the postnatal period.

COMMENTS

Background

The authors previously reported that electro-acupuncture can decrease hypothalamic corticotropin-releasing hormone (CRH) levels in a rat model of irritable bowel syndrome (IBS). However, it is still not known whether suspended moxibustion (SM) can decrease hypothalamic CRH levels. Previously, the authors reported that both SM and herb-partition moxibustion can decrease chronic visceral hypersensitivity (CVH) in a rat IBS model induced by mechanical colorectal irritation in the postnatal period. Moreover, the authors found that rats were relaxed or asleep during SM, indicating that the procedure was not stressful to the animals.

Research frontiers

The effects of CRH on different tissues are mediated *via* CRH receptors on the cell membrane. CRH receptors are expressed in different brain regions and in several peripheral organs. Both central CRH receptor 1 (CRH-R1) and peripheral CRH-R1 are believed to be responsible for colorectal distension-induced sensitization. Moreover, the activation of CRH-R2 reduces visceral sensitivity induced by colorectal distension in conscious rats.

Innovations and breakthroughs

This study is the first to report on the effects of SM on hypothalamic CRH levels in CVH rats.

Terminology

Moxibustion is an alternative or complementary therapy and is also used to treat IBS. Methods of moxibustion include SM (also named warming moxibustion), scarring moxibustion and herb-partition moxibustion. In the SM treatment, one ignited moxa-stick was suspended perpendicularly above the acupoints.

Peer review

The paper is very interesting. But there are some questions to be addressed before publication.

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S- Editor Shi ZF L- Editor Webster JR E- Editor Zheng XM

Narrow-band imaging endoscopy with and without magnification in diagnosis of colorectal neoplasia

Qing-Jie Zhou, Jian-Min Yang, Bao-Ying Fei, Qi-Shun Xu, Wei-Quan Wu, Hong-Jun Ruan

Qing-Jie Zhou, Jian-Min Yang, Bao-Ying Fei, Qi-Shun Xu, Wei-Quan Wu, Hong-Jun Ruan, Department of Gastroenterology, Zhejiang Provincial People's Hospital, Hangzhou 310014, Zhejiang Province, China

Author contributions: Zhou QJ and Yang JM contributed equally to this work; Yang JM designed the research; Yang JM, Zhou QJ, Fei BY, Xu QS, Wu WQ and Ruan HJ performed the research; Zhou QJ and Yang JM analyzed data and wrote the paper.

Correspondence to: Dr. Jian-Min Yang, Department of Gastroenterology, Zhejiang Provincial People's Hospital, Hangzhou 310014, Zhejiang Province, China. jianminyang@hotmail.com
Telephone: +86-571-85893430 Fax: +86-571-85131448

Received: August 16, 2010 Revised: October 22, 2010

Accepted: October 29, 2010

Published online: February 7, 2011

NBI endoscopy without magnification may also be used to distinguish neoplasia from non-neoplasia colorectal lesions.

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Key words: Narrow-band imaging; Colorectal neoplasia; Magnifying endoscopy; Non-magnifying endoscopy; Diagnosis

Peer reviewers: Dr. Benjamin Perakath, Professor, Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India; Chakshu Gupta, MD, FCAP, Pathology and Laboratory Medicine, Heartland Regional Medical Center, 5325 Faraon Street, St. Joseph, MO 64506, United States; Dr. John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent, ME20 7NH, United Kingdom

Abstract

AIM: To evaluate the diagnostic efficacies of narrow-band imaging (NBI) endoscopy with and without high magnification in distinguishing neoplasia from non-neoplasia colorectal lesions.

METHODS: A total of 118 patients with 123 colorectal lesions examined by NBI endoscopy in the Zhejiang Provincial People's Hospital from September 2008 to April 2010 were enrolled in this study. These lesions were classified by pit pattern and capillary pattern, and then assessed by histopathology.

RESULTS: Ten lesions not meeting the diagnostic criteria were excluded, the overall diagnostic accuracy of NBI endoscopy in distinguishing neoplasia from non-neoplasia colorectal lesions was 91.2% (103/113), and that of NBI endoscopy with and without high magnification was 93.0% (40/43) and 90.0% (63/70), respectively. Both were significantly higher than that of conventional colonoscopy reported in the literature ($P < 0.05$), but there was no significant difference between the two groups ($P > 0.05$).

CONCLUSION: Besides NBI magnifying endoscopy,

Zhou QJ, Yang JM, Fei BY, Xu QS, Wu WQ, Ruan HJ. Narrow-band imaging endoscopy with and without magnification in diagnosis of colorectal neoplasia. *World J Gastroenterol* 2011; 17(5): 666-670 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/666.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.666>

INTRODUCTION

Colorectal cancer is a common gastrointestinal malignancy with a slow process in occurrence and development involving multi-steps, multi-stages and multiple genes, and most of them arise from preexisting adenomas and have an adenoma-carcinoma sequence^[1,2]. Early detection and removal of colorectal adenomas may greatly reduce both the incidence of colorectal cancer and cancer-related death^[3].

Electronic colonoscopy is considered to be an effective examination for the detection of colorectal neoplastic lesions^[4]. However, it is difficult to assess pre-malignant and early neoplastic lesions precisely using conventional white light endoscopy. In chromoendoscopy, a biocompatible dye, such as indigo carmine, can strengthen the surface structure of epithelial lesions^[5], but the operation

is relatively cumbersome, time-consuming and costly, not conducive to observe the vascular structure, and may damage the DNA of epithelial cells.

Narrow-band imaging (NBI) is a novel technology that emerged in endoscopic diagnosis of early cancer, and it has better targeting for biopsy and higher diagnostic accuracy than conventional videoendoscopy by enhancing the visualization of surface mucosal and vascular patterns on the polyp surface^[6]. Our study aimed to verify the diagnostic accuracy of NBI endoscopy in distinguishing neoplasia from non-neoplasia colorectal lesions, and evaluate the diagnostic efficacies of NBI endoscopy with and without high magnification.

MATERIALS AND METHODS

Patients

The patients who have poor bowel preparation, familial adenomatous polyposis, infectious bowel disease, inflammatory bowel disease and colorectal cancer were excluded. A total of 118 patients with 123 colorectal lesions examined by NBI endoscopy in the Zhejiang Provincial People's Hospital from September 2008 to April 2010 were enrolled in this study. Forty-six and 77 lesions were examined by NBI endoscopy with and without high magnification.

NBI and colonoscopy

A standard videoendoscopy system with two light sources was used for examination. One light source was for the standard optical filter (broadband) and the other was for the NBI system. The control knob on the grip of the endoscope allows single touch exchange of the standard filter for the NBI filter. Olympus CV-260SL, CLV-260SL, CF240I, H260AZI and SONY LMD-2140MD were used respectively for the endoscopic host, source, conventional endoscopy, magnifying endoscopy and monitor.

Methods

Researching methods, observation tables and patient's informed consent were obtained before the study. Polyethylene glycol lavage solution and diprivan propofol were used for bowel preparation and intravenous anesthesia, respectively, and the whole examination process was managed by an experienced endoscopist (the second author). The scope was entered to the ileocecal part using conventional observation mode, and back with white light and NBI. The lesions were classified by pit pattern and capillary pattern with NBI immediately when they were detected, and then biopsied or resected for pathological diagnosis by an experienced pathologist. The endoscopist and pathologist were unaware of each other's diagnosis, and finally the NBI endoscopic diagnosis was assessed based on the pathological diagnosis.

The NBI endoscopic diagnostic criteria followed Kudo's^[7] classification of mucosal pit pattern and Sano's^[8] classification of capillary pattern. We developed the comprehensive diagnostic criteria: Pit II + CP1 as hyperplastic polyp, Pit III L + CP2 or Pit IV + CP2 as adenoma, and Pit V + CP3 as adenocarcinoma.

Table 1 Comparison of narrow-band imaging endoscopy and pathological diagnosis

	Narrow-band imaging diagnosis	Pathological diagnosis				Total
		Hyper-plastic polyp	Tubular adenoma	Villous adenoma	Adenocarcinoma	
Pit pattern	II	38	9	0	0	47
	III s	0	0	0	1	1
	III L	4	51	1	0	56
	IV	0	0	6	0	6
	V	0	0	0	13	13
Capillary pattern	CP1	35	4	0	0	39
	CP2	7	56	7	0	70
	CP3	0	0	0	14	14

Statistical analysis

Statistical differences of diagnostic accuracies were analyzed by the Mann-Whitney *U* test and χ^2 test. $P < 0.05$ was considered significantly different.

RESULTS

Among the 118 patients, 73 were males and 45 were females, with a mean age of 57.54 ± 14.01 years (range, 19-86 years). The location of the lesions was as follows: 53 in rectum, 22 in sigmoid colon, 14 in descending colon, 21 transverse colon and 13 in ascending colon, with a mean size of 10.13 ± 7.79 mm (range, 3-40 mm).

Pathologically, lesions were divided into non-neoplastic lesions, including hyperplastic and inflammatory lesions (42) and neoplastic lesions, including tubular adenoma (60), villous adenoma (7) and adenocarcinoma (14).

Based on the Kudo's classification of mucosal pit pattern and Sano's classification of capillary pattern, the lesions were classified with NBI and assessed by histopathology. The diagnostic accuracy, sensitivity and specificity to distinguish between non-neoplastic and neoplastic colorectal lesions were 94.7% (72/76), 88.9% (72/81) and 90.5% (38/42) for pit pattern delineation; and 91.7% (77/84), 95.1% (77/81) and 83.3% (35/42) for capillary pattern delineation, respectively (Table 1). According to the comprehensive diagnostic criteria, Pit II + CP1 (Figure 1A and B) was defined as hyperplastic polyp, Pit III L + CP2 (Figure 1C and D) or Pit IV + CP2 (Figure 1E and F) as adenoma, and Pit V + CP3 (Figure 1G and H) as adenocarcinoma. Ten lesions failed to meet the diagnostic criteria. The overall diagnostic accuracy of NBI endoscopy in distinguishing neoplastic from non-neoplastic colorectal lesions was 91.2% (103/113) and that of NBI endoscopy with and without high magnification was 93.0% (40/43) and 90.0% (63/70), respectively (Table 2). Both were significantly higher than that of conventional colonoscopy reported in the literature^[9] ($P < 0.05$), but there was no significant difference between the two groups ($P > 0.05$).

DISCUSSION

NBI is a novel imaging technology that uses special nar-

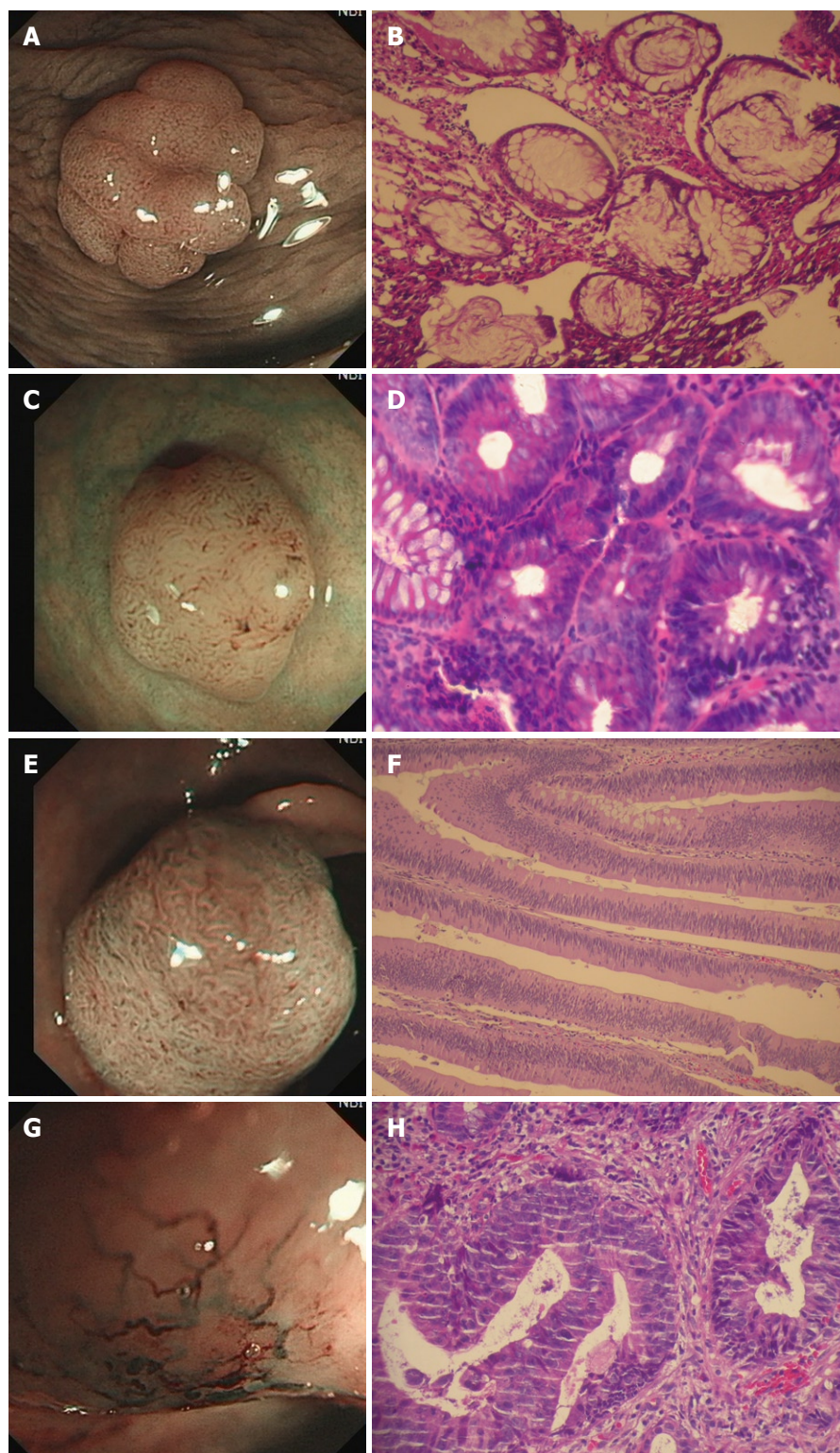


Figure 1 Narrow-band imaging endoscopic evaluation and the corresponding pathological images. A, B: A hyperplastic polyp with narrow-band imaging (NBI) magnifying endoscopy: the pits were asteroid, slightly larger than normal, with spacious space, and there was no significant microvascular structure. A: Pit II + CP1 (H260AZI); B: Hyperplastic polyp, 100 ×; C, D: A tubular adenoma with NBI conventional endoscopy: the pits were tubular, larger than normal, showing oval microvascular structure. C: Pit III L + CP2 (CF240I); D: Tubular adenoma, 400 ×; E, F: A villous adenoma with NBI conventional endoscopy: The pits were branching or gyrus-like, showing oval capillary. E: Pit IV + CP2 (CF240I); F: Villous adenoma, 100 ×; G, H: An adenocarcinoma with NBI magnifying endoscopy: the pits in the cancerous part completely disappeared, showing irregular angiogenesis network. G: Pit V + CP3 (H260AZI); H: Adenocarcinoma, 200 ×.

row-band filters in the endoscopic system, which allow for a more detailed visualization of the mucosal architecture and vascular pattern. Current NBI technology limits

the mucosal surface light penetration, thereby enhancing the visualization of the fine capillary vessel structure on the surface layer^[10]. According to a previous pilot study by

Table 2 Diagnostic efficacies of narrow-band imaging endoscopy with and without magnification

Pathological diagnosis	NBI without magnification		NBI with magnification	
	Consistent	Inconsistent	Consistent	Inconsistent
Hyperplastic polyp	21	3	12	1
Adenoma	37	4	20	2
Adenocarcinoma	5	0	8	0
Total	63	7	40	3

NBI: Narrow-band imaging.

Machida *et al*^[9], NBI with magnifying endoscopy achieved better visualization of the mucosal vascular network pattern than conventional white light imaging, and the diagnostic accuracy was higher than that of conventional colonoscopy and equivalent to chromoendoscopy. Furthermore, compared with chromoendoscopy, the NBI observation has the advantage of convenient application without the necessity of dye spraying, thus the procedure can be shortened in time and an overlooked lesion with accumulation of dark-blue dye at the dependent portion of colon can also be avoided^[11].

According to previous pathological studies, most of the colorectal cancers arise from preexisting adenomas and such an adenoma-carcinoma sequence, and the adenoma shares many architectural features with the carcinoma in terms of vascular architecture including vessel diameter and spatial distribution which is considerably different from that in the non-neoplastic portion of colonic mucosa^[12]. Therefore, NBI endoscopy has a significant advantage in the diagnosis of colorectal dysplasia accompanied with microvascular changes. In recent years, a number of researches^[9,13-15] have shown that the diagnostic accuracy of NBI endoscopy in distinguishing neoplastic from non-neoplastic colorectal lesions was higher than that of conventional colonoscopy and equivalent to chromoendoscopy. In our study, we used Kudo's classification of mucosal pit pattern and Sano's classification of capillary pattern, and the diagnostic accuracy, sensitivity and specificity to distinguish between non-neoplastic and neoplastic colorectal lesions were 94.7% (72/76), 88.9% (72/81) and 90.5% (38/42) for pit pattern delineation, which is similar to the data reported in the literature (92.7%, 95.7% and 87.5%), significantly higher than that of conventional endoscopy (82.9%, 80.0% and 81.8%)^[14]; and 91.7% (77/84), 95.1% (77/81), 83.3% (35/42) for capillary pattern delineation, which is slightly lower than the data reported in the literature (96.6%, 97.1% and 91.8%)^[16], but there was no significant difference. It may be related to the relatively small number of the cases in this study. In addition, it is possible that endoscopists who are familiar with NBI endoscopy may also improve the diagnostic accuracy, but this requires further investigation.

In recent years, as NBI combined with magnifying endoscopy could enhance the contrast detailed morphological changes in the mucosal surface and clearly visualize

the microvascular structures, most studies described the use of NBI endoscopy with magnification^[13,17-22], but few data about diagnostic accuracy of NBI endoscopy without magnification were reported. However, magnifying endoscopy is not clinically used as standard endoscopic equipment in most institutions, not only in China but also in Japan and some Western countries, because magnifying endoscopy is much more expensive. This greatly restricted the wide application of NBI magnifying endoscopy. In order to evaluate the diagnostic efficacies of NBI conventional endoscopy in distinguishing neoplastic from non-neoplastic colorectal lesions, we compared the diagnostic accuracy of NBI with and without high magnification. The results showed that the overall diagnostic accuracy of NBI endoscopy in distinguishing neoplastic from non-neoplastic colorectal lesions was 91.2% (103/113), and that of NBI endoscopy with and without high magnification was 93.0% (40/43) and 90.0% (63/70), respectively. Both were significantly higher than that of conventional colonoscopy reported in the literature, but there was no significant difference between the two groups. Therefore, we believe that NBI endoscopy without high magnification could also greatly improve the diagnostic accuracy and may also be used to distinguish neoplastic from non-neoplastic colorectal lesions instead of NBI magnifying endoscopy. However, as the sample was relatively small in our study, it was not clear whether NBI without high magnification could improve the detection rate in the mass population screening; this requires further study and investigation.

As a new non-invasive endoscopic method, the diagnostic efficacies of NBI combined with magnifying endoscopy in distinguishing neoplastic from non-neoplastic colorectal lesions has been confirmed by extensive literatures^[13,14,23-25]. We found in our study that the endoscopic system installed with the NBI system enables the clinicians to significantly improve the diagnostic accuracy. Therefore, even the institutions without the expensive magnifying endoscopy equipment can also use conventional NBI endoscopy to get an accurate diagnosis. Although these findings need to be confirmed in large prospective trials, this initial experience with conventional NBI endoscopy is encouraging and holds promise for future application in prospective studies. In addition, endoscopists, through training in NBI endoscopic practice, may also improve their own diagnostic accuracy and raise the detection rate of colorectal neoplasia^[26].

COMMENTS

Background

Narrow-band imaging (NBI) is a novel technology developed for endoscopic diagnosis of early cancer, and it has a higher diagnostic accuracy than conventional endoscopy by enhancing the visualization of surface mucosal and vascular patterns on the lesion surface.

Research frontiers

In recent years, as NBI combined with magnifying endoscopy could enhance the contrast detailed morphological changes in the mucosal surface and clearly visualize the microvascular structures, most studies described the use of NBI endoscopy with magnification, but few data about diagnostic accuracy of NBI endoscopy without magnification were reported.

Innovations and breakthroughs

Compared with NBI magnifying endoscopy, NBI endoscopy without magnification may be used in distinguishing neoplasia from non-neoplasia colorectal lesions.

Applications

NBI endoscopy enables clinicians to significantly improve their diagnostic accuracy. Even those institutions equipped without magnifying endoscope can also use NBI conventional endoscopy to get an accurate diagnosis.

Terminology

NBI is a novel imaging technology that uses special narrow-band filters in the endoscopic system, which allow for a more detailed visualization of the mucosal architecture and vascular pattern.

Peer review

This paper compares narrow band imaging with and without high magnification in 100 patient with colorectal lesions over an 18 mo period. The authors show that both of these techniques are more accurate than conventional colonoscopy in distinguishing between neoplastic and non-neoplastic lesions with similar sensitivity. A larger study would have had more value.

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S- Editor Tian L L- Editor Ma JY E- Editor Zheng XM

Fast-track rehabilitation program vs conventional care after colorectal resection: A randomized clinical trial

Gang Wang, Zhi-Wei Jiang, Jing Xu, Jian-Feng Gong, Yang Bao, Li-Fei Xie, Jie-Shou Li

Gang Wang, Zhi-Wei Jiang, Jian-Feng Gong, Yang Bao, Li-Fei Xie, Jie-Shou Li, Research Institute of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing 210002, Jiangsu Province, China

Jing Xu, Department of Medical Oncology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing 210002, Jiangsu Province, China

Author contributions: Wang G, Jiang ZW and Li JS designed the research; Wang G, Jiang ZW, Xu J, Gong JF, Bao Y and Xie LF performed the research; Wang G analyzed the data; Wang G and Xu J wrote the paper.

Supported by Social Development Fund of Jiangsu Province, No. BS2007054

Correspondence to: Zhi-Wei Jiang, MD, Research Institute of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, 305 Zhongshan East Road, Nanjing 210002, Jiangsu Province, China. surgery34@163.com

Telephone: +86-25-80860137 Fax: +86-25-84803956

Received: October 11, 2010 Revised: November 17, 2010

Accepted: November 24, 2010

Published online: February 7, 2011

Abstract

AIM: To compare the fast-track rehabilitation program and conventional care for patients after resection of colorectal cancer.

METHODS: One hundred and six consecutive patients who underwent fast-track rehabilitation program were encouraged to have early oral feeding and movement for early discharge, while 104 consecutive patients underwent conventional care after resection of colorectal cancer. Their gastrointestinal functions, postoperative complications and hospital stay time were recorded.

RESULTS: The restoration time of gastrointestinal functions in the patients was significantly faster after fast-track rehabilitation program than after conventional care (2.1 d vs 3.2 d, $P < 0.01$). The percentage of patients who developed complications was significantly lower 30 d after fast-track rehabilitation program than after

conventional care (13.2% vs 26.9%, $P < 0.05$). Also, the percentage of patients who had general complications was significantly lower 30 d after fast-track rehabilitation program than after conventional care (6.6% vs 15.4%, $P < 0.05$). The postoperative hospital stay time of the patients was shorter after fast-track rehabilitation program than after conventional care (5 d vs 7 d, $P < 0.01$). No significant difference was observed in the re-admission rate 30 d after fast-track rehabilitation program and conventional care (3.8% vs 8.7%).

CONCLUSION: The fast-track rehabilitation program can significantly decrease the complications and shorten the time of postoperative hospital stay of patients after resection colorectal cancer.

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Key words: Perioperative care; Fast track; Rehabilitation; Colorectal cancer resection

Peer reviewer: Luca Stocchi, MD, Desk A 30, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States

Wang G, Jiang ZW, Xu J, Gong JF, Bao Y, Xie LF, Li JS. Fast-track rehabilitation program vs conventional care after colorectal resection: A randomized clinical trial. *World J Gastroenterol* 2011; 17(5): 671-676 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/671.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.671>

INTRODUCTION

The concept of fast track rehabilitation program has been recently introduced with the intent to improve the management, stress, complications, shorten hospital stay time and reduce cost of patients after resection of colorectal cancer^[1-7]. Fast track rehabilitation program is basically a

multidisciplinary perioperative care strategy for patients after resection of colorectal cancer, including preoperative education, effective anesthesia, postoperative analgesia techniques, early oral nutrition and ambulation^[8-11]. However, the previous researches were mainly focused on the postoperative complications after conventional care rather than on the general complications after fast-track rehabilitation program. This study was to compare the complications, restoration of gastrointestinal functions, and hospital stay time of postoperative colorectal cancer patients after fast-track rehabilitation program and conventional care.

MATERIALS AND METHODS

Patients and procedures

Two hundred and thirty patients who underwent resection colorectal cancer in the Research Institute of General Surgery, Jinling Hospital (Nanjing, China) in July 2007 to August 2009 were enrolled in this study. Of the 230 patients, 115 who underwent resection of colorectal neoplastic disease served as a fast-track rehabilitation program group, and 115 who underwent resection of colorectal cancer served as a conventional care group. Nine patients with non-selective admission, preoperative distant metastasis, stoma, emergency situations, scheduled total colectomy or abdominoperineal resection, contraindications for epidural anesthesia or early ambulation were excluded from the fast-track rehabilitation program group, and 11 from the conventional care group. Finally, 106 patients in the fast-track rehabilitation program group and 104 patients in the conventional care group were analyzed in this study.

The contents of fast-track rehabilitation program include preoperative education of patients with no bowel preparation and fasting but with carbohydrate containing liquids 2 h before surgery, analgesia with routine oral non-steroidal anti-inflammatory medications and minimization of opioid pain management, avoidance of perioperative fluid overload, no routine use of nasogastric tubes, early removal of bladder catheters, early feeding and enforced ambulation on the day of surgery. In the fast track rehabilitation program group, minimal-access surgery or transverse curved incision used included right-sided hemicolectomy through a right horizontal incision above the umbilicus, sigmoid resection through a curved incision in the left iliac fossa and low anterior rectal resection through a mini-laparotomy in the subumbilicus which was extended toward the curvature if necessary. Principles of the perioperative care are shown in Table 1.

Discharge criteria for patients in both groups were the same, including tolerance to fluids and solid diet, adequate oral analgesia, passage of flatus or stool, and no surgical complication, basic self-care ability, and acceptance of discharge.

Clinical outcome

The intestinal function was defined as passage of flatus, morbidity requiring treatment during the first 30 postoperative days, postoperative hospital stay time, and readmis-

sion rate. No patient was lost during the follow-up. General complications were defined as those occurred in the cardiovascular, pulmonary, thromboembolic, urinary systems, while surgical complications were defined as wound complication, anastomotic leak, and bowel obstruction requiring reoperation as previously described^[12].

Statistical analysis

Statistical analysis, based on an intention-to-treat analysis, was performed with the SPSS version 16.0 (Chicago, IL, USA). Mann-Whitney test was used to compare the continuous variables. χ^2 test and Fisher's exact test were used to compare the discrete variables. $P < 0.05$ was considered statistically significant.

RESULTS

Of the 230 enrolled patients (115 in the fast track rehabilitation program group and 115 in the conventional care group), 210 were analyzed (106 in the fast track rehabilitation program group and 104 in the conventional care group). The relevant characteristics of patients and the types of surgery are shown in Table 2. No significant difference was observed in age, ASA status, types of surgery and tumor stages between the two groups.

The intestinal function of patients in the fast track rehabilitation program group and conventional care group became normal 2 d (range, 1-6 d) and 3 d (range, 1-8 d), respectively, after resection of colorectal cancer ($P < 0.01$). The median postoperative hospital stay time was 5 d (range, 2-41 d) and 7 d (range, 3-55 d), respectively, for the patients in the fast track rehabilitation program group and conventional care group ($P < 0.01$). The postoperative rehabilitation was also faster in patients of the fast track rehabilitation program group than in those of conventional care group. On the day of surgery, 11 patients (35%) in the fast track rehabilitation program group and no patient in the conventional care group were able to walk. On postoperative day 1, 56 patients (53%) in the fast track rehabilitation program group and 24 patients (23%) in the conventional care program group were able to walk. On postoperative day 2, 90 patients (85%) in the fast track rehabilitation program group and 61 patients (59%) in the conventional care group were able to walk ($P < 0.01$) (Table 3).

The urethral catheter in 81 patients (81%) of the fast track rehabilitation program group and in 21 patients (20%) of the conventional care group was removed on day 1 after resection of colorectal cancer ($P < 0.05$), and in 97 patients (92%) of the fast track rehabilitation program group and in 47 patients (45%) of the conventional care group on day 2 after resection of colorectal cancer ($P < 0.05$). Urinary retention occurred in 5 patients (5%) of the fast track rehabilitation program group and in 16 patients (15%) of the conventional care group. Urethral catheter was inserted again in 4 patients of the fast track rehabilitation program group and in 12 patients of the conventional care group.

Table 1 Principles of fast track rehabilitation program and conventional care

	Fast track rehabilitation program	Conventional care
Preoperative	Patients and their relatives were informed about the surgical procedure and postoperative course	Patient were educated in the standard manner
Day before surgery		
Bowel preparation	No bowel preparation was performed	Two oral sachets of fleet® bowel preparation
Carbohydrate load	4 units (preOp®)	No
Diet	Last meal 6 h before operation	Last meal at midnight
Day of surgery		
Pre-operative fasting	No, 2 units (preOp®) 2 h before surgery	Yes
Nasogastric tubes	No unless nausea and vomit	Routine placement
Pre-anesthetic medication	No	Oral diazepam 10 mg
Anesthesia	General anesthesia Remifentanyl 1 µg/kg per minute Propofol 2-4 mg/kg per hour Cisatracium 0.15 mg/kg Ondansetron 4 mg Bupivacaine 0.25% 20 mL (incision) Epidural catheter T10-T12 Test: 3 mL 2% lidocaine with epinephrine Bupivacaine 0.5% (6 + 6) mL	General anesthesia Remifentanyl 1 µg/kg per minute Propofol 2-4 mg/kg per hour Cisatracium 0.15 mg/kg Ondansetron 4 mg
Surgical management	Minimal invasive incision Infiltration of surgical wounds with Bupivacaine	Median laparotomy approach No infiltration of surgical wounds with local anesthetic drugs
Surgical drains	No, unless required in circumstances and discarded in early time (usually on postoperative day 1)	Routine placement usually discarded the day before discharge
Early post-operative care	Use of epidural catheter (0.125% Bupivacaine with 2.5 µg/mL Fentanyl) First oral drink 2 h after surgery IV infusion of Ringers lactate 1.5 L/d Mobilization in the evening (> 2 h out of bed)	Analgesia by bolus administration of diclofenac or morphine No oral application scheme IV infusion of Ringers lactate 2.5 L/d No mobilization scheme
Postoperative care		
Day 1 after surgery	Oral intake > 2 L (including 4 units CHL liquids) Semi-solid food intake Stop IV fluid administration Remove urine catheter Expand mobilization (> 6 h out of bed)	Diet increased on daily basis IV fluid administration (2.5 L/d) till adequate oral fluid intake Mobilization according to attending surgeon
Day 2 after surgery	Remove epidural add Diclofenac 3 × 50 mg/d Normal diet Expand mobilization (> 8 h) Plan discharge	Continue as on day 1 till discharge criteria fulfilled
Day 3 after surgery	Continue as on day 2 till discharge criteria fulfilled	Continue as on day 2 till discharge criteria fulfilled

Table 2 Characteristics of patients and their diagnosis

	Fast track rehabilitation group (n = 106)	Conventional care group (n = 104)	P value
Median age (range, yr)	57 (38-69)	55 (40-67)	0.462
Male/female	65/41	60/44	0.393
Colon/rectum	73/33	63/41	0.110
ASA score			0.384
I	27	32	-
II	60	56	-
III	19	16	-
Operation			0.721
Right hemicolectomy	30	24	
Left hemicolectomy	18	26	
Sigmoid colectomy	28	32	
Anterior resection	30	22	
TNM stage			0.741
I	19	17	
II	56	61	
III	31	26	

Table 3 Postoperative rehabilitation and hospital stay time of two groups n (%)

	Fast track rehabilitation group (n = 106)	Conventional care group (n = 104)	P value
Walk on surgery day	11 (35)	0 (0)	0.001
Walk on D 1	56 (53)	24 (23)	0.000
Walk on D 2	90 (85)	61 (59)	0.001
Days until flatus			0.001
mean ± SD	2.1 ± 2.0	3.2 ± 2.5	-
Median (range)	2 (1-6)	3 (1-8)	-
Hospital stay time (d)			0.001
mean ± SD	5.1 ± 3.1	7.6 ± 4.8	-
Median (range)	5 (2-41)	7 (3-55)	-

The nasogastric tube was maintained for 1-4 d in 3 patients (3%) of the fast track rehabilitation program group and for 1-11 d in 84 patients (81%) of the conventional

Table 4 General and surgical complications of two groups

	Fast track rehabilitation group (<i>n</i> = 106)	Conventional care group (<i>n</i> = 104)	<i>P</i> value
Overall complications	20	39	0.015
Patients with complications	14	28	0.016
General complications	10	23	0.042
Patients with general complications	7	16	0.048
Cardiac	2	5	-
Pulmonary	3	8	-
Thromboembolic	1	3	-
Urinary tract	2	5	-
Other	2	2	-
Overall surgical complications	10	16	0.221
Patients with surgical complications	7	12	0.230
Wound infection	4	7	-
Anastomotic leakage	4	2	-
Bowel obstruction	2	5	-
Death	2	1	0.572

care group ($P < 0.01$). The nasogastric tube was reinserted in 4 patients (4%) of the fast track rehabilitation program group and in 12 patients (11%) of the conventional care group due to nausea and vomit ($P < 0.05$).

No significant difference was observed in re-admission rate between the two groups within 30 d after resection of colorectal cancer. Four patients (4%) in the fast track rehabilitation program group were readmitted due to wound infection, and 9 patients (9%) in the conventional care group were readmitted due to bowel obstruction, vomit and wound infection.

The incidence of complications was 19% in patients of the fast track rehabilitation program group and 38% in those of the conventional care group ($P < 0.05$) during the first 30 postoperative days. One or more complications occurred in 14 patients (13%) of the fast track rehabilitation program group and in 28 patients (27%) of the conventional care group ($P < 0.05$). The overall incidence of general complications was lower in patients of the fast track rehabilitation program group than in those of the conventional care group ($P < 0.05$). The incidence of complications in the cardiac and pulmonary system was also significantly lower in patients of the fast track rehabilitation program group than in those of the conventional care group ($P < 0.05$). A significant difference was observed in surgical complications between the two groups. Two patients died in the fast track rehabilitation program group due to cardiovascular failure and multiple organ failure, respectively. One patient died in the conventional care group due to cardiovascular failure. The types of complications are listed in Table 4.

DISCUSSION

The results of the present study indicate that fast-track rehabilitation program can significantly accelerate the

restoration of gastrointestinal function and reduce the postoperative complications as well as hospital stay time of patients after resection of colorectal cancer. The results of this study show that preoperative education of patients, epidural anesthesia or regional anesthesia^[13], early ambulation and early postoperative oral nutrition are the important predictors for the rehabilitation of patients after resection of colorectal cancer.

Preoperative education of patients is regarded as one of the crucial factors for fast-track rehabilitation. It is necessary to explain the detailed treatment plan, different stages of fast-track rehabilitation program and relevant measures for recovery for the patients in order to make them better understand the importance of fast-track rehabilitation program. Better cooperation of patients can bring better outcomes of fast track rehabilitation program. Generally, since the gastric emptying time of solid meal and fluid is 6 and 2 h, respectively^[14], the patients should be encouraged to have liquid meal 2 h before operation instead of fasting. It has been shown that preoperative oral carbohydrate is safe and can efficiently reduce complications^[15-17].

The role of epidural anesthesia or regional anesthesia in fast-track rehabilitation program should be stressed. Postoperative epidural analgesia can avoid stress-induced neurological, endocrinological and homeostatic changes or the blocking of sympathetic nerve-related surgical stress response, reduce complications such as nausea, vomiting and enteroparalysis after operation, early ambulation, improve the intestinal function and shorten the hospital stay time of patients after resection of colorectal cancer^[18-24]. In this study, epidural analgesia significantly shortened the bedridden time and potentially reduced the cardiopulmonary and thromboembolic complications. The rate of cardiopulmonary and thromboembolic complications was much lower in patients of the fast track rehabilitation program group than in those of the conventional care group ($P < 0.05$).

Early postoperative oral nutrition also plays an essential part in fast-track rehabilitation program. Food intake can stimulate gastrointestinal peristalsis, and early feeding during the first 24 h after surgery promotes the recovery of ileus. It has been illustrated that early postoperative oral nutrition attenuates catabolism and potentially decreases infectious complications^[25,26]. Consistent with this, early postoperative oral nutrition has been suggested as a routine procedure of abdominal surgery^[26]. Enforced postoperative mobilization of patients can reduce protein loss due to long-term bedridden, pulmonary infection and venous thrombosis. In this study, complete analgesia, control of nausea and vomiting, early postoperative oral nutrition and early ambulation efficiently reduced the postoperative complication of ileus and improved the recovery of intestinal function.

In this study, the early removal of gastric tube and urethral catheter decreased not only the infectious complications in cardiopulmonary and urinary systems but also the symptoms of patients. The shortened fasting

time, preoperative carbohydrate load and intraoperative fluid restriction effectively protected against homeostasis in patients after resection of colorectal cancer. The outcome of fast-track rehabilitation program was better than that of conventional care.

Fast track rehabilitation program can improve the symptoms of patients after resection of colorectal cancer better than conventional care, thus benefiting their surgery, anesthesia, pain management, physical therapy and social work. The primary work of fast track rehabilitation program is the preoperative education of patients to make them understand the whole plan and the aim of each stage. Therefore, it is necessary to get the cooperation from nurses, because they need to work professionally and nicely. Although there must be lots of difficulties in fast track rehabilitation program, it is an inevitable stage to test a new set of rules and guidelines.

Recently, laparoscopic surgery, applied in treatment of colorectal and early gastric cancer, can significantly reduce trauma and speed up the rehabilitation of patients after surgery. It was reported that the hospital stay time is shorter and the morbidity and readmission rate are lower after laparoscopic surgery^[27,28]. However, these studies only compared open surgery with laparoscopic surgery rather than laparoscopic surgery with fast-track rehabilitation program^[27,28]. Therefore, further studies are needed to focus on the potential influence of laparoscopy-assisted surgery with or without fast-track rehabilitation program on the recovery of patients after resection of colorectal cancer. Laparoscopic surgery and fast-track rehabilitation program can effectively promote the recovery of patients after resection of colorectal cancer. We believe that laparoscopic surgery in combination with fast track rehabilitation program is significantly advantageous over other procedures for patients after resection of colorectal cancer.

In conclusion, fast track rehabilitation program plays an important role in the recovery of patients after resection of colorectal cancer, which can accelerate the restoration of their gastrointestinal function, decrease their postoperative complications, and shorten their hospital stay time.

COMMENTS

Background

Fast-track rehabilitation program, first reported by Kehlet *et al.*, can reduce the postoperative complications and hospital stay time of patients after resection of colorectal cancer without compromising the surgical outcome. The concept of fast track rehabilitation program has been recently introduced in colorectal surgery. It is basically a multidisciplinary perioperative care strategy for patients after resection of colorectal cancer.

Research frontiers

The previous studies seemed to compare the postoperative complications rather than the general complications of fast track rehabilitation program and conventional care.

Innovations and breakthroughs

The gastrointestinal function, postoperative complications, and hospital stay time of patients after resection of colorectal cancer were studied during their fast track rehabilitation program. The accelerated restoration of gastrointestinal

function and decreased postoperative complications may shorten the hospital stay time of patients after resection of colorectal cancer.

Applications

Surgical care has changed dramatically over the past half century and will continue to improve with the time. Extensive studies on the optimized care will allow us to develop more appropriate perioperative surgical care programs for patients after resection of colorectal cancer.

Terminology

Fast track rehabilitation program, basically a multidisciplinary strategy for patients after resection of colorectal cancer, is to optimize the preoperative, perioperative and postoperative factors for reducing their physiological and psychological stress surgery.

Peer review

This manuscript describes a prospective randomized trial comparing fast track rehabilitation program and conventional care for patients after resection of colorectal cancer. The data are sound support the hypothesis of the authors.

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S- Editor Tian L L- Editor Wang XL E- Editor Zheng XM

***In-vivo* characterization of DALM in ulcerative colitis with high-resolution probe-based confocal laser endomicroscopy**

Giovanni D De Palma, Stefania Staibano, Saverio Siciliano, Francesco Maione, Maria Siano, Dario Esposito, Giovanni Persico

Giovanni D De Palma, Saverio Siciliano, Francesco Maione, Dario Esposito, Giovanni Persico, Department of Surgery and Advanced Technologies, Center of Excellence for Technical Innovation in Surgery, Section of Diagnostic and Therapeutic Endoscopy, University of Naples Federico II, School of Medicine, 80131, Naples, Italy

Stefania Staibano, Maria Siano, Department of Biomorphological and Functional Sciences, Section of Pathology, University of Naples Federico II, School of Medicine, 80131, Naples, Italy

Author contributions: De Palma GD and Staibano S contributed to conception and design, analysis and interpretation of data, drafting the article, critical revision of the article, final approval of the article; Siciliano S, Siano M, Esposito D and Maione F contributed to data gathering, analysis and interpretation of the data, drafting the article, final approval of the article; Persico G contributed to critical revision of the article and final approval of the article.

Correspondence to: Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, Center of Excellence for Technical Innovation in Surgery, Section of Diagnostic and Therapeutic Endoscopy, University of Naples Federico II, School of Medicine, Via Pansini, 5, 80131, Naples, Italy. giovanni.depalma@unina.it

Telephone: +39-81-7462773 Fax: +39-81-7462752

Received: July 20, 2010 Revised: September 9, 2010

Accepted: September 16, 2010

Published online: February 7, 2011

showed specific alteration of crypt architecture, cellular infiltration, and vessel architecture with an excellent correlation between CLE and standard histological examination.

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Key words: Colonoscopy; Confocal laser endomicroscopy; Chronic ulcerative colitis; Dysplasia-associated lesional mass; Histology

Peer reviewer: Dr. Sanchoy Sarkar, Department of Gastroenterology, Royal Liverpool University Hospital, Prescot Street, Liverpool, L78XP, United Kingdom

De Palma GD, Staibano S, Siciliano S, Maione F, Siano M, Esposito D, Persico G. *In-vivo* characterization of DALM in ulcerative colitis with high-resolution probe-based confocal laser endomicroscopy. *World J Gastroenterol* 2011; 17(5): 677-680
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i5/677.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i5.677>

Abstract

Recently, the use of confocal laser endomicroscopy (CLE) in the diagnosis of chronic ulcerative colitis (CUC) was reported. In this brief report we aimed to assess the application of probe-based CLE to characterize colonic mucosa and dysplasia in CUC. The study involved a patient presenting long-standing CUC. Confocal imaging of both the inflamed mucosa, a circumscribed lesion (dysplasia-associated lesional mass), and adjacent colonic mucosa are demonstrated and the correlation between the CLE and histological images. Inflamed mucosa and dysplasia

INTRODUCTION

The term dysplasia-associated lesional mass (DALM) has been adopted to describe the group of endoscopically visible lesions, within the colitic colon (in the course of IBD), that refers to a heterogeneous population of lesions that demonstrate plaque-like, mass, stricture, sessile, or pedunculated morphology, that have an associated dysplasia in the surrounding mucosa^[1-3].

With recent rapid advances in videoendoscopic instrument systems, improved endoscopic skills, and improved detection techniques such as pan-colonic dye spraying, the proportion of lesions that are discovered macroscopically is likely to increase^[4].

Recently, the use of confocal laser endomicroscopy

(CLE) in the diagnosis of chronic ulcerative colitis (CUC) was reported^[5,6].

This report describes the CLE findings in a case of DALM in CUC in correlation with histopathology diagnosis.

CASE REPORT

A 48-year-old woman with a 23 years history of left-sided ulcerative colitis underwent a surveillance colonoscopy.

A mild anemia (hemoglobin level, 110 g/L) with low serum iron levels (210 µg/L; normal value, 53-167 µg/L) and a high erythrocyte sedimentation rate (37 mm/L per hour; normal value, 1-10 mm/L per hour) were the only blood chemistry abnormalities identified.

Colonoscopy revealed a left-sided Mayo CU-1 CUC with a 2 cm plaque-like lesion at the sigmoid colon (Figure 1A and B). A morphological characterization of the lesion with CLE was undertaken.

Probe-based CLE procedure

The procedure was performed using the Cellvizio® Endomicroscopy System (Mauna Kea Technologies, Paris, France) by a Coloflex UHD-type probe (1 µm lateral resolution; 12 frames/s).

This system uses a 2.5-mm catheter probe (Coloflex UHD-type probe) that is inserted through the endoscope-working channel to obtain dynamic imaging of the mucosa. This probe has a field of view of 240 µm × 200 µm, with a lateral resolution of 1 µm. Probe-based CLE (pCLE) imaging data were collected at a scan rate of 12 frames/s with a scanning field of 30 000 pixels. Single video frames were reconstructed into 1 larger static image (4 mm × 2 mm) by a special computer software ("mosaicing" Mauna Kea Technologies).

Five milliliters of 10% sodium fluorescein were injected intravenously before CLE image acquisition as a contrast agent.

Confocal imaging of both the inflamed mucosa, the circumscribed lesion (DALM), and adjacent colorectal mucosa was performed by placing the tip of the probe in direct contact with the target tissue site.

Mucosal biopsy specimens were collected from the observation sites using biopsy forceps. Fixed samples were embedded in paraffin and sectioned transversely, and stained with hematoxylin-eosin to facilitate the comparison between confocal images and histology.

pCLE Images

pCLE imaging of inflamed mucosa showed dilation of crypt openings, more irregular arrangement of crypts, and enlarged spaces between crypt, crypt destruction, and/or crypt fusion and crypt abscess. Microvascular alterations with fluorescein leaks into the crypt lumen (therefore making the lumen brighter than the surrounding epithelium) were observed (Figure 2A and B).

DALM was characterized by "dark" cells, with mucin depletion and goblet cell/crypt density attenuation; the

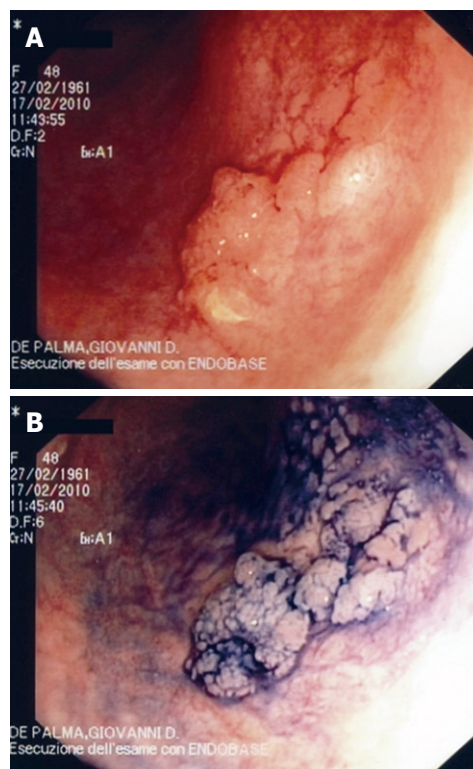


Figure 1 Conventional "white light" imaging of a plaque-like lesion of sigmoid colon in ulcerative colitis (A) and 0.5% indigo carmine chromoscopy of the lesion (B). The lesion is "unmasked" and clearly delineated.

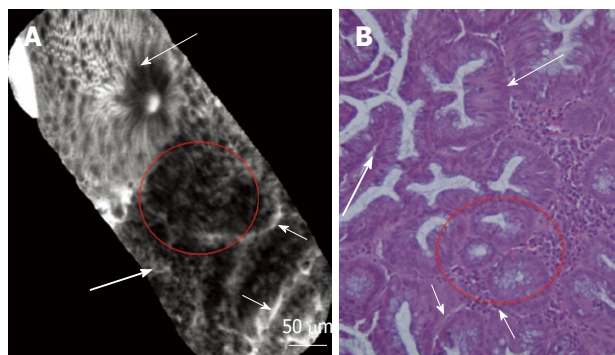


Figure 2 Confocal (A) and histological (B) images of colonic mucosa showing the switch from normal mucosa to inflamed mucosa. Normal crypt architecture is classically represented by ordered and regular crypt orifices covered by a homogeneous epithelial layer with visible "black-hole" goblet cells within the subcellular matrix (long thin arrows). Inflamed mucosa showing irregular arrangement of crypts, crypt fusion (red circles) and capillaries alterations (short arrows) and inflammatory cells (lymphocytes: long thick arrows). Magnification, × 200.

architectural pattern was irregular, as well as the epithelial thickness, with villiform structures and "dark" epithelial border. The blood vessels were dilated and irregularly-branching, with poor orientation to adjunct tissue, and fluorescein extravasation (Figure 3A and B).

pCLE imaging of colorectal mucosa adjacent to lesion (1 to 2 cm around DALM) showed the switch from the inflamed mucosa, to the neoplastic mucosa as evidenced by DALM (Figure 4).

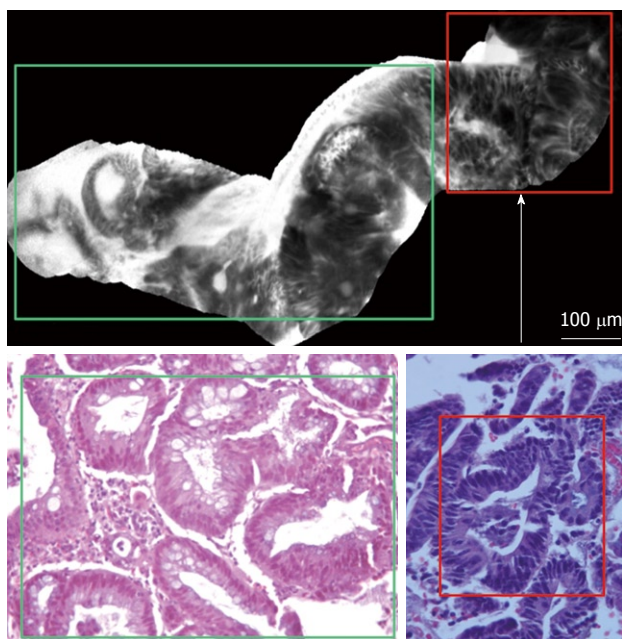


Figure 3 Confocal images of colonic mucosa evidencing the switch from the inflamed mucosa, to the neoplastic mucosa. Inflamed mucosa (green rectangle) is characterized by dilation of crypt openings, enlarged spaces between crypt, and microvascular alterations with fluorescein leaks into the crypt lumen (white arrow) therefore making the lumen brighter than the surrounding epithelium. Dysplastic mucosa (red rectangle) is characterized by “dark” cells, irregular architectural patterns with villiform structures and a “dark” epithelial border. Histology images show high-power hematoxylin and eosin stain of the tissue sampled, evidencing respectively inflamed area with features suggestive of chronic ulcerative colitis (green rectangle) and low grade dysplasia (red rectangle). Magnification, $\times 200$.

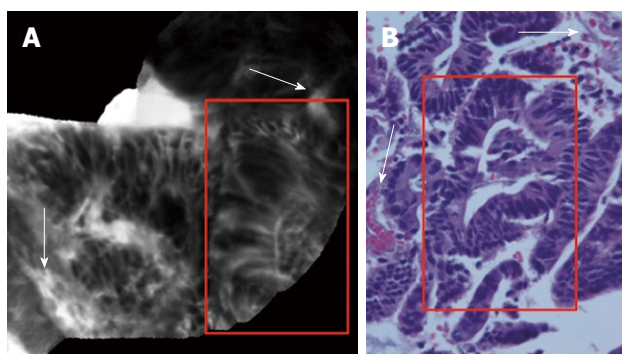


Figure 4 Confocal (A) and histological (B) images of dysplasia-associated lesion mass showing “dark” cells, with mucin depletion and goblet cell/crypt density attenuation; the architectural pattern is irregular, as well as the epithelial thickness, with villiform structures and “dark” epithelial border (red rectangles). There is gross distortion of the vascular architecture with tortuous and dilated vessels (white arrows). The hematoxylin and eosin stain histology shows a low grade dysplasia (red rectangle; hematoxylin and eosin staining; original magnification, $\times 200$).

DISCUSSION

CLE is a new technology that has enabled endoscopists to collect real-time *in vivo* histological images or “virtual biopsies” of the gastrointestinal mucosa during endoscopy.

CLE can be performed currently with 2 devices: one integrated into an endoscope (Pentax, Japan, herein termed

eCLE) and one as a stand-alone probe (herein termed pCLE) capable of passage through the accessory channel of most endoscopes (Cellvizio, Mauna Kea Technologies, Paris, France)^[7-9]. There are no data, at present, comparing pCLE with eCLE to demonstrate the superiority of any one system. pCLE has several advantages and disadvantages compared with eCLE. Advantages include the greater versatility of pCLE probes, which can be used in conjunction with virtually any endoscope (high-resolution endoscopes, NBI, cholangioscope, *etc.*), ad hoc usage (such as when a lesion is detected with a normal endoscope) and acquisition at video frame rate of 12 frames/s. allowing *in vivo* imaging of capillary flow. Disadvantages include a slightly lower resolution (approximately 1 μm compared with 0.7 μm for eCLE) and smaller field of view (240-600 μm).

Recently, the use of eCLE in the diagnosis of CUC was reported. Watanabe *et al.*^[5] and Li *et al.*^[6] reported on real-time inflammation activity assessment by CLE. The inflammation activity assessment includes crypt architecture, cellular infiltration, and vessel architecture. These studies evidenced that images taken with the CLE provided information that was equivalent to conventional histology, differentiating between active and non-active CUC patients during ongoing endoscopy.

Hurlstone *et al.*^[10] assessed the clinical applicability and predictive power of the CLE for the *in vivo* differentiation of ALM and DALM in CUC. The study evidenced that ALM and DALM can be differentiated with a high overall accuracy, enabling the safe selection of patients suitable for endoluminal resection *vs* immediate referral for surgery.

To the best of our knowledge, this is the first report that addressed the application of pCLE for the *in vivo* characterization of colonic mucosa and DALM in a patient with CUC during ongoing videocolonoscopy.

Our study showed that the pCLE system permits high-quality cellular, subsurface vascular and stromal imaging *in vivo*, with an excellent correlation between CLE and standard histopathologic examination for both inflammation and dysplasia in ulcerative colitis.

Post-acquisition specifically-developed software (“mosaicing”, Mauna Kea Technologies) was used to reconstitute the dynamic high-resolution pictures into a larger static image. By the use of mosaicing, the image area could be increased 2- to 4-fold, and image definition could be further enhanced to allow finer detail visualization. As a result of the large static image comprising of many single pictures of a video sequence, evaluation of the examination is easier and more efficient. Thereby, these features lead to an excellent correlation between CLE and standard histopathologic examination (Figures 1-4).

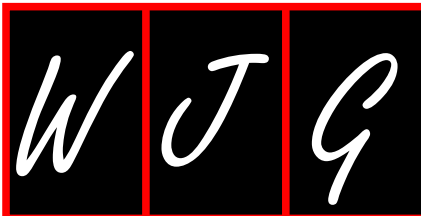
The main aspect of inflamed mucosa consisted of dilation of crypt openings, enlarged spaces between crypt, and microvascular alterations with fluorescein leaks into the crypt lumen (therefore making the lumen brighter than the surrounding epithelium); DALM showed typical neoplastic features of Mainz CLE criteria for prediction of intraepithelial neoplasia^[11] (Figure 4).

In conclusion, this is the first study to address the novel applicability of pCLE for the *in vivo* characterization of mucosal inflammation and dysplasia in CUC. With appropriate training and careful patient selection, CLE imaging may become a suitable imaging modality in patients with CUC. The ability to target biopsies to areas suggestive of dysplasia *in vivo* allows rapid, highly accurate diagnosis “on table”, reducing inappropriate, non-significant histopathology.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Luigi Bonavina, Professor, Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Xian-Ming Chen, MD, Associate Professor, Department of Medical Microbiology and Immunology, Creighton University, 2500 California Plaza, Omaha, NE 68178, United States

Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, #301, Nok-Ji Building, Seoul 136-701, South Korea

T Choli-Papadopolou, Dr., Associate Professor, Department of Biochemistry, Aristotle University of Thessaloniki, School of Chemistry, Thessaloniki, 55124, Greece

Ashraf Dahaba, Dr., MD, PhD, MSc, Professor, Department of Anaesthesiology, Medical University of Graz Medical University, Auenbruggerplatz 29, A-8036 Graz, Austria

Robert JL Fraser, Associate Professor, Investigations and Procedures Unit, Repatriation General Hospital, Daw Park, Australia

Pankaj Garg, Dr., Consultant, Department of General Surgery, Fortis Super Speciality Hospital, Mohali, Punjab, Panchkula, 134112, India

Jon C Gould, MD, FACS, Associate Professor of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States

Pietro Invernizzi, MD, PhD, Division of Internal Medicine and Hepatobiliary Immunopathology Unit, IRCCS Istituto Clinico Humanitas, via A. Manzoni 113, 20089 Rozzano, Milan, Italy

Bhupendra Kumar Jain, Dr., MS, Professor of Surgery and Head, Department of Surgery, GTB Hospital and University College of Medical Sciences, Delhi 110 095, India

Ingmar Königsrainer, MD, Department of General, Visceral and Transplant Surgery, Hoppe Seyler Str. 3, 72076 Tübingen, Germany

Rupert Leong, Associate Professor, Director of Endoscopy, Concord Hospital, ACE Unit, Level 1 West, Hospital Rd, Concord NSW 2139, Australia

Fabrizio Montecucco, MD, Assistant, Division of Cardiology, Department of Internal Medicine, University of Geneva, Avenue de la Roseraie 64, 1211 Geneva, Switzerland

Patrick O'Dwyer, MB, BCh, BAO, FRCS (I), MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

Josep M Pique, MD, Department of Gastroenterology, Hospital Clinic of Barcelona, Villarroel, 170, Barcelona 08036, Spain

Marco Romano, MD, Professor, Dipartimento di Internistica Clinica e Sperimentale-Gastroenterologia, II Policlinico, Edificio 3, II piano, Via Pansini 5, 80131 Napoli, Italy

Norbert Senninger, Professor, Department of General Surgery, University Clinics, Westphalian-Wilhelm's-University, Waldeyerstrasse 1, D-48149 Muenster, Germany

Paul E Sijens, PhD, Associate Professor, Radiology, UMCG, Hanz-eplein 1, 9713GZ Groningen, The Netherlands

Hiroaki Takeuchi, MD, PhD, Kochi Medical School, Nankoku-City, Kochi 783-8505, Japan

Hans L Tillmann, Professor, Medizinische Klinik und Poliklinik II, University Leipzig, Philipp Rosenthal, Str. 27, Leipzig 04103, Germany

Frank I Tovey, OBE, ChM, FRCS, Honorary Research Fellow, Department of Surgery, University College London, London, United Kingdom

Li Yuyuan, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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Name of journal

World Journal of Gastroenterology

CSSN

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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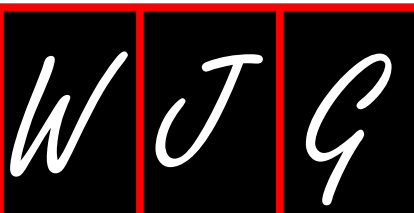
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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Multifaceted nature of membrane microdomains in colorectal cancer.
World J Gastroenterol 2011; 17(6): 681-690
<http://www.wjgnet.com/1007-9327/full/v17/i6/681.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
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SPONSOR
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EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
February 14, 2011

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

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Multifaceted nature of membrane microdomains in colorectal cancer

Kristina A Jahn, Yingying Su, Filip Braet

Kristina A Jahn, Yingying Su, Filip Braet, Australian Centre for Microscopy and Microanalysis, The University of Sydney, NSW 2006, Australia

Author contributions: Braet F, Jahn KA and Su Y prepared the manuscript for publication; Jahn KA and Su Y carried out the experiments presented under Figure 1; Braet F composed Figure 2.

Supported by The Australian Research Council through Linkage Infrastructure, Equipment and Facilities grants, No. LE0775598; and the ARC/NHMRC FABLS Research Network, No. RN0460002

Correspondence to: Filip Braet, Associate Professor, Australian Centre for Microscopy and Microanalysis, Madsen Building F09, The University of Sydney, NSW 2006, Australia. filip.braet@sydney.edu.au

Telephone: +61-2-93517619 Fax: +61-2-93517682

Received: August 2, 2010 Revised: November 23, 2010

Accepted: November 30, 2010

Published online: February 14, 2011

Abstract

Membrane microdomains or lipid rafts are known to be highly dynamic and to act as selective signal transduction mediators that facilitate interactions between the cell's external and internal environments. Lipid rafts play an important mediating role in the biology of cancer: they have been found in almost all existing experimental cancer models, including colorectal cancer (CRC), and play key regulatory roles in cell migration, metastasis, cell survival and tumor progression. This paper explores the current state of knowledge in this field by highlighting some of the pioneering and recent lipid raft studies performed on different CRC cell lines and human tissue samples. From this literature review, it becomes clear that membrane microdomains appear to be implicated in all key intracellular signaling pathways for lipid metabolism, drug resistance, cell adhesion, cell death, cell proliferation and many other processes in CRC. All signal transduction pathways seem to originate directly from those peculiar lipid islands, thereby orchestrating the colon cancer cells' state and fate. As confirmed by recent animal and preclinical studies in different CRC

models, continuing to unravel the structure and function of lipid rafts - including their associated complex signaling pathways - will likely bring us one step closer to better monitoring and treating of colon cancer patients.

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Key words: Actin; Caveolae; Cytoskeleton; Combined imaging; Detergent-resistant membranes; Drug targeting; Electron microscopy; Lipid domains; Membrane rafts; Prognosis; Staging; Tomography; Lipid-mediated therapy

Peer reviewer: Antonio Picardi, MD, PhD, Department of I Medicine, University Campus Bio-Medico of Rome, Via. À. Del Portillo 200, Rome 00128, Italy

Jahn KA, Su Y, Braet F. Multifaceted nature of membrane microdomains in colorectal cancer. *World J Gastroenterol* 2011; 17(6): 681-690 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/681.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.681>

INTRODUCTION

In recent years, high-speed multidimensional live-cell confocal laser imaging^[1], often combined with combinatorial labeling approaches^[2] and/or subsequent correlative electron microscopy studies^[3], has allowed high-throughput studies that add missing pieces of temporal and spatial resolution and detail to the cell membrane puzzle. At the nanometer length scale, semi-automated transmission electron tomography (TET) has enabled generation of accurate three-dimensional models of the fine architecture of the cell membrane and its associated proteins^[4,5]. Meanwhile, advances in the high-throughput analyses of chemical and molecular biology have allowed researchers to catalogue the lipidomics of the cell with hitherto unseen sensitivity^[6,7], complementing recent computer simulations of lipid membranes^[8,9]. Over the last decade, this

progress in analytical chemistry and in rapid microscopy-based imaging has underpinned the exponential growth in our understanding of the dynamic and multifaceted role of the cell membrane in various physiological processes.

The cell membrane is unquestionably one of the most studied subcellular components in the modern era of cell biology^[10-12]. This is not simply because it is an attractive target for drug designers^[13], but also because it acts as a selective protector of the interior of the cell^[14]. Key regulatory roles for the cell membrane have been demonstrated in cell homeostasis and differentiation, cell death and cell survival pathways, inter- and intracellular signaling, cell development and movement, and trans- and intercellular transport mechanisms^[15,16]. Of special interest are unique lipid domains within the cell membrane that have been shown to be involved, directly or indirectly, in such lipid-mediated cell regulation. These small domains, also known as lipid rafts, were initially described by Palade through the use of electron microscopy^[17] and later carefully identified by Simons *et al.*^[18] through a combined biochemistry and imaging approach. These rafts lie as discrete patches, known as detergent-resistant membrane structures, in the plasma membrane of cells and are rich in sphingolipids and cholesterol^[18,19]. The fatty acid chains of lipids within these rafts tend to be tightly packed, creating ordered lipid domains that float in a sea of poorly-ordered lipids within the membrane^[20]. Lipid rafts are highly dynamic and temperature sensitive, are able to form large clusters and to interact with the cell's internal molecular and structural compartments^[21,22]. From recent studies, it is becoming ever more evident that rafts act as highly dynamic and selective guardians between the cell's external and internal worlds, which makes researchers view them as important structural and molecular targets for altering cell function and behavior (for a review^[23]).

With regard to the biology of cancer, it has been demonstrated that lipid rafts play important mediating roles in cell migration, metastasis, cell survival and tumor progression^[24,25]. The literature of the past five years contains over one thousand original research papers that have studied the role of these peculiar lipid islets in cancer. This illustrates their importance and their perceived potential in future cancer cures and/or as markers for tumor staging and, hence, diagnosis and prognosis. This literature also shows the presence of lipid rafts in almost all existing experimental cancer models, *in vitro* and *in vivo*. The presence and role of rafts have also been highlighted within relevant human and clinical settings^[26,27], including in colorectal cancer (CRC)^[28,29]. Lipid rafts in CRC cells were observed initially in 1998 by Orlandi and Fishman^[30] and studied extensively by many others subsequently (see next section). So far, however, no dedicated paper has addressed "raft biology and pathobiology" in CRC. Here, therefore, we carefully review the current state of knowledge by highlighting some of the pioneering and recent raft studies carried out on different CRC cells and tissue samples.

MEMBRANE MICRODOMAINS IN COLORECTAL CANCER CELLS

Researchers have shown, for example, that lipid rafts in CRC cells act as go-betweens for cell death-mediated signaling^[31,32], as portals for bioactive compounds^[33], and as congregation regions for adhesion proteins and major histocompatibility complex class 1 (MHC-1) molecules^[34]. However, despite the abundance of literature available on the biology and pathobiology of lipid rafts in cancer^[35], only a few dedicated papers presently address the importance of these membrane domains in the process of CRC. While lipid rafts have been thoroughly described in other malignant tumor models such as cancers of the breast, lung and prostate, this does not imply that we should overlook their important role in the onset and development of CRC (see below). Unraveling their structure and function - including their associated complex signaling pathways - could eventually form the basis for future therapeutic interventions.

Labeling and morphometric imaging approaches

Although lipid rafts have been studied extensively through dedicated labeling and microscopy techniques^[36], relatively little is known about their fine structure in CRC cells or tissue at the nanometer scale^[37]. The majority of CRC membrane raft studies use immunofluorescence microscopy, often in conjunction with protein blotting and/or flow cytometry. Microscopic identification of lipid rafts in CRC cells is frequently performed by direct labeling against specific molecular components of membrane rafts^[32,38]; the classic example is the use of fluorescently-labeled cholera toxin against ganglioside II3-N-acetylneuraminosyl-gangliotetraosylceramide (GM1)^[39-41]. Indirect identification approaches are frequently used as well, such as staining against molecular targets or proteins that are supposed to associate with CRC lipid microdomains^[42]. However, combinatorial protein blotting studies on isolated raft protein fractions must be an essential part of this approach to unambiguously identify the association of raft-specific proteins with lipid rafts. The different caveolin (Cav) isoforms - predominantly Cav-1 and Cav-2 - are also popular protein targets for raft detection^[43,44], but should be used with caution because the degree of Cav expression in cancer cells depends on the differentiation status of the cells and the cancer model studied^[35].

Depending on the imaging approach applied, lipid rafts have been reported to range from between 30 and 100 nanometers up to almost one micrometer in size^[20,45,46]. We have applied correlative fluorescence and electron microscopy (CFEM) to confirm that the same holds true for CRC cells^[37]. CFEM analysis of Caco-2 cells labeled for GM1 allowed the direct observation of fluorescently-labeled lipid rafts by light-optical microscopy and by electron microscopy (Figure 1A). We observed that the smaller lipid rafts could be easily resolved under electron microscopy, but could not be clearly seen by confocal imaging. In some

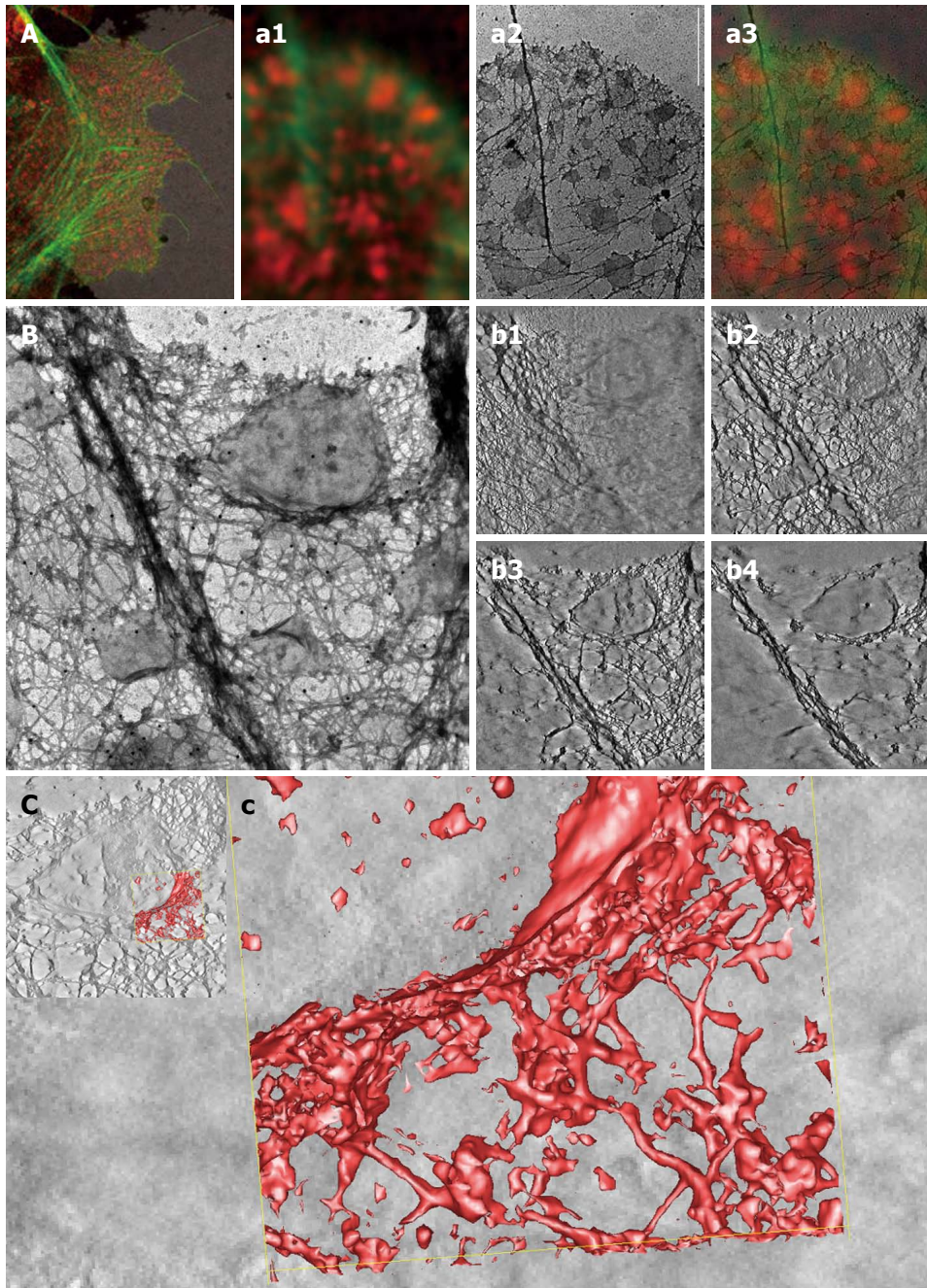


Figure 1 High-resolution imaging of membrane domains in human Caco-2 colorectal cancer cells. These data come from high-resolution correlative fluorescence and transmission electron microscopy (CFEM) studies on whole mounts. Human colorectal cancer (CRC) cells (Caco-2) were cultured on formvar-coated nickel grids and then treated with Triton X-100 in cytoskeleton stabilization buffer, leaving detergent-resistant membranes behind^[37]. These membrane fractions were labeled with the membrane raft marker GM1 CTxB-594 (red) and the actin cytoskeleton was stained with phalloidin-488 (green)^[48]. A: Low-magnification overview of the peripheral cytoskeleton reveals actin-rich lamellipodia and associated filopodial extensions. Note the numerous GM1-positive lipid rafts interspersed throughout the extracted cytoplasm. The corresponding high-magnification CFEM analysis (a1-a3) of one of these lamellipodium-filopodial regions reveals the complex architectural nature of the leading edge, showing an intricate cytoskeleton-rich matrix and the close structural relationship with the lipid rafts (a1). Subsequent TEM analysis of exactly the same region not only allowed us to determine the exact size and shape of lipid rafts, but also provided an accurate idea of whether the rafts were located on top or beneath the lamellipodium: i.e. apical or basal (a2). The corresponding merged information (a3) clearly shows the additional value of applying different imaging techniques on the same cell: the electron-microscope data reveals small detergent-resistant membrane islands that could not be resolved by advanced confocal microscopy. Scale bar, 2 μ m; For the electron tomographic analysis of a detergent-resistant membrane island and the surrounding cytoskeletal matrix (shown in B and C), whole mounts of Caco-2 cells were prepared as outlined under A. Then we performed transmission electron tomography imaging at 1.5° incremental steps under dual-axis tilting (B) and then carried out subsequent segmenting (b1-b4) of the XYZ-tilting series. The single-image slices obtained via the IMOD tomography software show the sample at different heights from bottom to top (b1-b4) spanning a height of about 300 nm; C: An area of the entire tomogram was next selected (c) and a three-dimensional model generated of the membrane raft-cytoskeleton interface (c), showing the close interaction of fine cytoskeletal fibers at the rim of the detergent-resistant membrane.

cases, no fluorescent label could be detected at all, supporting earlier observations regarding the heterogeneous size and composition of lipid rafts^[46,47]. The samples prepared

for CFEM investigation were also readily used for subsequent TET^[48], which generated a stack of virtual XYZ sections through the structure of interest (Figure 1B). When

combined with computer modeling, we obtained a three-dimensional view of the structure at a typical resolution of approximately 5-8 nm (Figure 1C). From these tomographic data, it became apparent that a web of fine cytoskeletal fibers, derived from the surrounding cytoskeletal matrix, accumulates at the circumference of rafts. These cytoskeleton-raft interactions probably indicate that membrane rafts require an intact cytoskeleton lattice for proper functioning of raft-associated subcellular processes^[49].

Raft-mediated cell death

There has been an extraordinary increase in research activity aimed at understanding the mechanisms and processes that underlie cell death, which occurs in different forms. The best-studied cell death mechanisms are apoptosis and necrosis (for a recent review^[50]). Whether by apoptosis or necrosis, cell death is initiated, triggered and regulated by a cascade of signaling pathways that involve different molecules. As we continue to unravel the pathways of cell death, novel findings emerge concerning the complex role that the cell membrane - and in particular rafts and their associated molecules - play in the tightly coordinated process of programmed cell death and cell survival^[25]. A few reports unambiguously illustrate that rafts are key signal transducers when it comes to death of CRC cells. Remacle-Bonnet *et al.*^[51] showed that lipid rafts segregate pro- from anti-apoptotic insulin-like growth factor-I (IGF- I) receptor signaling in various human colon adenocarcinoma cell lines (HT29-D4, HRT-18, HCT-116, HCT-15, COLO-205, SW480, SW620, HCT-8/E11 and HCT-8/E11R1) when exposed to tumor necrosis factor- α (TNF- α). However, the pro-apoptotic effect of IGF-1 was not observed in all CRC cell lines tested, and SW480, SW620, HRT-18 and COLO-205 cells seem to be exceptions. The authors found that the paradoxical pro-apoptotic action of IGF- I is conveyed *via* the phosphoinositide 3-kinase (PI3K)/Akt pathway and that integrity of lipid rafts is necessary for proper anti-apoptotic cell signaling (Figure 2). In contrast, the activation of the Erk 1/2 and p38 MAPK pathways that transmit the IGF- I anti-apoptotic signaling is independent of lipid rafts.

These unexpected findings, obtained by incubating the different cell lines with the cholesterol-depleting agent methyl- β -cyclodextrin (Me- β -CD), showed the complicated functions that lipid rafts can display, depending on the molecules they are exposed to in the tumor microenvironment. Here, lipid rafts acted to precisely regulate whether CRC cells would survive or die. This might also partially explain the different and conflicting data reported in CRC cell death studies.

Another groundbreaking finding comes from a careful comparative *in vitro* and *in vivo* study in which sugar-cholestanol derivatives provoked cell death in COLO-201, HT29 and Colon-26 cells, including Balb/c mice that contain Colon-26 tumors^[38]. In this study, Hahimoto *et al.* showed that chemically-synthesized sugar-cholestanols, with mono-, di- and tri-saccharides attached to cholestanol, are transported into the cell's interior *via* membrane microdomains; however, cholestanol without sugar moi-

eties was not taken up. Biochemical analysis revealed that all N-acetyl-D-glucosamine-based sugar-cholestanols accumulate quite rapidly within the mitochondria of CRC cells, gradually increasing the release of cytochrome C from these organelles within the cytoplasm. Subsequent studies performed with time-pulse DNA ladder fragmentation assays and Western blotting demonstrated that cell death occurred *via* the caspase-9/caspase-3 apoptotic pathway (Figure 2). In their animal studies, the authors validated the potential anti-cancer effect of sugar-cholestanol derivatives when administered intraperitoneally at different time intervals: Balb/c mice showed a significant reduction in tumor growth and had prolonged survival. This is one of the first CRC *in vivo* studies that unambiguously demonstrated the importance of membrane microdomains as a molecular target for cancer therapy (see also next section).

Other studies have shown that food-derived biochemical compounds can induce substantial cell death in CRC. In 2003, resveratrol - a polyphenol found in various food products - was reported to trigger apoptosis in SW480 human CRC cells^[42]. By combining microscopy, cell sorting and protein blotting, the authors established the direct involvement of the caspase-8/caspase-3-mediated apoptotic cascade (Figure 2). Furthermore, resveratrol exposure induced a specific redistribution of the cell death receptor Fas (i.e. CD95) within membrane microdomains, and caused formation of the death-inducing signaling complex. Intriguingly, no interaction between the cell death receptor ligand (i.e. FasL) and Fas was required for the resveratrol-induced cell death. The authors, therefore, postulated that resveratrol, which is abundantly found in grape skin, holds strong potential as a chemoprotective and therapeutic agent for CRC and other malignant tumors. In another study, quercetin - a plant-derived flavonoid, plentiful in apples and red onions - was reported to induce apoptosis in HT-29 and SW-620 cells, although by a different apoptosis signaling pathway^[40]. It was found that quercetin enhanced apoptosis caused by the TNF-related apoptosis-inducing ligand (TRAIL) through redistributing the death receptors (DR) DR4 and DR5 into membrane microdomains (Figure 2). The application of nystatin, a cholesterol-sequestering agent, prevented (1) quercetin-induced clustering of death receptors; and (2) sensitization to TRAIL-induced apoptosis in CRC cells. The involvement of the mitochondrial-dependent death pathway was demonstrated by the activation of related pro-apoptotic molecules and the subsequent release of cytochrome C to the cytosol. These data suggest that membrane microdomain localization of death receptors is probably required for optimal cytotoxicity of quercetin and/or TRAIL.

Cisplatin or cisplatin is a well-known chemotherapeutic drug, widely used to treat various types of cancers. Rebillard *et al.*^[32] demonstrated that cisplatin-induced apoptosis in human CRC cells involves cell membrane fluidification *via* the inhibition of the Na⁺/H⁺ membrane exchanger-1. Inhibition leads to an overall intracellular acidification and the subsequent activation of acidic sphingomyelinase, which generate ceramides that finally affect membrane fluidity. The team also found that this

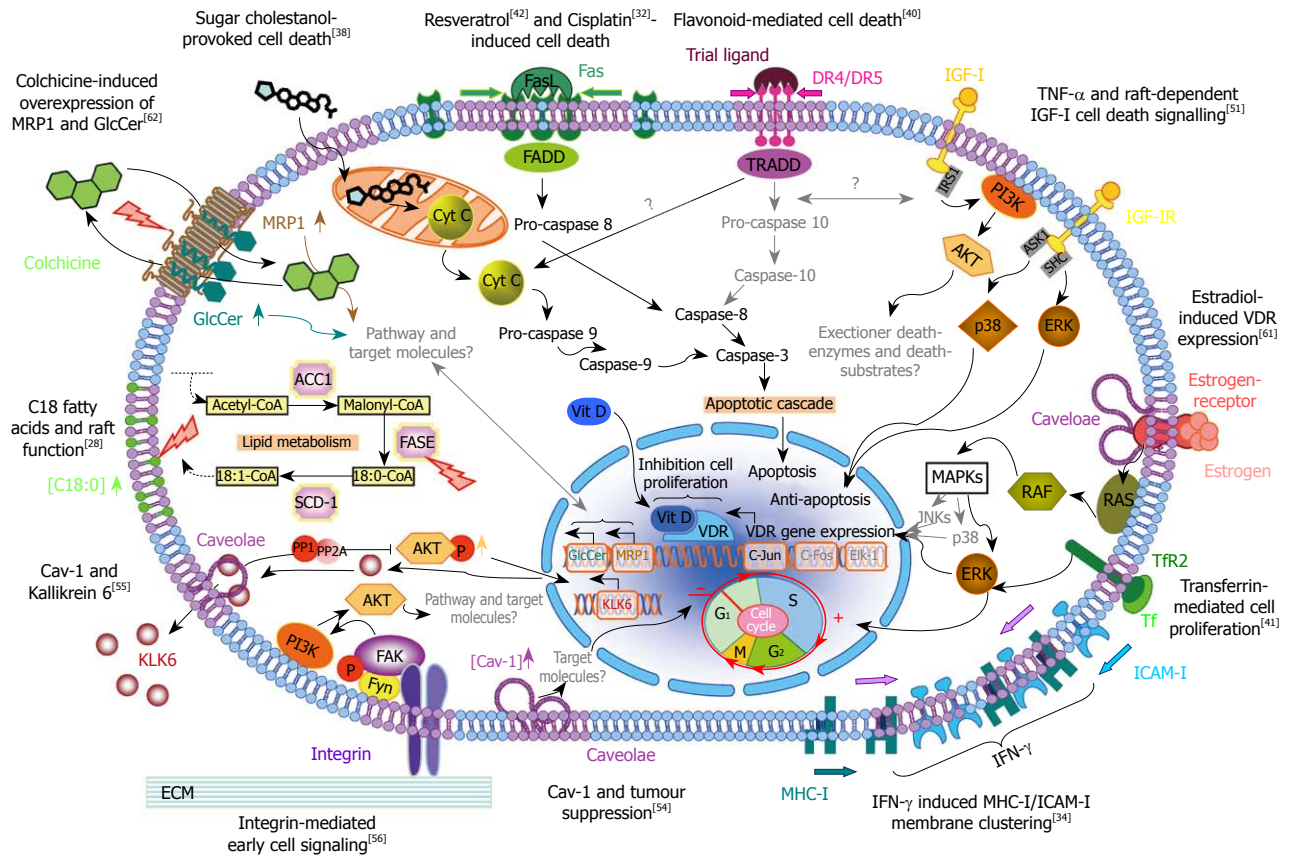


Figure 2 Scheme outlining the various membrane microdomain-mediated intracellular signaling pathways in colorectal cancer. This diagram summarizes what has been reported to date in the literature about the different intracellular signaling pathways that are mediated by lipid rafts and the implications of these paths for the colon cancer cells' state and fate. Briefly, the different observations of colorectal cancer (CRC) lipid rafts can be generally categorized under the following main topics of investigation: cell death-mediated mechanisms, caveolae in cancer cell growth and function, unique structure-function molecular associations, and intervention studies with bioactive compounds. Note that the text and connector arrows as shown in black are confirmed observations, whereas the gray denotes postulated signaling pathways and/or unknown molecular targets. The lipid bilayer of the cell membrane is depicted in light blue, membrane microdomains or lipid rafts in light purple, and the pear-shaped caveolae associated with these rafts in dark purple. For detailed descriptions of each of the individual cell signaling pathways, refer to the corresponding sections in this paper. The numbers in superscript refer directly to the original published papers. MRP: Multidrug-resistance protein; GlcCer: Glucosylceramide; FADD: Fas-associated protein with death domain; TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain protein; PI3K: Phosphoinositide 3-kinase; AKT: Serine/threonine protein kinase; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; IRS1: Insulin receptor substrate 1; ASK1: Apoptosis signal-regulating kinase 1; SHC: Src homology 2 domain; TNF- α : Tumor necrosis factor- α ; IGF-I: Insulin-like growth factor-1; VDR: Vitamin D receptor; Vit D: Vitamin D; RAF: Proto-oncogene serine/threonine-protein kinase; RAS: Rat sarcoma; Tfr2: The second transferrin receptor; Tf: Transferrin; JNKs: c-Jun N-terminal kinases; ICAM-1: Intercellular adhesion molecule 1; IFN- γ : Interferon- γ ; MHC-I: Major histocompatibility complex 1; FAK: Focal adhesion kinase; ECM: Extracellular matrix; FASE: Fatty acid synthase; SCD-1: Stearoyl-coenzyme A desaturase 1; ACC1: Acetyl-CoA carboxylase; Cav: Caveolin.

rapid increase in membrane fluidity after cisplatin treatment was inhibited by membrane stabilizing agents, such as excess cholesterol or monosialoganglioside-1 treatment. Furthermore, these lipid-interfering compounds prevented the early aggregation of the Fas receptor and of membrane microdomains on the cell surface of HT-29 cells. As a result, significant inhibition of cisplatin-induced apoptosis was observed, without altering the intracellular drug uptake or the formation of cisplatin-DNA adducts. Hence, cisplatin-induced cell death in CRC cells seems to be mediated, in part, *via* the Fas-signaling pathway, the cell death receptors of which reside within lipid rafts (Figure 2). This concept was elegantly demonstrated through a variety of analytical tools, including GM1 labeling studies, in the course of lipid fluidity studies on isolated lipid rafts, and biochemical assays to detect caspase-3 activity and determine the extent of apoptosis.

Caveolin at the raft interface

There is widespread evidence that caveolin proteins associate structurally and functionally with membrane microdomains, interacting closely with numerous microdomain-associated molecules and thereby regulating cell signaling pathways that control cell function and cell fate^[23]. Although all types of caveolins (Cav-1, Cav-2 and Cav-3) are structurally similar and associate with cholesterol and sphingolipids in parts of the cell membrane to form caveolae, Cav-1 and Cav-2 are most prominently upregulated and/or downregulated in oncogene-transformed cells^[35]. However, a certain degree of variability in Cav-1 and Cav-2 expression seems to occur within various classes of cancer and across different types of cells or tissues. Furthermore, the amount of Cav-1 and Cav-2, together with the ultrastructural presence of caveolae, not only depends on the tumor model studied, but also on the stage and grade of

the cancer. This is particularly relevant for CRC in which the expression of Cav-1 mRNA is four to five times lower or two times lower, respectively, for Cav-2 mRNA in HT-29 or in COLO-205 CRC cell lines. These data were obtained by directly comparing the relative expression of caveolins in more than 55 commonly-used human cancer cell lines from different tissues (for an overview^[35]). However, others have demonstrated, *via* classical immunocytochemical staining^[52] and reverse transcriptase-polymerase chain reaction (RT-PCR)^[53] in human colon tumor tissue, that Cav-1 was significantly enhanced compared to normal colon epithelium. In addition, caveolin-mediated raft signaling has been demonstrated to be pivotal in various experimental CRC models, especially in the strong anti-proliferative action of Cav-1. Bender *et al.*^[54] showed that Cav-1 possesses a strong tumor suppression activity in CRC cells, but the exact signaling mechanism responsible is not yet clear (Figure 2). Experiments involving immunoblotting, RT-PCR and microarrays disclosed that expression of Cav-1 in HT-29 and DLD-1 cells delayed or blocked tumor formation in nude mice. Likewise, Cav-1 levels were significantly reduced in colon tumors from human patients. Another interesting finding was that increased levels of Cav-1 were observed in multidrug-resistant HT-29 cells. The authors concluded from their studies that Cav-1 modulates a variety of signaling pathways, but that we still require a better understanding of target molecules affected by the expression of this all-round protein in malignant cells.

Henkhaus *et al.*^[55] demonstrated a direct link between Cav-1-mediated expression and secretion of kallikrein 6 (KLK6) in HCT116 human CRC cells. Sucrose-gradient subcellular fraction analysis revealed that Cav-1 and KLK6 co-localize to lipid rafts. Deactivation of Cav-1 - through interference in the Src-mediated phosphorylation pathway - decreases KLK6. In addition, immunoblotting, ELISA and RT-PCR studies revealed that Cav-1 controls KLK6 expression *via* the Src, Akt and phosphatases (i.e. PP1/PP2A) signaling pathway (Figure 2). Kallikreins are serine proteases, and the various subtypes of kallikreins are considered to hold promise as specific biomarkers for different cancers. Furthermore, once secreted by the cell, these serine proteinases have the ability to degrade the surrounding extracellular matrix (ECM). The authors postulated that KLK6-mediated degradation of the ECM enhances CRC cell invasiveness.

Raft-associated molecular expression

Besides the presence of cell death receptors and caveolar proteins within membrane microdomains, other key molecules have been shown to have close structure-function associations with rafts, thereby controlling a variety of other vital cellular processes in CRC, such as cell adhesion and motility, intracellular transport and cellular exchange, immune tolerance, and numerous hormone-mediated cellular responses^[23]. For example, Baillat *et al.*^[56] demonstrated how focal adhesion kinase (FAK) and Src family protein tyrosine kinases (SFKs) work together in lipid rafts during the initial stage of CRC cell adhesion. It is well known that elevated expression and activity of SFKs in CRC often ac-

company disease progression. In cancer development, tumor cells acquire migration capability and effective homing ability in the body's host environment. Cell adhesion molecules, such as integrins that link components of the ECM with the cytoskeleton, are pivotal during this process. By applying a combination of cell transfection methods, protein-blotting assays and membrane raft ultracentrifugation to Me- β -CD-treated and/or cholesterol-treated SW480 cells, the team found that the formation of raft-associated FAK/Fyn complexes and the activation of Akt-1 *via* PI3K occur simultaneously during early contact with the ECM (Figure 2). Fyn is a tyrosine-specific phospho-transferase and member of the SFKs. Akt-1 is a serine threonine kinase that is considered to be an oncogene and is often activated in human cancers, thereby contributing to tumor progression and metastasis. The team concluded that, during the very early stage of cell adhesion, FAK is transiently co-located with Fyn thereby inducing raft-dependent Akt-1 signaling. This study also showed, for the first time, that FAK in membrane microdomains can act as a signaling intermediate to control various aspects of tumor cell behavior during cell adhesion.

Major histocompatibility complex I (MHC- I) and intercellular adhesion molecule I (ICAM- I) are crucially involved in the functioning of the immune system and are implicated in inflammatory bowel diseases and CRC. Cytokines used in therapy, such as interferon γ (IFN- γ), are known to modulate the expression of MHC- I and/or ICAM- I, so it is no surprise that these cell surface receptors are under investigation in an attempt to cure various gastrointestinal diseases. Bacsó *et al.*^[34] showed that exposing LS-174-T colon carcinoma cells to IFN- γ significantly increased the cell surface density of MHC- I and ICAM- I. Flow cytometric fluorescence energy-transfer measurements of immunolabeled LS-174-T cells and confocal microscopy of GM1-labeled LS-174-T cells revealed that both receptor types cluster in the nanometer range and that amounts of both substantially increase, within GM1-positive membrane microdomains, upon IFN- γ treatment (Figure 2). Moreover, the team found that the relative size of the lipid rafts increased, while the total cell size and membrane surface remained unchanged. Another interesting observation was that MHC- I and ICAM- I form sterically tight hetero-associates such that ICAM-1, with its long protrusion above the cell membrane, can readily bind to a cytotoxic lymphocyte (CTL) and simultaneously MHC- I can favorably present its peptide directly to the CTL. As both receptors are co-localized in lipid rafts, which are considered as pre-formed cell-signaling sites, all steric conditions are present for rapid trans-membrane signal transduction. These data imply that IFN- γ treatment can alter the surfaces of CRC cells to make them better target for CTLs.

Examination of resected tumor tissue has demonstrated that expression of the second transferrin receptor (TfR2) occurs in human colon carcinomas^[41]. These authors also showed the presence of TfR2 in three different CRC cell lines (HT29, HCT116 and SKCO1) as assessed by immunolabeling, flow cytometry and Western blot analysis. This is intriguing given that TfR2 expression in

normal tissues is restricted to the liver, where it mediates cellular uptake of transferrin (Tf)-bound iron. The authors also found evidence that TfR2 expression induces a rapid and pronounced ERK1/ERK2 phosphorylation, indicating involvement of the Ras-dependent ERK1/ERK2 MAP-kinase signaling pathway (Figure 2). This pathway has a central role in controlling cell proliferation and is frequently activated in cancers, including CRC. It was concluded that TfR2 present within lipid rafts of CRC cells might contribute to the growth advantage of these cells.

Recently, Taïeb *et al.*^[57] demonstrated the presence of prominin 1 (CD133) within Caco-2 and HT-29-D4 cells. CD133 is a trans-membrane protein that has been shown, in different experimental tumor models, to make cancer cells transplantable, resistant against radiation therapy, and highly likely to initiate tumors. Researchers also believe CD133-ganglioside interactions are crucial for the recruitment and/or the phenotype of cancer stem cells. For this reason, there is obvious interest in understanding the molecular biology of CD133⁺ cancer cells. With the aid of a novel anti-CD133 antibody, Taïeb *et al.*^[57] demonstrated that CD133-immunolabeling progressively decreased to undetectable levels in postconfluent CRC cell cultures, possibly through ganglioside-mediated epitope masking, because the staining was partially recovered after chemical disruption of lipid rafts. It is noteworthy that the N-terminal epitope of CD133 belongs to a ganglioside-binding domain and that blocking experiments with various gangliosides, including purified GM1, resulted in negative labeling. The authors proposed that synthetic soluble ganglioside analogues, which act as competitors and specifically affect CD133⁺-mediated signaling pathways, deserve thorough evaluation in the development of new therapeutic approaches to CRC.

Bioactive compounds and raft function

Since the mid-1990s, hundreds of papers have examined the interference of drugs, pharmacological agents and other chemical or natural compounds with membrane microdomains^[26]. Recent headway in animal disease models has provided knowledge as to how compounds affect membrane raft function, which might help to cure diseases such as ischemic heart impairment^[58], keratitis^[59] and colitis^[60]. In colorectal studies, findings are limited so far to experimental *in vitro* models (see below). However, despite the limited data, the results all indicate that CRC membrane microdomains appear to be an important entryway for anti-cancer drugs, hormone(-like) molecules and dietary components. As reviewed above (see “Raft-Mediated Cell Death”), the bioactive compounds cisplatin^[32], sugar cholestanol^[38], flavonoids^[40] and resveratrol^[42] have all been shown to possess a strong adverse cellular effect that is mediated *via* membrane raft signaling. Besides these classes of cell death-inducing compounds, other molecules have been described that control CRC cell function and fate.

For instance, in a study of the estrogen-induced vitamin D receptor (VDR) expression model, Gilad *et al.*^[61] elegantly illustrated how vitamin D (Vit D) controls CRC cell proliferation. By combining agents that interfere with lipid

and intracellular signaling with subsequent protein immunoblotting studies, the authors unraveled that the estrogen 17 β -estradiol (E2) binds to estrogen receptors (ERs) confined to lipid rafts or caveolae in HT29 cells, thereby activating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway *via* the protein kinases Ras/Raf. This affected transcriptional activity and finally resulted in upregulation of expression of the VDR gene. The MAPK/ERK pathway links intracellular responses to the binding of hormones and/or cell growth factors to cell membrane receptors (Figure 2). A direct functional association of ERs with lipid rafts was shown by using the cholesterol-binding agent Me- β -CD that blocked ERK phosphorylation concomitantly with VDR upregulation. E2 treatment did not affect proliferation of HT29 cells, while Vit D exposure significantly inhibited cell proliferation, and the combined treatment resulted in potentiation of Vit D activity. This anti-proliferative effect of Vit D, mediated *via* membrane microdomain receptor signaling, again illustrates the significance of lipid rafts in the regulation of tumor growth. It also emphasizes the importance of a well-balanced diet, including the daily intake of essential vitamins such as Vit D abundant in dairy products and fish oils, for controlling health.

Multidrug resistance (MRD) is a major challenge for drug designers and cancer cell biologists. When continuously exposed to chemotherapeutic compounds over long periods, cancer cells tend to acquire MDR by overexpressing proteins that belong to the superfamily of ATP-binding cassette (ABC) transporter proteins. Klappe *et al.*^[62] generated a new HT29^{col} cell line that, during colchicine-induced acquisition of MDR, increases their glucosylceramide (GlcCer) content and upregulates multidrug-resistance protein 1 (MRP-1). The overexpression of sphingolipids, such as GlcCer, appears to be a rather general aspect of MDR cancers and has even been proposed as a candidate marker for MDR⁺ malignant tumor cells. Furthermore, the tightly coordinated upregulation of MRP-1 - a member of the ABC transporter proteins that displays strong drug-efflux properties - and GlcCer were both enriched in lipid rafts (Figure 2). The authors also demonstrated that GlcCer upregulation did not appear to be necessary for MRP-1 function, given the absence of effects of inhibition of GlcCer biosynthesis on MRP-1-mediated drug efflux and cell survival. They, therefore, concluded that GlcCer appears to play a structural role in membrane microdomain organization instead and, as such, might help to accommodate the excess MRP-1 expressed in membrane lipid islets in CRC cells.

The enzyme fatty acid synthase (FAS) synthesizes only saturated fatty acids and overexpression has been shown to be involved in numerous human malignancies, including CRC. Rakheja *et al.*^[28] demonstrated, through gas chromatography and mass spectrometry, a statistically significant increase in saturated C18:0 fatty acid (stearic acid) in colonic adenocarcinoma, compared to adjacent normal colonic mucosa tissue. None of the thirteen patients investigated had received any pre-operative chemotherapy and/or radiotherapy, thereby excluding artifactual read-

ings or false-positive measurements caused by treatment. Although the authors did not present any immunohistochemical data on normal or cancerous tissues, they postulated that the increase in the proportion of saturated fatty acids will most likely affect the functional properties of lipid rafts in CRC cells (Figure 2), and particularly might impair intracellular signaling mechanisms as discussed earlier (i.e. cell growth and cell death). In support of this proposal, it is known that the relative abundance of saturated fatty acids is a principle reason for the liquid-ordered state of lipid rafts and that the inhibition of FASE mainly affects the synthesis of raft-associated lipids. The authors concluded that dietary intervention to normalize the balance between saturated and unsaturated lipids within the cell membranes of colonic mucosa might be seen as a preventive or even therapeutic cure (see next section).

POTENTIAL DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS OF MEMBRANE MICRODOMAINS IN CRC

Many benign and malignant tumors synthesize and secrete compounds (i.e. tumor-associated proteins) that can be detected histopathologically on tissue sections or biochemically by chemical pathology analysis of blood or other bodily fluids. As the majority of these compounds are produced within tumors, they are said to be tumor-derived and so provide direct evidence of the tumor's existence (i.e. they are tumor-associated markers). It is highly probable that determination of raft composition or the detection of raft-associated markers, *via* proteomic and/or lipidomic approaches, could be extremely helpful in diagnosing malignancy in a patient with symptoms, preferably during the early stages of tumor formation^[63,64]. The measured marker concentration or a combination of markers should directly correlate with the mass and/or activity of the tumor, and ideally might even help to fine-tune the formulation and dosages of anti-cancer drugs. Taking the currently available information on CRC rafts into account, the ratio of Cav-1/GM⁺ and/or the presence of other molecules - such as TfR2, CD133 or KLK6 - have proven to be extremely useful candidate cancer markers that could aid in the diagnosis, staging and prognosis of CRC. However, despite initially high enthusiasm when the first experimental tumor marker-based assays appeared, only rarely does a marker exhibit sufficient specificity and sensitivity to be of any practical use in the clinic. While one single marker will not be sufficient to make a reliable diagnosis and prognosis, mass-spectrometry-based lipidomics^[64] seems to hold great promise as a novel diagnostic approach to detect the unique "lipid fingerprint" of cancer cells. When combined with existing biomarker assays, lipidomics brings us one step closer to better monitoring and treating of cancer patients.

As it stands, we still have a long way to go when it comes to implementing our practical knowledge of lipid rafts to treat patients. Although the first animal studies showed much promise - as briefly mentioned before - in helping find a cure for diverse diseases, we still await the

first full translational (pre-)clinical studies for curing diseases by targeting lipid rafts and their associated molecules. This, however, does not mean that raft-mediated therapy is entirely impossible or merely wishful thinking. It is just a reflection of the early days of membrane raft biology. When it comes to cancer and membrane raft-mediated therapeutic intervention, commonalities in proposed therapeutic approaches can be found that are independent of the cancer model studied. The majority of work targets trans-membrane proteins present in lipid rafts that control cell death *via* the programmed pathway (i.e. apoptosis; for a review^[65]). However, this enthusiasm must be tempered by caution, because cancer cells have the ability to change the expression pattern of membrane-associated cell death receptors, depending on their cellular environment and/or progression stage. CRC cells have the ability, for example, to switch between the cell death receptor Fas and its corresponding ligand (i.e. FasL), depending on their microenvironment or the chemokines they meet^[66,67]. A therapeutic intervention that uses smart drug complexes to target several different cell death molecules, including membrane raft-disrupting compounds, therefore is the approach most likely to result in successful outcomes.

Another interesting field concerns the modulation of membrane raft composition *via* dietary intake, or altering lipid synthesis *via* pharmacologic intervention within malignant cells^[68]. Both are meant to change the cholesterol content within the lipid membranes in the hope of impairing the membrane raft signaling that controls tumor development and growth. Altered levels of membrane cholesterol and cholesterol-rich membranes have been shown to influence the aggressiveness and progression of cancers^[69]. In CRC, this approach has a considerable chance of success because of the cancer's position within the digestive tract, which allows clinicians to directly expose the cancer cells to significant amounts of dietary or pharmacological compounds. In animal studies of various tumor models, dietary supplementation with long-chain polyunsaturated fatty acids combined with anti-cancer drugs significantly decreased tumor size and resulted in prolonged survival (for a review^[70]). These fatty acids have been shown to be beneficial in various colon-related diseases, so an important role has been postulated for membrane microdomains in controlling tumor growth; this is currently a topic of active investigation^[29,71]. Other examples of potential dietary therapies, as previously discussed, include the reduction of CRC tumor growth mediated by sugar-cholestanols^[38], or the beneficial effects of polyphenols, found in grape skin and various other food products^[42], or of quercetin, which is present in apples and red onions^[40]. Whatever the ultimate therapeutic approach will be, it is most likely that a combination of drugs, combining classic anti-cancer pharmacological agents with anti-membrane raft compounds, has the best chance for success against the cancer cells' unfortunate ability to change their cell membrane composition to resist anti-cancer drugs. Indeed, it is not inconceivable that the same molecular drift will occur in membrane microdomains of cancer cells after continuous exposure to a certain class of drugs. The quest to identify

stable membrane raft molecular markers and/or target molecules within the different stages of tumor development and progression is the greatest challenge that faces cancer membrane biologists today.

CONCLUSION

Membrane microdomains or lipid rafts in CRC cells appear to be involved in all key cellular regulatory processes that control tumor development, growth, progression and regression. Signaling pathways for lipid metabolism, drug resistance, cell death, cell division and many other processes all seem to diverge from those peculiar lipid islands, thereby orchestrating the cancer cells' state and fate. This demonstrates once more the multifaceted nature of lipid rafts. In the near future, we foresee that manipulating the structural and functional integrity of lipid rafts with anti-cancer drugs might result in the direct inhibition of CRC cell adhesion, the arrest of cancer cell division, or might even totally eliminate cancer cells. One can only dream.

ACKNOWLEDGMENTS

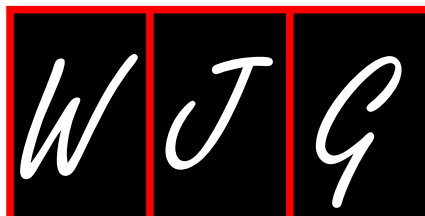
The authors acknowledge the facilities, and technical and administrative assistance from staff, of the AMMRF at the Australian Centre for Microscopy & Microanalysis (ACMM), the University of Sydney. The authors are also thankful to Mr. Xavier Heiligenstein (Structural and Computational Biology - Dr. A Frangakis, EMBL Heidelberg, Germany) for his proficient assistance in running the electron tomography experiments and to Dr. Kyle Ratnac (AKCMM) for editing this manuscript.

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S- Editor Tian L L- Editor Logan S E- Editor Lin YP



State-of-the-art imaging techniques in endoscopic ultrasound

Adrian Săftoiu

Adrian Săftoiu, Department of Gastroenterology, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Romania, Craiova, Dolj 200349, Romania
Author contributions: Săftoiu A solely contributed to this paper.
Supported by "Utility of EUS and OCT for the minimal invasive evaluation of tumour neo-angiogenesis in the patients with digestive cancers" financed by the Romanian Ministry of Education and Research - National University Research Council (UEFISCSU - Ideas Program), No. 239/2007

Correspondence to: Adrian Săftoiu, MD, PhD, Professor, Department of Gastroenterology, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Romania, 2-4 Petru Rares Str., Craiova, Dolj 200349, Romania. adrian.saftoiu@webmail.umfcv.ro

Telephone: +40-251-522458 Fax: +40-251-310287
Received: April 23, 2010 Revised: May 28, 2010
Accepted: June 5, 2010

Published online: February 14, 2011

Abstract

Endoscopic ultrasound (EUS) has recently evolved through technological improvement of equipment, with a major clinical impact in digestive and mediastinal diseases. State-of-the-art EUS equipment now includes real-time sono-elastography, which might be useful for a better characterization of lesions and increased accuracy of differential diagnosis (for e.g. lymph nodes or focal pancreatic lesions). Contrast-enhanced EUS imaging is also available, and is already being used for the differential diagnosis of focal pancreatic masses. The recent development of low mechanical index contrast harmonic EUS imaging offers hope for improved diagnosis, staging and monitoring of anti-angiogenic treatment. Tridimensional EUS (3D-EUS) techniques can be applied to enhance the spatial understanding of EUS anatomy, especially for improved staging of tumors, obtained through a better assessment of the relationship with major surrounding vessels. Despite the progress gained through all these imaging techniques, they cannot replace cytological or histological diagnosis. However, real-time optical his-

tological diagnosis can be achieved through the use of single-fiber confocal laser endomicroscopy techniques placed under real-time EUS-guidance through a 22G needle. Last, but not least, EUS-assisted natural orifice transluminal endoscopic surgery (NOTES) procedures offer a whole new area of imaging applications, used either for combination of NOTES peritoneoscopy and intraperitoneal EUS, but also for access of retroperitoneal organs through posterior EUS guidance.

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Key words: Endoscopic ultrasound; Real-time sono-elastography; Contrast-enhancement; Tridimensional (3D); Hybrid imaging; Endoscopic ultrasound-guided fine needle aspiration

Peer reviewer: Richard Hu, MD, MSc, Division of Gastroenterology, Department of Medicine, Olive view-UCLA Medical Center, 14445 Olive View Drive, Los Angeles, CA 91342, United States

Săftoiu A. State-of-the-art imaging techniques in endoscopic ultrasound. *World J Gastroenterol* 2011; 17(6): 691-696
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/691.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.691>

INTRODUCTION

Endoscopic ultrasound (EUS) has evolved in recent years into a technique with a major clinical impact in digestive and mediastinal diseases. Thus, EUS determines a change in the diagnosis in approximately a quarter of patients, as well as a change in management in half of the patients examined^[1]. A major step in the development of the EUS imaging techniques (Table 1) was represented by the appearance of electronic linear EUS scopes, which allowed a significant improvement in image quality, as well as the

development of several EUS-guided or EUS-assisted procedures, which start with the real-time targeted placement of a fine-needle aspiration needle under direct imaging by ultrasound guidance^[2].

REAL-TIME SONO-ELASTOGRAPHY

Technique

Real-time sono-elastography (RTSE) represents a technique which allows the calculation and visualization of tissue strain and hardness based on the average tissue strain in a selected region of interest^[3]. The technique allows the real-time visualization of the calculated strain values (Figure 1A), displayed in a transparent layout over the gray-scale images, in a similar fashion with color Doppler imaging^[4]. Several generations of software led to the improvement of image quality, reduced artifacts, but more important to the possibility of averaging through several cycles and calculation of semi-quantitative values of tissue strain inside a defined region of interest (for e.g. a lymph node or a focal pancreatic mass). By obtaining average hue histogram values inside a region of interest, the system displays the average strain inside a defined region of interest, as a semi-quantitative value that estimates tissue elasticity at that level.

Applications

Several applications were described for RTSE, as a technique that offers additional information as compared with gray-scale EUS images^[5]. The technique allows the selection of the most probable lymph nodes to be malignant, as well as the identification of lymph nodes that are most probable to be benign^[5-7]. This was suggested to be helpful for the selection and guidance of EUS-guided fine needle aspiration (FNA) for staging purposes in lung cancer or other digestive and mediastinal cancers (including esophageal, gastric or pancreatic cancer). EUS elastography was also reported to be useful for the differentiation of focal pancreatic masses, especially in pseudotumoral chronic pancreatitis and pancreatic cancer, in the presence of negative (false-negative) EUS-guided FNA and a strong suspicion of pancreatic cancer^[8-11]. The results of initial studies were recently validated in two multicentre studies^[12,13]. Both studies indicated similar values for sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy (92.6% *vs* 92.3%, 71.7% *vs* 80%, 76% *vs* 77.4%, 90.9% *vs* 93.3% and 87.4% *vs* 89.2%, respectively). It was thus suggested that the overall accuracy of 85-90% of EUS elastography might change current clinical decision making algorithms for the patients with focal pancreatic masses, especially in false-negative cases of EUS-FNA, when the suspicion of pancreatic cancer is still strong^[12]. This warrants a more aggressive approach in negative EUS-FNA cases where EUS elastography suggests a hard mass, with the patients referred directly to surgery or to repeat EUS-guided FNA.

The method was also tested in initial feasibility studies in diffuse pancreatic diseases like early chronic pancreatitis or autoimmune pancreatitis^[14,15].

Future usage of RTSE as a technique that simulates virtual palpation might include distant transmission of information and simulation of tele-palpation by using haptic devices and systems. This could lead to a better educational tool in order to simulate intra-operative palpation, and could also help provide guidance for remote surgical laparoscopic and robotic techniques.

CONTRAST-ENHANCEMENT

Technique

The development and subsequent approval of blood-pool contrast agents was a major step forward for the development of contrast specific ultrasound techniques^[16]. Several contrast agents are clinically available, including Albunex, Levovist and Echovist (first generation), as well as SonoVue, Sonazoid and Optison (second generation), *etc.* All of them are quite safe, without severe complications or long-lasting side-effects. The usage of second-generation microbubble contrast agents further improved the diagnostic capabilities, through a strong increase in ultrasound backscatter and enhancement of echogenicity during the dynamic assessment of small volume and slow velocity blood flow. The advantage of second generation microbubble contrast agents is that they are able to pass through the lungs, thus remaining confined to the intravascular space for a longer time. Also, because of the low solubility they are more stable with favorable resonance at low acoustic pressures, hence longer specific imaging in real-time.

Initial applications used spectral (pulsed) Doppler, color or power Doppler imaging, with contrast agents used as vascular signal enhancers^[17]. Contrast agents can thus rescue non-diagnostic Doppler examinations by increasing the intensity of weak flow signals to detectable levels. The appearance of contrast specific ultrasound modes further allowed the cancellation of tissue signals and utilization of the non-linear response of microbubbles (especially the second generation harmonic). The development of low-mechanical index techniques consequently led to a significant improvement consisting of visualization of the dynamic enhancement pattern in real-time. The main advantage is the absence of motion artifacts caused by cardiac or respiratory movements, including also flash and blooming (overpainting) artifacts.

Conventional imaging applications

One specific use of contrast-enhancement techniques in EUS was to detect low-velocity, low-volume flow of pancreatic tumors, with emphasis on the differential diagnosis between focal pancreatitis and pancreatic cancer^[18-23]. An initial feasibility study in a pig model showed that the use of contrast agents is possible during EUS, leading to improved visualisation of the splanchnic vasculature^[18]. Several studies further showed that contrast-enhanced power Doppler EUS can be successfully used for the differential diagnosis of chronic pseudotumoral pancreatitis and pancreatic cancer, with a sensitivity and specificity higher than 90%, in the presence of hypovascular malignant tumors^[19-23]. An initial study that used Optison in combina-

Table 1 Comparative assessment of new endoscopic ultrasound imaging techniques

EUS technique	Advantage	Disadvantage	Cost	Invasiveness
Real-time sono-elastography	Improved diagnosis of focal pancreatic masses	Assessment in large RCTs needed	Average	Minimal
Contrast-enhanced EUS	Improved diagnosis and staging of focal pancreatic masses	Assessment in large RCTs needed	Average	Minimal
3D-EUS	Moderate improvement of staging in pancreatic area	Limited assessment in clinical applications	Average	Minimal
Optical diagnosis	Improvement of real-time diagnosis	Limited assessment in clinical applications	High	Average
EUS-NOTES	Improvement of therapeutic options	Limited assessment in clinical applications	High	High

EUS: Endoscopic ultrasound; RCTs: Randomized clinical trials; 3D-EUS: Tridimensional EUS; NOTES: Natural orifice transluminal endoscopic surgery.

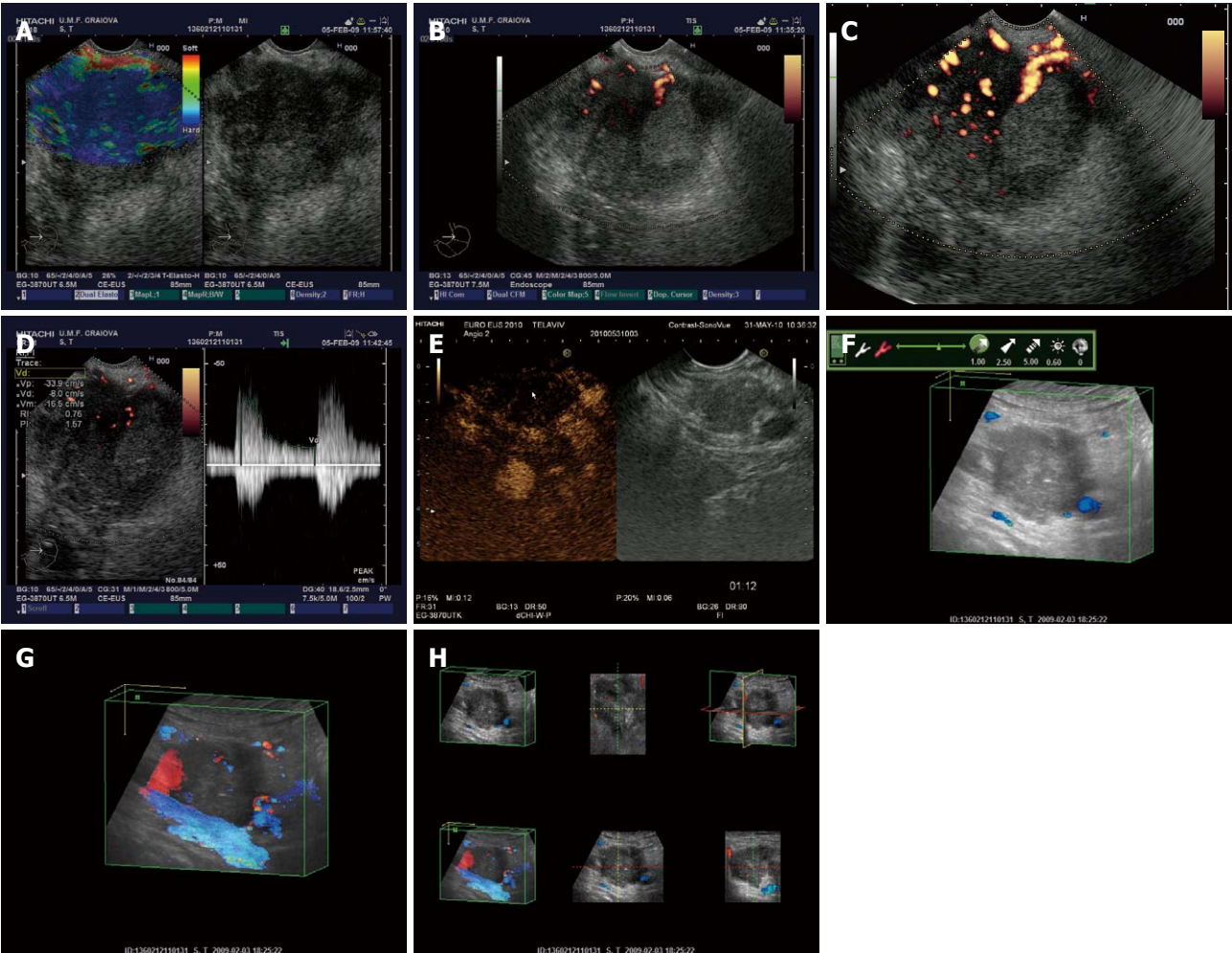


Figure 1 Pancreatic carcinoma at the level of pancreatic head depicted by different endoscopic ultrasound imaging techniques. A: Real-time elastography showing an in-homogenous hard mass; B: Power Doppler endoscopic ultrasound (EUS) without contrast-enhancement; C: Power Doppler EUS after contrast-enhancement with Sono-Vue, showing a hypovascular mass; D: Pulsed Doppler (triplex mode) after contrast-enhancement, with high resistivity and pulsatility indexes of intratumoral arteries; E: Contrast-enhanced low-mechanical index EUS harmonic imaging, showing a hypovascular appearance in the late (venous) phase; F: Tridimensional EUS showing an enhanced image in the opacity mode; G: Transparency mode obtained after contrast-enhancement with Sono-Vue; H: Multiview tridimensional (3D)-EUS display of the pancreatic tumor.

tion with power Doppler contrast-enhanced EUS showed a sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy of 94%, 100%, 100%, 88% and 96%, respectively^[19]. Another study used different criteria for benign and malignant lesions, after contrast-enhancement with Sono-Vue and power Doppler EUS examinations, in combination with pulsed (spectral) Doppler. Malignant lesions were defined by the presence

of irregular arterial vessels over a short distance and no detectable venous vessels inside the lesion, while benign lesions included regular appearance of vessels over a distance of at least 20 mm after injection of SonoVue and detection of both arterial and venous vessels^[20]. By using this methodology, the sensitivity and specificity for the detection of pancreatic cancer were 91.1% and 93.3%, with an overall accuracy of 91.9%. Two other studies con-

firmed these results, although they used Levovist, a first generation contrast agent, in combination with color or power Doppler^[21,22]. By using power Doppler vascularity index values calculated by special software (Figure 1B-D), a recent study also depicted values of sensitivity and specificity of 90.5% and 90.1% for the characterization and differentiation of focal pancreatic masses after contrast-enhancement with Sono-Vue^[23].

Harmonic imaging applications

Contrast harmonic imaging based on the second harmonic, in combination with microbubble specific software, allows an improved visualization of vascular and parenchymal phases, in a similar approach with computer tomography (CT) or magnetic resonance (MR) techniques. However, contrast harmonic imaging has several advantages and differences as compared with contrast-enhanced CT or MR, due to the different pharmacokinetics and containment inside the intra-vascular space of the ultrasound contrast agents^[16]. The most important advantage is that contrast-enhancement patterns can be followed in real-time, with a very good temporal resolution (Figure 1E), while the administration can be easily repeated. Because the technology has recently become available for use during EUS examinations, a few studies have already assessed the value of contrast-enhanced harmonic EUS, based on second-generation contrast agents (mostly Sono-Vue)^[24,25]. An initial feasibility study showed that harmonic EUS with low mechanical index can be used for the differential diagnosis of pancreatic cancer and chronic pancreatitis^[24]. By using a mechanical index of 0.4 in conjunction with harmonic EUS with a low mechanical index, both real-time visualization of finely branching vessels of the pancreas, as well as intermittent parenchymal perfusion images could be obtained^[25]. The method showed irregular “network like” structures inside the pancreatic carcinoma masses, with hypovascular heterogeneous perfusion images in the intermittent mode. This contrasted with focal masses in chronic pancreatitis that were homogenous iso- or hypervascular, thus allowing a correct differential diagnosis.

The use of microbubble contrast agents has already been recommended for the monitoring of the response to anti-angiogenic treatment, because the conventional criteria [Response Evaluation Criteria in Solid Tumors (RECIST) and World Health Organization size criteria] do not show changes in the tumor parenchymal perfusion, hence they cannot predict response in the presence of tumor necrosis without volume changes^[16]. Thus, a clear correlation has been proven between histological intratumoral microvessel density, vascular endothelial growth factor (VEGF) and microvessels visualized by contrast-enhanced ultrasound in pancreatic ductal carcinoma^[26]. By using a similar approach, contrast-enhanced EUS might prove very useful for the real-time monitoring of the efficacy of antiangiogenic treatments. Contrast-enhanced harmonic EUS would certainly have the advantage of an improved resolution and decreased artifacts induced by bowel air and obesity.

Targeted imaging and targeted treatment

Microbubbles used as ultrasound contrast agents can be targeted *in vivo* to specific endothelial cell surface receptors^[27]. Thus, different ligands can be conjugated to the outer surface of microbubbles and directed selectively towards endothelial cells. Microbubbles can be linked with monoclonal antibodies directed against VEGF receptor 2 (VEGFR2), thus allowing the binding to tumor-associated epithelium *in vivo*^[28]. This allows *in vivo* quantification of VEGFR2 expression in tumor vessels, permitting both the selection of antiangiogenic drugs (e.g. bevacizumab which blocks specifically the VEGF-VEGFR2 pathway), as well as monitoring of treatment response. Multitarget quantification and visualization of targeted contrast-enhanced ultrasound microbubbles conjugated with either VEGFR2 and/or $\alpha(v)\beta(3)$ integrin were tested in initial experimental designs^[29]. Targeted treatment during targeted contrast-enhanced ultrasound has been recently proven interesting due to the enhanced cellular uptake of drugs and genes in the presence of ultrasound and especially contrast-enhanced ultrasound, a process called sonoporation^[30]. New microbubbles incorporating chemotherapeutic or gene vectors can be delivered at a cellular level through the formation of transient porosities in the cell membrane.

3D-EUS

Tridimensional EUS was recently reviewed in a separate paper^[31]. The method enhances the spatial understanding of EUS anatomy, especially for the pancreatobiliary area. The method can better depict the relationship with major surrounding vessels, consequently improving staging and resectability, mainly for pancreatic tumors (Figure 1F-H). Contrast-enhanced 3D-EUS can also be performed, allowing a better calculation of the vascular index that might offer important prognostic information, linked with the status before and after antiangiogenic treatment.

REAL-TIME OPTICAL DIAGNOSIS

Real-time optical pathological diagnosis might be achieved based on recent advances in single fiber-based optical techniques, the best example being confocal laser endomicroscopy^[32]. Miniaturization of a confocal laser endomicroscopy miniprobe allowed the EUS-guided placement of a miniprobe through a 22G needle, inside different organs/lesions located in the vicinity of the digestive tract, e.g. pancreas, spleen, adrenal, liver, *etc.* The method has been shown to be feasible, yielding high quality confocal laser endoscopy images, equivalent of real-time histopathology images.

EUS-NATURAL ORIFICE TRANSLUMINAL ENDOSCOPIC SURGERY

The combination of EUS and natural orifice transluminal endoscopic surgery (NOTES) has already been described as a combination of NOTES peritoneoscopy and intra-peritoneal EUS through transgastric and transcolonic ap-

proaches^[33]. Thus, intraperitoneal EUS is considered safe and feasible, allowing adequate visualization of 4 sections of liver. Although objective landmarks for EUS were absent, intraperitoneal EUS could replace laparoscopic US, while NOTES peritoneoscopy can successfully replace laparoscopy. EUS-guided NOTES procedures were proven to be useful in a comparative sequential study which assessed mediastinoscopy/thoracoscopy, gastrojejunostomy and adrenalectomy^[34]. EUS-guided access was useful mainly to obtain initial access or to identify structures in difficult areas, especially in the mediastinal or retroperitoneal regions. Furthermore, both an anterior and a posterior approach of the pancreas are possible through EUS-NOTES procedures, indicating a possible role for these combined techniques^[35]. The aim was to improve pancreatic cancer staging of borderline cases and minimal invasive therapy of pancreatic diseases. Peritoneoscopy based on a EUS-assisted anterior transgastric approach, as well as EUS-guided posterior transgastric access to the pancreas, were both shown to be possible in this small non-survival animal study. Different therapeutic procedures like gastrojejunostomies and cholecysto-gastrostomies were also shown to be possible after initial EUS-assisted procedures. Future survival studies with randomized design should establish clearly the clinical role of these procedures.

CONCLUSION

EUS reached maturity as an imaging technique, as compared with the initial description in 1980. With a superior resolution as compared with cross-sectional imaging and with the addition of recent techniques like real-time sonoelastography, contrast-enhancement and 3D reconstructions, EUS seems likely to represent the technique of choice for early diagnosis, staging and stratification of prognosis. EUS-guided FNA or EUS-assisted procedures are also considered procedures of choice for the pathological confirmation of advanced cases, as well as for targeted treatment procedures. All of these procedures lead to a significant clinical impact of EUS, especially due to the improved clinical decision making algorithms, which nowadays incorporate routine EUS-guided or EUS-assisted procedures. The transition of these procedures to real-time optical diagnosis might offer additional value, allowing the immediate initiation of minimal invasive therapeutic procedures. Also, the appearance of combined EUS-NOTES procedures might enhance the safety and success of recent NOTES applications.

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S- Editor Wang YR L- Editor O'Neill M E- Editor Lin YP



Primary gastrointestinal lymphoma

Prasanna Ghimire, Guang-Yao Wu, Ling Zhu

Prasanna Ghimire, Guang-Yao Wu, Ling Zhu, Department of Magnetic Resonance Imaging, Zhongnan Hospital, Wuhan University, 169 East Lake Road, Wuhan 430071, Hubei Province, China
Author contributions: Ghimire P designed, performed the literature search and wrote the review; Wu GY and Zhu L assisted in revising the manuscript.

Supported by Hubei Province Natural Science Fund of China, No. 2009CDA071; and Wuhan University Independence Fund of China, No. 303275894

Correspondence to: Guang-Yao Wu, MD, PhD, Department of Magnetic Resonance Imaging, Zhongnan Hospital, Wuhan University, 169 East Lake Road, Wuhan 430071, Hubei Province, China. wuguangy2002@yahoo.com.cn

Telephone: +86-27-67813187 Fax: +86-27-67813188

Received: September 22, 2010 Revised: November 11, 2010

Accepted: November 18, 2010

Published online: February 14, 2011

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Key words: Gastrointestinal lymphoma; Epstein-Barr virus; *Helicobacter pylori*; Celiac disease; Diffuse large B-cell lymphoma

Peer reviewer: Dr. Takuya Watanabe, Department of Intern Medicine and Gastroenterology, The Nippon Dental University School of Life Dentistry at Niigata, 1-8 Hamauracho, Chu-o-ku, Niigata 951-8580, Japan

Ghimire P, Wu GY, Zhu L. Primary gastrointestinal lymphoma. *World J Gastroenterol* 2011; 17(6): 697-707 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/697.htm>
DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.697>

Abstract

Gastrointestinal tract is the most common extranodal site involved by lymphoma with the majority being non-Hodgkin type. Although lymphoma can involve any part of the gastrointestinal tract, the most frequent sites in order of its occurrence are the stomach followed by small intestine and ileocecal region. Gastrointestinal tract lymphoma is usually secondary to the widespread nodal diseases and primary gastrointestinal tract lymphoma is relatively rare. Gastrointestinal lymphomas are usually not clinically specific and indistinguishable from other benign and malignant conditions. Diffuse large B-cell lymphoma is the most common pathological type of gastrointestinal lymphoma in essentially all sites of the gastrointestinal tract, although recently the frequency of other forms has also increased in certain regions of the world. Although some radiological features such as bulky lymph nodes and maintenance of fat plane are more suggestive of lymphoma, they are not specific, thus mandating histopathological analysis for its definitive diagnosis. There has been a tremendous leap in the diagnosis, staging and management of gastrointestinal lymphoma in the last two decades attributed to a better insight into its etiology and molecular aspect as well as the knowledge about its critical signaling pathways.

INTRODUCTION

Gastrointestinal tract is the most common extranodal site involved by lymphoma accounting for 5%-20% of all cases^[1]. Primary gastrointestinal lymphoma, however, is very rare, constituting only about 1%-4% of all gastrointestinal malignancies. Gastrointestinal lymphoma is usually secondary to the widespread nodal diseases. Although virtually lymphoma can arise from any region of the gastrointestinal tract, the most commonly involved sites in term of its occurrence are the stomach followed by small intestine and ileocecal region^[2]. Histopathologically, almost 90% of the primary gastrointestinal lymphomas are of B cell lineage with very few T-cell lymphomas and Hodgkin lymphoma. Certain histological subtypes have been noted to have a relative predilection site as mucosa-associated lymphoid tissue (MALT) lymphoma in stomach, mantle cell lymphoma (MCL) in terminal ileum, jejunum and colon, as well as enteropathy-associated T-cell lymphoma (EATL) in jejunum, and follicular lymphoma (FL) in duodenum with a geographic variation in its distribution^[3]. Multifocality, however, has been noticed particularly in MALT lymphoma and follicular lymphoma. Certain risk factors have been implicated in the pathogenesis of gastrointestinal lymphoma including *Helicobacter pylori* (*H. pylori*) infection, human immunodeficiency

virus (HIV), celiac disease, *Campylobacter jejuni* (*C. jejuni*), Epstein-Barr virus (EBV), hepatitis B virus (HBV), human T-cell lymphotropic virus-1 (HTLV-1), inflammatory bowel disease and immunosuppression^[4,5]. Marker expression and translocations of common histological types of gastrointestinal lymphoma are depicted in Table 1.

Dawson's criteria are used for labeling primary gastrointestinal lymphoma, that include (1) absence of peripheral lymphadenopathy at the time of presentation; (2) lack of enlarged mediastinal lymph nodes; (3) normal total and differential white blood cell count; (4) predominance of bowel lesion at the time of laparotomy with only lymph nodes obviously affected in the immediate vicinity; and (5) no lymphomatous involvement of liver and spleen^[6]. Ann Arbor staging with Musshoff modification is commonly employed to stage gastrointestinal lymphoma and the international prognostic index has been used to define the prognostic subgroups and Paris staging has increasingly gained its significance. Accurate diagnosis and staging of gastrointestinal lymphoma are detrimental for the stratification of treatment in this heterogeneous group of malignancies. The different procedures employed for the pre-treatment staging include endoscopic ultrasound (EUS), endoscopic biopsies, computed tomography (CT), magnetic resonance imaging (MRI), 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) or molecular markers^[7,8]. Contrast-enhanced techniques and functional imaging such as perfusion CT can also help the monitoring, assessment, and prediction of response. New promising techniques such as hybrid PET-CT imaging and new PET tracers like 18F-fluoro-thymidine may significantly benefit the overall management of lymphomas^[9].

In the following sections, the commonly involved sites of gastrointestinal lymphoma and its clinical, pathological and radiologic features are discussed with stress laid on the different histological subtypes based on the predilection. The characteristic features of gastrointestinal lymphoma in different regions of gastrointestinal tract are shown in Table 2.

CLINICAL/PATHOLOGICAL/IMAGING CHARACTERISTICS

Oropharyngeal lymphoma

The head and neck region is the second most common site for extra-nodal lymphoma accounting for 10%-15% of all cancers in this region. Approximately 2.5% of all malignant lymphomas originate from the oral and paraoral region, and the majority of them in the Waldeyer's ring include adenoids, palatine tonsils, base of tongue and oropharyngeal walls. Tonsil is the most frequently involved site (> 50%) of tumors, followed by nasopharynx and base of tongue^[10]. Several factors are known to increase the risk of oropharyngeal lymphoma including EBV. The affected patients are usually at the age of over 50 years with a predilection of males. The most common clinical presentations of oropharyngeal lymphoma include airway obstruction, hearing pain, progressive enlarging painless local mass, dysphagia and foreign body sensation in the

throat. Cervical lymphadenopathy is present in over 50% patients with tonsillar lymphoma^[11].

More than 80%-90% of oropharyngeal lymphomas belong to the B-cell lineage of non-Hodgkin lymphoma (NHL)^[12]. Diffuse large B-cell lymphoma (DLBCL) is the most common type of primary oral and paraoral NHL with a small percentage of thymic T-cell type. Histologically, DLBCL, composed of intermediate-large cells which may be noncleaved, cleaved and immunoblastic, shows B-cell lineage with expression of pan-B-cell antigens (CD19, CD20, CD22, CD79A, and PAX5/BSAP), and is less commonly positive for germinal centre cell markers (CD10 and BCL6) and negative for T-cell antigens. A small number of cases show a translocation between the *BCL-2* gene on chromosome 18 and the *IgH* gene on chromosome 14, t (14;18)^[13]. Other lymphomas involving the Waldeyer's ring include 15% B-cell lymphomas in extranodal marginal zone of MALT, 8% peripheral T-cell lymphomas, 6% follicular lymphomas, and 3% MCLs. Hodgkin lymphoma (HL) involving the oropharynx is very rare accounting for about 1%-5% of all Hodgkin diseases. The majority of oropharyngeal HL are of lymphocyte predominant and nodular sclerosis type on histopathology with a common immunophenotype of Reed Sternberg cells positive for CD15, CD30 and negative for CD45, CD20, and EMA, which can rule out the diagnosis of NHL^[14].

Radiologically, oropharyngeal lymphoma typically appears in barium studies as a lobular mass near the base of tongue in the palatine fossa with the overlying mucosa usually being nodular. The appearance of oropharyngeal lymphoma can be hard to differentiate from more common pharyngeal carcinomas. Because the signal intensity of lymphoma is similar to that of normal tissue, the MR signal characteristics cannot reliably show the early lymphomatous involvement at these sites. CT or PET with FDG and CT (PET/CT) has proved their usefulness both in diagnosis and staging of the disease and in assessment of its response to therapies^[15]. Certain features that may favor the diagnosis of NHL on imaging are the short clinical history and a large homogeneous mass which displaces rather than invades local structures and large homogeneous non-necrotic cervical nodes^[16].

Esophageal lymphoma

The esophagus is a rarely involved site, accounting for < 1% of all gastrointestinal lymphomas. Esophageal involvement usually results from metastasis from cervical or mediastinal lymph nodes or extension from gastric lymphoma. Primary esophageal lymphoma is extremely rare, with less than 30 cases reported in the literature^[17-19]. The majority are the DLBCL type of NHL. Only few cases of MALT lymphoma, MCL, T-cell lymphoma and HL involving the esophagus have been reported^[19-22]. The etiology of esophageal lymphoma is unknown and the role of EBV in its pathogenesis is controversial. It has been shown that esophageal lymphoma is most common in immunocompromised patients, with HIV infection as a probable risk factor^[17]. The age of presentation is variable. The common symptoms

Table 1 Expression of common markers and translocations in histological subtypes of gastrointestinal lymphomas

Type	CD5	CD10	CD19	CD20	CD22	CD23	CD43	CD79a	CD3	CD7	CD4	CD8	CD30	CD15	CD45RO	Additional features
Diffuse large B-cell lymphoma	-(+)	-(+)	+	+	+	-	-	+	-	-	-	-	-(+)	-	-(+)	Bcl-6+(-), Bcl-2+(-), t (14;18), t (3;14), t (8;14)
MALT lymphoma	-	-	+	+	+	-	-(+)	+	-	-	-	-	-	-	-(+)	t (11;18), t (14;18), t (1;14), t (3;14)
Follicular lymphoma	-	+(-)	+	+	+	-(+)	-	+	-	-	-	-	-(+)	-	-	Bcl-2+, Bcl-6+, t (14;18)
Burkitt lymphoma	-	+	+	+	+	-(+)	+	+	-	-	-	-	-	-	-	C-myc, t (8;14)
Mantle cell lymphoma	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	Cyclin D1+, t (11;14)
Peripheral T-cell lymphoma-unspecified	+(-)	-(+)	-	-	-	-	+(+)	-	+(-)	-(+)	+(-)	-(+)	-(+)	-(+)	-(+)	-
Extranodal NK/T cell lymphoma	-(+)	-	-	-	-	-	+	-	-(+)	-(+)	-(+)	-	-(+)	-(+)	-	EBV+, gains of 2q, 15q, 17q, 22q, losses of 6q, 8p, 11q, 12q, 13q
EATL	-	-	-	-	-	-	+/-	-	+	+	-(+)	+(-)	+(-)	-	+(-)	TIA1+, gains of 1q, 5q, 7q, 9q, losses of 8p, 9p, 13q
Hodgkin disease	+(-)	-	-	-(+)	-	-	-	-(+)	+(-)	-(+)	-	-	+(-)	+(-)	-(+)	Variable

+: ≥ 90% positive; +(-): > 50% positive; -(+): < 50% positive; -: < 10% positive cases. MALT: Mucosa-associated lymphoid tissue; EATL: Enteropathy-associated T-cell lymphoma; EBV: Epstein-Barr virus.

of patients with esophageal lymphoma include dysphagia, odynophagia, weight loss, chest pain or present as a result of complications such as hemorrhage, obstruction or perforation with a tracheoesophageal fistula. Constitutional B symptoms (fever, night sweats) are not typically present.

Almost all cases of primary esophageal lymphoma are DLBCLs with positive surface markers of tumor cells on immunofluorescent staining for immunoglobulin G and κ light chain. MALT lymphoma of the esophagus, however, unlike that of stomach, is not associated with *H. pylori*. HL of the esophagus is extremely rare. Follicular lymphoma affecting the esophagus is a part of multifocal presentation in the gastrointestinal tract.

Radiological and endoscopic findings in esophageal lymphoma vary greatly and are nonspecific, which poses diagnostic challenges when it is differentiated from other benign and malignant lesions. Radiographic patterns of esophageal lymphoma, described in the literature^[18-20], include stricture, ulcerated mass, multiple submucosal nodules, varicoid pattern, achalasia-like pattern, progressive aneurysmal dilatation, and tracheoesophageal fistula formation, and none of them is diagnostic. The morphological features seen at endoscopy are nodular, polypoidal, ulcerated or stenotic^[21]. EUS has gained clinical acceptance for the assessment of lymphoma and preoperative staging, because it can accurately depict the structural abnormalities and depth of invasion of the lesions. EUS findings, however, are not pathognomonic, with presentation varied as anechoic, hypoechoic or even hyperechoic masses^[22]. CT findings in esophageal lymphoma are nonspecific and not diagnostic, with features such as thickening of the wall mimicking other common tumors, such as esophageal carcinoma. CT, however, they are valuable for the evalua-

tion of the extraluminal component of esophageal mass, its mediastinal extension, fistula formation, and status of lymph nodes, thus playing a role in staging disease, assisting in stratification of available treatment modalities, evaluating treatment responses, monitoring disease progression, and detecting relapses^[23]. Recently, incorporation of PET/CT has emerged as an indispensable tool in staging the disease and following up the patients with extranodal involvement of Hodgkin's and non-Hodgkin's lymphoma, with an increased sensitivity and specificity. Diffuse large B-cell non-Hodgkin lymphoma of the esophagus is manifested as circumferential thickening of the wall, with diffuse increased FDG uptake. However, the intensity of FDG uptake in lymphoma is influenced by various intrinsic tumor factors such as histological features and grade, as well as various extrinsic factors. FDG PET/CT can also detect the indolent lesions that are undetectable on conventional cross-sectional imaging^[24].

Gastric lymphoma

Stomach is the most commonly involved site (60%-75%) in gastrointestinal tract followed by small bowel, ileocecal region and rectum^[25]. Gastric lymphoma accounts for 3%-5% of all malignant tumors of the stomach^[26]. Although the incidence of gastric carcinoma has been reduced, the incidence of primary gastric lymphoma is increasing^[27]. *H. pylori* play a role in the development of most MALT lymphomas. However, its exact mechanism has not been fully understood, although a chronic inflammation may enhance the probability of malignant transformation via B cell proliferation in response to *H. pylori* mediated by tumor-infiltrating T cells^[28]. *H. pylori* may play a similar role in development of DLBCL and few studies have shown

Table 2 Characteristic features of gastrointestinal lymphomas

Region	Age (yr)	GIL (%)	Sex	Predilection site	Etiological/risk factors	Presenting symptoms	Common pathological subtypes	Radiographic features
Oropharyngeal	> 50	-	M > F	Tonsil, nasopharynx ¹ , base of tongue	EBV	Dysphagia, dyspnea, painless mass, ulcer, oral/hearing pain, B symptoms rare	DLBCL, EMZL/MALT, PTCL, FL, MCL, ENKL, HD	Lobular mass, ulcers
Esophagus	Variable	< 1	-	Mid and lower third	EBV, HIV	Dysphagia, odynophagia, weight loss, epigastric/chest pain, pneumonia, bleeding rare, B symptoms rare	DLBCL, MALT lymphoma, HD, MCL, T-cell lymphoma	Stricture, ulcerated mass, submucosal nodules, varicoid-like, achalasia-like, aneurysmal, fistula formation
Stomach	> 50	60-75	M > F	Antrum	<i>H. pylori</i> (MALT lymphoma), HTLV-1, HBV (DLBCL), EBV, HCV	Epigastric pain, dysphagia, nausea, vomiting, weight loss, abdominal mass, gastrointestinal bleeding, obstruction, perforation, B symptoms rare	DLBCL, MALT, PTCL	Ulcers, polypoid mass, thickened fold, mucosal nodularity, linitis plastica-like
Small intestine	Variable	20-30	Usually, M > F	Ileum, jejunum, duodenum, multiple sites	Celiac disease (EATL), <i>C. jejuni</i> (IPSID), EBV, HIV/AIDS	Abdominal pain, nausea, vomiting, weight loss, GI bleeding, obstructive symptoms, intussusceptions, perforation, diarrhea (in IPSID), B symptoms rare	DLBCL, MALT, EATL, MCL, Burkitt lymphoma, FL, IPSID, PTCL, ENKL	Polypoid mass, multiple nodules, infiltrative form, ulcer, excavation, fistulization, extraluminal mass, mucosal thickening, strictures
Colon/rectum	50-70	6-12	M > F	Caecum, ascending colon, rectum	Celiac disease (EATL), EBV, <i>H. pylori</i> (MALT lymphoma)	Abdominal pain, weight loss, abdominal mass, lower GI bleeding, obstruction, perforation	DLBCL, MALT, EATL, MCL, PTCL, Burkitt lymphoma	Polypoid mass, ulcers, mucosal nodularity, cavitory mass, mucosal thickening, strictures, aneurysmal

¹Included here though usually not applicable. GIL: Gastrointestinal lymphoma; EBV: Epstein Barr virus; DLBCL: Diffuse large B cell lymphoma; EMZL: Extranodal marginal-zone lymphoma; PTCL: Peripheral T cell lymphoma; FL: Follicular lymphoma; EATL: Enteropathy-associated T-cell lymphoma; MCL: Mantle cell lymphoma; IPSID: Immunoproliferative small intestinal disease; HD: Hodgkin's disease; HTLV-1: Human T-cell lymphotropic virus-1; HCV: Hepatitis C virus; HBV: Hepatitis B virus; *H. pylori*: *Helicobacter pylori*; *C. jejuni*: *Campylobacter jejuni*; HIV: Human immunodeficiency virus; AIDS: Acquired immune deficiency syndrome; ENKL: Extranodal NK/T-cell lymphoma.

complete remission after eradication therapy alone^[28]. It has been shown that individuals with positive HBsAg have an increased risk of developing NHL^[29]. It was reported that HBV plays a role in the development of B-cell NHL^[30]. In contrast, primary gastric lymphoma with a T-cell phenotype is relatively rare, accounting for only 7% of primary gastric lymphomas in HTLV-1 infected endemic areas and a relatively large number of such cases are secondary gastric involvement of adult T-cell leukemia. Primary gastric T-cell lymphoma without HTLV-1 infection is rare, and sporadic cases have been reported^[31]. The age of most gastric lymphoma patients is over 50 years with a relative predilection in males. Clinical symptoms of gastric lymphoma are nonspecific and indistinguishable from other benign and malignant conditions. The most common complaints of gastric lymphoma patients are epigastric pain, weight loss, nausea and vomiting. Occasionally, an abdominal mass is palpable. Lymphadenopathy is rare and its patients often have no physical signs. Perforation, bleeding, or obstruction is very uncommon. Unlike nodal lymphoma, B constitutional symptom is not common.

Although all histological kinds of nodal lymphoma can arise from the stomach, the majority of them are of the B-cell origin, and MALT lymphoma and DLBCL ac-

count for over 90%. MALT lymphoma comprises up to 50% of all primary lymphomas involving the stomach. Histologically, the most significant finding is the presence of a variable number of lymphoepithelial lesions defined by evident invasion and partial destruction of mucosal glands by the tumor cells. MALT lymphoma shows the immunophenotype of B cells in the normal marginal zone of spleen, Peyer's patches and lymph nodes. The tumor B-cells can express the surface immunoglobulin and pan-B antigens (CD19, CD 20, and CD79a), the marginal zone-associated antigens (CD35 and CD21, and lack CD5, CD10, CD23) and cyclin D1. MALT lymphoma can be divided into *H. pylori* positive or negative based on the presence of *H. pylori*. *H. pylori* negative MALT lymphoma tends to have a higher positive rate for t (11;18) (q21;q21) translocation than *H. pylori* positive MALT lymphoma^[32]. DLBCL, a heterogeneous group of tumors which are clinically, histologically, immunophenotypically, cytogenetically variable, can be divided into 3 subgroups, namely germinal-center B-cell-like, activated B-cell-like, and primary mediastinal DLBCL according to the gene expression patterns with each having a different prognostication. The most commonly seen translocations as mentioned earlier include t (14;18) (q32;q21) with BCL2-rearrange-

ment, t (3;14) (p27;q32) with BCL6-rearrangement and t (8;14) (q24;q32) with MYC rearrangement, respectively. Variability has been observed in CD45, CD5 and CD10 expression, with the CD10 expression in particular referred as a prognostic indicator^[33].

Endoscopy cannot distinguish gastric lymphoma from the more common gastric carcinoma. The three main patterns that can be recognized at endoscopy include ulceration, diffuse infiltration, and polypoid mass, which are, however, not specific^[34]. Endoscopy, however, is an indispensable tool for the initial diagnosis and follow-up of cases as well as for obtaining biopsy specimens. EUS can assess the extent of lesion and its invasion. Lesions are usually hypoechoic although few hyperechoic cases have been reported^[34]. Infiltrative carcinoma tends to have a vertical growth in gastric wall, while lymphoma tends to show mainly a horizontal extension and more involvement of perigastric lymph nodes^[35]. EUS is highly accurate in detecting the depth of lymphomatous infiltration and the presence of perigastric lymph nodes, thus providing additional information for treatment planning, and can differentiate lymphoma from carcinoma both in early stage and in advanced stage^[36].

Radiographic patterns of gastric lymphoma observed in double-contrast UGI studies include ulcers, polypoid mass, thickened fold, mucosal nodularities or infiltrating lesions, which are not conclusive, thus posing a diagnostic challenge while differentiating from other malignant and benign lesions, hence requiring pathological confirmation. Preservation of gastric distensibility and pliability, despite the extensive infiltration with gastric fold thickening, is a finding more suggestive of lymphoma. Gastric wall thickening is much less severe in low-grade lymphoma than in high-grade lymphoma on CT images, and abdominal lymphadenopathy is less common in low-grade lymphoma. Preservation of the fat plane with no invasion of surrounding structures may be suggestive of lymphoma, although it is, however, not specific. Transpyloric spread and extension of lymphadenopathy below the renal hilum and the presence of bulky lymph nodes are more suggestive of lymphoma than carcinoma^[37]. The patterns of gastric involvement observed can be segmental or diffuse infiltration, or localized polypoid. Tumor infiltration is usually homogeneous although areas of low attenuation may be present in larger tumors. Diffuse infiltration involving more than 50% of the stomach and segmental infiltration are the most common features of gastric NHL on CT images^[38]. The MRI features include irregularly thickened mucosal folds, irregular submucosal infiltration, annular constricting lesion, exophytic tumor growth, mesenteric masses and mesenteric/retroperitoneal lymphadenopathy. The tumors are usually homogeneous and intermediate in signal intensity on T1-weighted images. Heterogeneously increased signal intensities are noted on T2-weighted images. The enhancement is usually mild-moderate after intravenous administration of gadolinium dimeglumine^[39]. Application of 18F-FDG PET/CT in diagnosis of gastric lymphoma is challenging due to the physiologic FDG activity in the stomach and variability

in the degree of uptake in various histologic subtypes. It was reported that aggressive gastric lymphoma has more intense uptake than low grade MALT lymphoma^[40].

Small intestine lymphoma

Primary malignant tumors of the small intestine are very rare, accounting for less than 2% of all gastrointestinal malignancies. Lymphoma constitutes 15%-20% of all small intestine neoplasms and 20%-30% of all primary gastrointestinal lymphomas. Ileum is the most common site (60%-65%) involving small intestine lymphoma followed by jejunum (20%-25%), duodenum (6%-8%) and other sites (8%-9%)^[41]. The age of presentation varies with the histological subtype of lymphoma. The clinical presentation of small intestinal lymphoma is non specific and the patients have symptoms, such as colicky abdominal pain, nausea, vomiting, weight loss and rarely acute obstructive symptoms, intussusceptions, perforation or diarrhea^[42].

Primary small intestine lymphomas that are more heterogeneous than those in stomach include MALT lymphoma, DLBCL, EATL, MCL, follicular lymphoma and immunoproliferative lymphoma, and can be divided into immunoproliferative small intestinal disease (IPSID)^[43]. IPSID, also known as alpha chain disease, is a MALT-associated lymphoma due to *C. jejuni* infection and characterized by "centrocyte like" mucosal infiltration with plasma cells that secrete monotypic and truncated immunoglobulin, a heavy chain lacking of an associated light chain. IPSID mainly affects older children and younger adults with a predominant involvement of proximal small intestine, the symptoms of its patients are diarrhea and abdominal pain^[44]. MCL primarily affects individuals at the age of over 50 years, and involves terminal ileum and jejunum appearing as numerous polyps, hence called multiple lymphomatous polyposis^[45]. The prototype MCL is positive for pan B-cell antigens, although few cases of CD5-MCL have been reported^[44]. Cytogenetic analysis of MCL has shown the rearrangement of bcl-1 locus on chromosome11 due to t (11;14) (q13;q32) translocation, accompanying cyclin D1 antigen overexpression. Few cases of cyclin D1-negative MCL, however, have been reported with up-regulated cyclin D2 or D3^[46]. Burkitt's lymphoma mainly affects children and is associated with EBV and HIV/AIDS^[47]. T cell lymphoma of the small intestine accounts for approximately 10%-25% of all primary intestinal lymphomas primarily occurring as enteropathy-associated T cell lymphoma, and most of them are often complicated by Crohn's disease^[48,49]. Although follicular lymphoma is very rare, it expresses SIg (frequently IgM) and pan B-cell antigens with CD10 and bcl-2 expressed in almost 90% of cases. It is negative for CD5 and cyclin D1 differentiating it from MCL. IgH/BCL2 rearrangement with t (14;18) (q32;q21) can be demonstrated by FISH or PCR analysis in the majority of cases^[50]. Lymphocytic lymphoma (chronic lymphocytic leukemia) rarely arises primarily from the gastrointestinal tract.

Evaluation of the small intestinal lymphoma has been revolutionized since the introduction of capsule endoscopy (CE) and double-balloon technique of push-and-

pull enteroscopy which is capable of enabling biopsies as well as performing interventions, and limiting major surgical interventions. Small intestine lymphoma appears as a mass, polyp and ulcer on CE which cannot be distinguished from other lesions^[51]. Radiologic findings of small intestinal lymphoma are not specific, thus posing a difficulty in distinguishing it from other benign and malignant lesions. The common features of small intestine lymphoma seen in barium studies and CT include polypoid form, multiple nodules, infiltrative form, endoexoenteric form with excavation and fistulization, and mesenteric invasive form with an extraluminal mass. The radiological findings usually do not correlate to its pathological subtypes. Certain features are, however, peculiarly noted. MCL, follicular lymphoma and MALT lymphoma rarely present with multiple polyps (multiple lymphomatous polyposis)^[52]. Burkitt lymphoma usually presents as a bulky mass in the right lower quadrant. IPSID tends to affect proximally with a disseminated nodular pattern leading to mucosal fold thickening, irregularity and speculation. EATL, usually proximal or diffuse, shows nodules, ulcers or strictures^[53]. PTCL preferentially involves the jejunum with an increased tendency to perforate^[54].

Colorectal lymphoma

Colorectal lymphoma constitutes 6%-12% of all gastrointestinal lymphomas. Most colorectal lymphomas are secondary involvement of the wide spread diseases. Primary colorectal lymphoma is very rare, constituting only 0.2% of all malignant tumors arising from the colorectal region with caecum, ascending colon and rectum more often affected^[55]. The disease predominantly affects males in the fifth-seventh decade of life with abdominal pain, loss of weight, palpable abdominal mass or lower gastrointestinal bleeding. Obstruction and perforation are relatively rare in patients with colorectal lymphoma^[56].

Lymphoma of the colorectal region is mostly the B-cell lineage as other sites of the gastrointestinal tract. Primary colorectal lymphoma comprises low grade B-cell lymphoma arising from MALT, MCL and T-cell lymphoma besides large B cell lymphoma. The role of *H. pylori* in the pathogenesis of colorectal lymphoma has not been fully established^[57]. Colorectal MALT-lymphoma is less common in colon and rectum than in small intestine. MCL in the colorectal region presents usually in the setting of diffuse systemic diseases. Peripheral T-cell lymphoma is rare in Western countries with an increasing frequency in many Asian countries, and is more aggressive in nature than other types with perforation as its common feature, and its prognosis is poor^[58].

Endoscopically, lymphoma appears to be fungating, ulcerative, infiltrative, ulcerofungating, and ulceroinfiltrative types, with fungating and ulcerofungating types being more common^[59]. The radiologic appearances of colorectal lymphoma are variable and significantly overlapped with other benign and malignant condition of the colorectal region. The imaging findings during double-contrast barium enema can be divided into focal and diffuse lesions. The observed focal lesions include polypoid mass,

circumferential infiltration with smooth mucosal surface or extensive ulceration, cavitary mass, mucosal nodularity, and mucosal fold thickening. Diffuse lesions encompass diffuse ulcerative and nodular lesions. Peripheral T-cell lymphoma presents as a diffuse or focal segmental lesion with extensive mucosal ulceration similar to that observed in granulomatous conditions as Crohn's disease or tuberculosis. MALT lymphoma is manifested as multiple mucosal nodularity^[60,61].

TREATMENT

The treatment strategy for gastrointestinal lymphoma is dependent on the age of patients, clinical scenario, histological subtype, extent and burden of the disease, and comorbidity, besides other factors. Surgery, chemotherapy, radiotherapy and radioimmunotherapy are the different modalities for its management and can be applied in different combinations. A detailed discussion on the treatment of all subtypes is beyond the scope of this article, thus the most common treatment modalities are highlighted in brief based on the region of presentation.

Oropharyngeal lymphoma

The definite management protocol for oropharyngeal lymphoma has not yet been established. Unlike the majority of other malignancies in this region, surgery does not play a primary role in the management of oropharyngeal lymphoma^[62]. Combined chemotherapy and radiotherapy for localized oropharyngeal lymphoma is recommended in most studies^[14]. Advanced oropharyngeal lymphoma is usually treated with aggressive chemotherapy with or without radiotherapy.

Esophageal lymphoma

Due to the rarity of esophageal lymphoma, no standardized approaches to its management have been formulated. Secondary lymphoma involving the esophagus can be treated with chemotherapy, while primary esophageal lymphoma can be managed with surgery, chemotherapy and radiotherapy or their combination. Treatment protocols vary depending on its histological subtypes and extent. Although surgery is the initial treatment modality, it has been recently reserved for cases with their diagnosis not possibly made at endoscopic biopsy or for those who warrant surgical intervention due to complications such as perforations. Esophageal lymphoma can be treated with local resection and chemotherapy with or without radiotherapy as its initial therapy. However, chemotherapy or radiotherapy alone can be also used as its initial therapy. The commonly employed chemotherapy regimen is CHOP in combination with Rituximab. It was reported that external beam radiation at the dose of 40 Gy can also be used^[63].

Gastric lymphoma

Treatment strategies for gastric lymphoma have changed dramatically over the last two decades. However, they are still very controversial. The most widely recommended

strategy for the management of early stage *H. pylori* positive MALT type of gastric lymphoma is to eradicate *H. pylori* with antibiotics and proton pump inhibitors. Antibiotic therapy can achieve a long-term remission in 60%-100% patients with localized *H. pylori*-positive MALT lymphoma without t (11;18) chromosomal translocation. Histological assessment of treatment response, however, faces the problem of standardization, thus mandating serial follow-up. The GELA histologic evaluation system is commonly employed at certain centers. It has been shown that monoclonal B-cells still exist in almost half of the patients despite histological and endoscopic remission following antibiotic therapy^[64].

No definite guidelines have been advocated for the treatment of advanced or *H. pylori* negative MALT-type of gastric lymphoma. Although surgery has been used as its initial treatment, recent studies showed that radiotherapy alone can achieve a complete remission with a 5-year disease free period^[20]. Thus, "involved-field" irradiation at the total dose of 30 Gy for over 4 wk has become the treatment of choice for stages I and II MALT lymphoma without *H. pylori* or with persistent lymphoma following therapy. Surgery is, at present, reserved only for those with complications such as perforation, hemorrhage or obstruction that cannot be treated with other alternative therapies. Systemic therapy similar to that for indolent and advanced lymphoma must be taken into consideration in patients with their disease spread. Treatment options include chemotherapy and use of monoclonal antibodies. Diffuse large B-cell lymphoma of the stomach is treated with aggressive poly-chemotherapy, which is usually combined with Rituximab. Thus, gastric lymphoma should be treated with the front-line chemioimmunotherapy with 3-4 cycles of standard R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) followed by "involved-field" radiotherapy. A complete remission can be achieved in advanced gastric lymphoma patients after 6-8 cycles of R-CHOP as their nodal counterparts. Recent studies have demonstrated that anti-*H. pylori* therapy can achieve the remission of indolent lymphoma, *H. pylori* negative MALT lymphoma and DLBCL^[26,65].

Intestinal lymphoma

The treatment outcome of intestinal lymphoma is relatively poorer than that of gastric lymphoma depending on their histologic subtypes. Lymphoma primarily located in the small intestine usually warrants laparotomy with the affected segment removed both for its diagnosis and for its treatment. Low-grade B-cell lymphoma of the small intestine (stage IE) only requires surgical resection. Although few studies have reported its benefit for localized intestinal lymphoma particularly that of the duodenum and rectum^[66], radiotherapy in particular is not beneficial for intestinal lymphoma due to the multifocal involvement and its spread. No therapeutic guidelines are available for MALT lymphoma involving the small intestine with various modalities depending on the disease burden and other clinical parameters. Local intestinal lymphoma can be managed with surgical or endoscopic resection, while

some cases of colonic MALT lymphoma can benefit from *H. pylori* therapy alone. Multi-agent chemotherapeutic strategy is warranted for advanced stage intestinal lymphoma with multifocal presentation of MALT lymphoma. A wait and watch policy for indolent FL at stage IE is advocated by some authors until they are symptomatic or show evidence of its progression due to a comparable relapse rate in treated patients and the progression of FL in untreated patients^[50,66]. Symptomatic cases, or advanced disease of FL necessitates surgery, chemotherapy (CHOP) and/or irradiation intervention. Although Rituximab is beneficial for FL, its true value has not been well ascertained^[66]. MCL treatment response and prognosis are poor with a short unmaintained remission after chemotherapy. Treatment is stratified based on the eligibility of patients for stem cell transplantation (SCT). Those who are eligible for grafting are previously induced with R-CHOP or R-HyperCVAD (Rituximab, cyclophosphamide, vincristine, doxorubicin and dexamethasone). Chemotherapy regimen, consisting of Rituximab alone or purine nucleoside analogs with Rituximab, can be applied to those ineligible for stem cell transplantation. The mammalian target of rapamycin inhibitors, antibodies, bendamustine or radioimmuno conjugates, can achieve a promising outcome in patients with relapse or refractory setting single-agent bortezomib, temsirolimus and ibritumomab tiuxetan^[67,68]. IPSID in early stage responds to antibiotics such as tetracycline or combined metronidazole and ampicillin, with a remission occurring within 6-12 mo. IPSID at intermediate or advanced stage responds to anthracycline-based chemotherapy, with added antibiotics such as tetracycline. Surgery plays a limited role in the majority of cases due to diffuse involvement, although it may be required for accurate diagnosis. It has been reported that radiotherapy as an adjuvant or palliative treatment is beneficial for some cases^[66]. High grade lymphoma frequently presents with complications, thus mandating surgical intervention. No optimized therapeutic protocol is available for Burkitt lymphoma which usually requires an aggressive approach. High intensity chemotherapeutic agents for a short duration, such as cyclophosphamide, vincristine, doxorubicin, methotrexate and cytarabine, can significantly improve the treatment outcome. High dose chemoradiotherapy and hematopoietic SCT are beneficial for almost 50% of Burkitt lymphoma patients^[69]. Radiotherapy is not beneficial for DLBCL involving the small intestine^[70]. Systemic treatment with anthracycline-based chemotherapy followed by radiotherapy is proposed for wide spread advanced intestinal lymphoma which cannot be removed. Some studies have shown that post surgery chemotherapy is beneficial for some patients^[68]. The overall response of non surgical patients with intestinal B cell lymphoma to chemotherapy is better than that of those with the intestinal T cell subtype^[71]. No guidelines are available for the management of EATL although anthracyclin-based chemotherapy is a mainstay treatment modality for overt EATL with a poor response. In view of the poor performance and complications related to chemotherapy, such as perforation, multimodal approaches including curative or debulking surgery

Table 3 Paris staging system for primary gastrointestinal lymphomas

Stage	Gastrointestinal lymphomas
TX	Lymphoma extent not specified
T0	No evidence of lymphoma
T1	Lymphoma confined to the mucosa/submucosa
T1m	Lymphoma confined to mucosa
T1sm	Lymphoma confined to submucosa
T2	Lymphoma infiltrates muscularis propria or subserosa
T3	Lymphoma penetrates serosa (visceral peritoneum) without invasion of adjacent structures
T4	Lymphoma invades adjacent structures or organs
NX	Involvement of lymph nodes not assessed
N0	No evidence of lymph node involvement
N1	Involvement of regional lymph nodes
N2	Involvement of intra-abdominal lymph nodes beyond the regional area
N3	Spread to extra-abdominal lymph nodes
MX	Dissemination of lymphoma not assessed
M0	No evidence of extranodal dissemination
M1	Non-continuous involvement of separate site in gastrointestinal tract (e.g. stomach and rectum)
M2	Non-continuous involvement of other tissues (e.g. peritoneum, pleura) or organs (e.g. tonsils, parotid gland, ocular, adnexa, lung, liver, spleen, kidney, breast, <i>etc.</i>)
BX	Involvement of bone marrow not assessed
B0	No evidence of bone marrow involvement
B1	Lymphomatous infiltration of bone marrow
TNM	Clinical staging: status of tumor, node, metastasis, bone marrow
pTNMB	Histopathological staging: status of tumor, node metastasis, bone marrow
pN	The histological examination will ordinarily include six or more lymph nodes

are recommended to remove the gross EATL in all cases, if it is tolerable prior to chemotherapy^[72]. It was reported that 66% of EATL patients undergoing surgical resection followed by combination chemotherapy and autologous stem cell transplantation can achieve a sustained complete response^[73].

STAGING, PROGNOSTICATION AND RESTAGING

Staging of gastrointestinal lymphoma is a matter of debate due to various available staging systems. Although the modified Ann Arbor classification is feasible and relevant for prognosis, certain demerits in terms of disseminated and incurable infiltration of the gastrointestinal tract prompted development of the Paris staging system, which can differentiate distant lymphoma manifestations depending on the involved organ, and further subdivide lymph node involvement (Table 3)^[74]. In general, comprehensive history taking and physical examination may reveal the possible etiologies of some specific lymphoma types and provide information for their further assessment and management. Minimal laboratory investigations performed include complete blood count, liver and renal function test, measurement of lactate dehydrogenase, blood glucose, serum uric acid, potassium, calcium, and phosphorus levels. Bone marrow aspirate with a biopsy is

Table 4 International prognostic index

Adverse risk factors
Age > 60 yr
≥ 2 extranodal sites
Ann arbor stage III-IV
Performance status ≥ 2 (ECOG)
High lactate dehydrogenase
Risk
Low (<i>n</i> = 0-1)
Low-intermediate (<i>n</i> = 2)
High-intermediate (<i>n</i> = 3)
High (<i>n</i> = 4-5)

performed for involvement of lymphoma cells and monitoring of treatment response. Other investigations include serum protein electrophoresis and identification of paraprotein in certain types of lymphoma. Additional serological tests are often employed for etiological recognition in various types of lymphoma. CT scan of the chest, abdomen and pelvis is employed to stage gastrointestinal lymphomas with a marked sensitivity and specificity. Incorporation of FDG-PET has a significant advantage in staging of DLBCL, follicular lymphoma and MCL with a sensitivity of 80% and a specificity of 90%, although it has no added benefit for MALT lymphomas. EUS has gained momentum as an integral tool in the diagnosis, locoregional staging, and monitoring response of gastrointestinal lymphoma to treatment. EUS is superior to CT scan for the T- and N-staging by providing vivid details for any invasion to the mucosa, submucosa, muscularis propria or beyond serosa. The value of EUS and CT, however, is a matter of debate in the follow-up of patients as it is well established that histological remission precedes the normalization of wall changes in patients with lymphoma^[74,75], thus precluding the necessity for endoscopic biopsy follow-up. Gastric MALT lymphoma, though indolent, often warrants a more meticulous staging procedure because it is usually multifocal, transforms to the DLBCL variant, and is difficult to diagnose due to normal endoscopic findings in the majority of cases as well as involvement of multiple organs. Endoscopic biopsies are therefore usually taken from multiple sites of the stomach and duodenum encompassing both normal and abnormal regions^[75].

The international prognostic index (IPI) developed for DLBCL is, at present, the most valuable and widely used for the stratification of almost all subtypes of NHL (Table 4). However, the IPI does not hold the same predictive value for patients treated with immunochemotherapy. Moreover, the IPI is less useful for follicular lymphoma because a significant number of patients with a poor prognosis are not recognized, thus warranting development of follicular lymphoma IPI^[76]. Reevaluation of patients who have completed the whole planned treatment is an integral part in the management of lymphoma patients. The most important prognostic factor for the management is the assessment of complete remission of the disease because salvage treatment with a high dose and autologous or allogeneic bone marrow transplantation may be contem-

plated in those who fail to initial therapy. The different parameters are compared with the prior treatment values and evaluated. A possible necessity of histopathological assessment by follow-up biopsy may be required in certain atypical situations. Nuclear studies and PET in particular have been recommended for the evaluation of recurrence of various lymphomas^[77].

FUTURE PERSPECTIVE

There has been a tremendous leap in the diagnosis, staging and management of gastrointestinal lymphoma in the last two decades. With a better insight into its etiology and molecular aspect, various critical signaling pathways provide an impetus with greater benefits. Identification of the cell surface antigens has led to the introduction of monoclonal antibodies like Rituximab and radioimmunotherapy that can result in a more targeted approach with a significant impact for the overall management of lymphoma. A deep understanding of the role of monoclonal antibodies in the pathogenesis of gastrointestinal lymphoma has led to development of the second and third generations of anti CD-20 antibodies (ofatumumab, veltuzumab, ocrelizumab), anti CD-22 antibodies such as Epratuzumab, anti CD-30 antibodies such as SGN-30, anti CD-40 antibody SGN-40, and anti vascular endothelial growth factor (VEGF) antibody bevacizumab^[78]. Furthermore, addition of cytokines and other immune modulators has a boon resulting from a better understanding of the antibody activities at targeted tissues. Agents targeting the Bcl-2, Syk and the PI3K/AKT/mTOR pathways have emerged as a more biologically- focused management with further development in this field^[79].

Another important aspect to be considered is the increasing sensitivity and specificity of imaging techniques like EUS and PET-CT in the diagnosis of lymphomas. An emerging field is the molecular imaging with a variety of new radiopharmaceutical agents that target the up-regulated specific receptors in cancer cells^[80].

CONCLUSION

The epidemiology, clinical presentation, histopathologic subtypes, as well as radiological presentation of gastrointestinal lymphomas are highlighted in this review, with emphasis laid on the need for accurate diagnosis, staging, treatment of the disease with the promising novel techniques.

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S- Editor Sun H L- Editor Wang XL E- Editor Lin YP

Association of core promoter mutations of hepatitis B virus and viral load is different in HBeAg(+) and HBeAg(-) patients

Andi Utama, Marlinang Diarta Siburian, Sigit Purwantomo, Mariana Destila Bayu Intan, Tri Shinta Kurniasih, Rino Alvani Gani, Wenny Astuti Achwan, Arnelis, Syafruddin AR Lelosutan, Benyamin Lukito, Tantoro Harmono, Nasrul Zubir, Julius, Soewignjo Soemohardjo, Laurentius Adrianus Lesmana, Ali Sulaiman, Susan Tai

Andi Utama, Marlinang Diarta Siburian, Sigit Purwantomo, Mariana Destila Bayu Intan, Tri Shinta Kurniasih, Susan Tai, Molecular Epidemiology Division, Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan, Lippo Karawaci, Tangerang, 15810, Indonesia

Rino Alvani Gani, Laurentius Adrianus Lesmana, Ali Sulaiman, Hepatology Division, Department of Internal Medicine, Faculty of Medicine, University of Indonesia, Jakarta, 10430, Indonesia

Wenny Astuti Achwan, Soewignjo Soemohardjo, Department of Internal Medicine, Mataram General Hospital, Mataram, 83127, Indonesia

Arnelis, Nasrul Zubir, Julius, Gastroentero-Hepatology Division, Department of Internal Medicine, Dr. M. Jamil Hospital, Padang, 25127, Indonesia

Syafruddin AR Lelosutan, Gastroentero-Hepatology Division, Department of Internal Medicine, Dr. Gatot Soebroto Hospital, Jakarta, 10410, Indonesia

Benyamin Lukito, Department of Internal Medicine, Siloam Hospital Lippo Village, Tangerang, 15810, Indonesia

Tantoro Harmono, Department of Internal Medicine, Dr. Moewardi Hospital, Surakarta, 57126, Indonesia

Author contributions: Utama A, Siburian MD and Purwantomo S designed and performed the experiments; Intan MDB and Kurniasih TS performed some experiments; Tai S was involved in experiment design and editing the manuscript; Gani RA, Achwan WA, Arnelis, Zubir N, Julius, Soemoharjo S, Lelosutan SAR, Lukito B, Harmono T, Lesmana LA and Sulaiman A coordinated and provided the collection of human materials and were involved in editing the manuscript.

Supported by MRIN Funding (Budget No. cc041/2009)

Correspondence to: Andi Utama, PhD, Molecular Epidemiology Division, Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan, Jalan Boulevard Jend. Sudirman 1688, Lippo Karawaci, Tangerang 15810, Banten, Indonesia. autama@mrinstitute.org

Telephone: +62-21-54210123 Fax: +62-21-54210110

Received: September 1, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: February 14, 2011

Abstract

AIM: To identify the prevalence of hepatitis B e antigen (HBeAg) and to assess the association of hepatitis B virus (HBV) core promoter mutations and viral load in Indonesian patients.

METHODS: Sixty-four patients with chronic hepatitis, 65 with liver cirrhosis and 50 with hepatocellular carcinoma were included in this study. HBeAg and hepatitis B e antibody (HBeAb) tests were performed using enzyme-linked immunosorbent assay and the mutations were analyzed by sequencing. Viral load was measured by real-time polymerase chain reaction.

RESULTS: Of 179 patients, 108 (60.3%) were HBeAg(-) and 86 (79.6%) of these HBeAg(-) patients had been seroconverted. The A1896 mutation was not found in HBeAg(+) patients, however, this mutation was detected in 70.7% of HBeAg(-) patients. This mutation was frequently found when HBeAg was not expressed (87.7%), compared to that found in HBeAg seroconverted patients (65.1%). The A1899 mutation was also more prevalent in HBeAg(-) than in HBeAg(+) patients ($P = 0.004$). The T1762/A1764 mutation was frequently found in both HBeAg(+) and HBeAg(-) patients, however, the prevalence of this mutation did not significantly differ among the two groups ($P = 0.054$). In HBeAg(+) patients, the T1762/A1764 mutation was correlated with lower HBV DNA ($P < 0.001$). The A1899 mutation did not correlate with HBV DNA ($P = 0.609$). In HBeAg(-) patients, the T1762/A1764 mutation alone was not correlated with HBV DNA ($P = 0.095$), however, the presence of either the T1762/A1764 or A1896 mutations was associated with increased HBV DNA ($P < 0.001$).

CONCLUSION: The percentage of HBeAg(-) patients is high in Indonesia, and most of the HBeAg(-) patients

had been seroconverted. The A1896 mutation was most likely the major cause of HBeAg loss. The T1762/A1764 mutation alone was associated with lower viral loads in HBeAg(+) patients, but not in HBeAg(-) patients.

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Key words: Hepatitis B e antibody; Hepatitis B e antigen; Hepatitis B virus; Indonesia; Precore/core promoter mutations; Viral load

Peer reviewer: Tong-Chuan He, MD, PhD, Associate Professor, Molecular Oncology Lab, University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637, United States

Utama A, Siburian MD, Purwantomo S, Intan MDB, Kurniasih TS, Gani RA, Achwan WA, Arnelis, Lelosutan SAR, Lukito B, Harmono T, Zubir N, Julius, Soemohardjo S, Lesmana LA, Sulaiman A, Tai S. Association of core promoter mutations of hepatitis B virus and viral load is different in HBeAg(+) and HBeAg(-) patients. *World J Gastroenterol* 2011; 17(6): 708-716 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/708.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.708>

INTRODUCTION

More than 2 billion people are infected with hepatitis B virus (HBV) and 350 million of them are chronic carriers of the virus^[1]. Indonesia has a moderate to high endemicity of HBV infection, which is perhaps due to the lack of proper health facilities, poor economical status and less public awareness^[2]. HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carriers, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)^[3]. Patients with chronic hepatitis B are typically hepatitis B e antigen positive [HBeAg(+)] with detectable HBV DNA in serum. Generally, seroconversion from HBeAg to hepatitis B e antibody (HBeAb) positive correlates with reduced HBV replication in the liver and low infectivity during the natural course of infection^[4,5]. In some patients, however, the immune pressure associated with seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage^[6].

Most infected patients that are HBeAg(-) harbor HBV variants with mutations in the precore or core promoter region^[7]. The predominant precore variation is a G-to-A change at A1896, which creates a premature stop codon and which abolishes the synthesis of HBeAg^[8-10]. The most common core promoter mutations involve a two-nucleotide substitution at T1762 and A1764 (T1762/A1764 mutation)^[7,11]. Several transfection studies showed that the T1762/A1764 mutation decreased the level of precore mRNA by 50% to 70% and led to reduced HBeAg synthesis^[12-14]. A previous study has also demonstrated that HBeAg may be a target antigen on HBV-infected hepatocytes^[15].

Failure to produce a target antigen may allow the infected cell to evade immune clearance.

The prevalence of HBeAg(-) patients is likely to vary across geographic areas. The total number of HBeAg(-) chronic hepatitis B patients is higher in the Mediterranean region and is estimated to be up to 33%. However, the prevalence of HBeAg(-) patients who achieved HBeAg seroclearance was higher in Asian patients (36%) than in Mediterranean patients (24%)^[16]. Furthermore, HBeAg(-) patients have a higher rate of active liver disease in Asian patients, however, no data from Indonesia is available at present. In a recent study, we analyzed the genotype and core promoter mutations of HBV isolates in Indonesian carriers and patients^[17]. The present study identifies the prevalence of HBeAg and assesses the association of HBV core promoter mutations and viral load in Indonesian patients.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 179 HBV-associated liver disease patients, comprising 64 patients with chronic hepatitis B (CH), 65 patients with liver cirrhosis (LC), and 50 patients with HCC. Sera from CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati "Professor Ali Sulaiman", Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Moewardi Hospital, Surakarta, Mataram General Hospital, Mataram, and M. Jamil Hospital, Padang, Indonesia, during the period of May 2006 to March 2010. All sera were persistently seropositive for HBsAg for at least 6 mo. The study was approved by the Institutional Ethics Committee and informed consent was obtained from each patient.

HBeAg and HBeAb tests

HBeAg and HBeAb from all plasma were tested using the MicroLISA™-HBeAg Test and MicroLISA™-HBeAb Test kits (Amgenix, San Jose, CA, USA), according to the manufacturer's instructions.

Analysis of HBV genotype and precore/core promoter mutations

HBV DNA was extracted from 200 µL serum using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80 µL eluted DNA was stored at -70°C until use. HBV genotype was identified based on S gene sequence or genotype-specific polymerase chain reaction (PCR)^[17,18]. Precore and core promoter mutations were analyzed by direct sequencing of the corresponding regions, as described previously^[17]. However, for samples in which the precore sequence appeared inconsistent with the HBeAg test, amplification products were inserted into pBluescript II SK(+), and at least ten independent clones of each were sequenced.

HBV viral load measurement

HBV DNA was first extracted from 200 µL serum with

Table 1 Demographics and characteristics of patients enrolled in this study

Characteristics	Total	CH	LC	HCC	P-value		
					CH vs LC	CH vs HCC	LC vs HCC
n (%)	179 (100.0)	64 (35.8)	65 (36.3)	50 (27.9)	-	-	-
Gender (male/female) (% male)	129/50 (72.1)	38/26 (59.4)	47/18 (72.3)	44/6 (88.0)	0.121	< 0.001	0.040
Age (yr, mean \pm SD)	45.8 \pm 12.3	39.7 \pm 13.3	49.9 \pm 10.8	48.4 \pm 9.5	< 0.001	< 0.001	0.495
AFP [ng/mL, median (min-max)]	13.2 (0.1-3295000.0)	3.2 (0.1-5039.0)	11.8 (1.0-444718.0)	704.8 (1.2-3295000.0)	< 0.001	< 0.001	< 0.001
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	39.5 (7.0-481.0)	66.0 (15.0-297.0)	138.0 (9.0-3618.0)	< 0.001	< 0.001	< 0.001
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47 (6-748)	46 (9-216)	64 (1-860)	0.480	0.481	0.123
AST/ALT	1.28 (0.1-120.6)	0.9 (0.1-2.9)	1.4 (0.2-6.0)	2.1 (0.1-120.6)	< 0.001	< 0.001	< 0.001
Serum HBV DNA (log ₁₀ IU/mL, mean \pm SD)	5.6 \pm 2.0	6.1 \pm 2.1	5.8 \pm 1.4	4.6 \pm 2.2	0.334	< 0.001	0.003
All HBeAg(+), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
All HBeAg(-), (%)	108 (60.3)	28 (43.8)	46 (70.8)	34 (68.0)			
HBeAg(+); HBeAb(-), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
HBeAg(-); HBeAb(+), (%)	86 (48.0)	22 (34.4)	40 (61.5)	24 (48.0)	0.002	0.141	0.039
HBeAg(-); HBeAb(-), (%)	22 (12.3)	6 (9.4)	6 (9.2)	10 (20.0)	0.978	0.105	0.098
Genotype							
B, n (%)	132 (73.7)	47 (73.4)	48 (73.8)	37 (74.0)	0.958	0.946	0.985
C, n (%)	47 (26.3)	17 (26.6)	17 (26.2)	13 (26.0)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α -fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of internal control (1 μ L/10 μ L sample) from ARTUS HBV (Qiagen, Hilden, Germany). From 50 μ L of eluted DNA, 20 μ L was then quantified by ARTUS real-time PCR assay according to the manufacturer's instructions (Qiagen, Hilden, Germany). The range of HBV DNA detection was 10^1 to 10^5 IU/mL.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). Significance differentiations for continuous variables were analyzed using *t*-test analysis. The categorical variables were analyzed using the Fisher's exact test and χ^2 test. *P*-values of < 0.05 were considered significant.

RESULTS

Characteristics of patients

Patients' characteristics are summarized in Table 1. Of the 179 patients, 129 (72.1%) were male and the male/female ratio was significantly increased from CH to HCC and from LC to HCC, but not from CH to LC. The mean age of all patients was 45.8 ± 12.3 years, and significantly increased from CH to LC and to HCC, but not from LC to HCC. The median level of AST and ALT were 66.0 and 50.0 IU/mL, respectively. The level of AST, but not ALT, was significantly increased according to the severity of liver disease. The mean level of HBV DNA was 5.6 ± 2.0 log₁₀ IU/mL, and was significantly lower in HCC (4.6 ± 2.2) than in LC (5.8 ± 1.4) and CH (6.1 ± 2.1). Seventy-one (39.7%) and 108 (60.3%) of patients were HBeAg(+) and HBeAg(-), respectively. The percentage of HBeAg(+) samples was significantly higher in CH (56.3%) than in

LC (29.2%) or HCC (32.0). Eighty-six of 179 patients (48.0%) were HBeAg seroconverted, and the percentage of HBeAg seroconversion was higher in LC (61.5%) than HCC (48.0%) and CH (34.4%). There was no difference in HBV genotype prevalence between samples of different clinical diagnosis.

Comparison between HBeAg(+) and HBeAg(-)

Table 2 demonstrates the comparison between HBeAg(+) and HBeAg(-) groups. The mean age of patients was significantly higher in the HBeAg(-) group (49.2 ± 11.7) than in the HBeAg(+) group (40.7 ± 11.5) ($P < 0.001$), indicating a longer period of disease in the HBeAg(-) group. No significant difference in the male/female ratio between the two groups was observed ($P = 0.106$). Surprisingly, there was also no significant difference in ALT levels between the two groups ($P = 0.535$). As shown in Table 3, 79.6% (86/108) of HBeAg(-) patients were HBeAg seroconverted [HBeAb(+)]. There was no significant difference in ALT levels in the samples prior to HBeAg seroconversion [i.e. HBeAg(+), HBeAb(-)] vs after HBeAg seroconversion [i.e. HBeAg(-), HBeAb(+)] ($P = 0.200$). However, ALT levels in the HBeAg seroconverted group were significantly higher than those in the group that did not express HBeAg (60.0 IU/L vs 43.0 IU/L, $P = 0.023$). The AST/ALT ratio was significantly higher in HBeAg(-) than in HBeAg(+) patients (1.4 vs 1.1, $P = 0.022$), suggesting the presence of other factors involved in HBeAg(-) patients. HBV DNA was significantly higher in the HBeAg(+) (6.5 ± 1.8 log₁₀ IU/mL) group compared to the HBeAg(-) (5.0 ± 1.9 log₁₀ IU/mL) group ($P < 0.001$) (Table 2). The percentage of samples with HBV DNA load ≥ 20000 IU/mL was much higher in HBeAg(+) patients (91.5%) than in HBeAg(-) patients (68.5%). There was no significant difference in levels of HBV DNA between the HBeAg sero-

Table 2 Comparison of hepatitis B e antigen (+) and hepatitis B e antigen (-) patients

Characteristics	Total	HBeAg(+)	HBeAg(-)	P-value
n (%)	179 (100.0)	71 (39.7)	108 (60.3)	-
Gender (male/female) (% male)	129/50 (72.1)	47/24 (66.2)	82/26 (75.9)	0.106
Age (yr, mean \pm SD)	45.8 \pm 12.3	40.7 \pm 11.5	49.2 \pm 11.7	< 0.001
AFP [ng/mL, median (min-max)]	13.2 (0.1-3295000.0)	10.9 (0.2-230472.0)	19.9 (0.1-3295000.0)	0.008
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	55.0 (12.0-635.0)	71.0 (7.0-3618.0)	0.028
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47.0 (1.0-748.0)	52.0 (6.0-860.0)	0.535
AST/ALT	1.3 (0.1-120.6)	1.1 (0.1-30.8)	1.4 (1.4-120.6)	0.022
Serum HBV DNA (log ₁₀ IU/mL, mean \pm SD)	5.6 \pm 2.0	6.5 \pm 1.8	5.0 \pm 1.9	< 0.001
< 20000 IU/mL, n (%)	40 (22.3)	6 (8.5)	34 (31.5)	< 0.001
\geq 20000 IU/mL, n (%)	139 (77.7)	65 (91.5)	74 (68.5)	
Clinical status				
CH, n (%)	64 (35.8)	36 (50.7)	28 (25.9)	0.003
LC, n (%)	65 (36.3)	19 (26.8)	46 (42.6)	
HCC, n (%)	50 (27.9)	16 (22.5)	34 (31.5)	
Genotype				
B, n (%)	132 (73.7)	50 (70.4)	82 (75.9)	0.259
C, n (%)	47 (26.3)	21 (29.6)	26 (24.1)	

CH: Chronic hepatitis; LC: Liver cirrhosis; AFP: α -fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

Table 3 Comparison of patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion

Characteristics	Group 1	Group 2	Group 3	P-value		
	HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	1 vs 2	1 vs 3	2 vs 3
n (%)	71 (39.7)	86 (48.0)	22 (12.3)	-	-	-
Gender (male/female) (% male)	47/24 (66.2)	71/15 (82.6)	11/11 (50.0)	0.018	0.171	0.001
Age (yr, mean \pm SD)	40.7 \pm 11.5	49.8 \pm 11.9	46.9 \pm 10.6	< 0.001	0.028	0.296
AFP [ng/mL, median (min-max)]	10.9 (0.2-230472.0)	17.1 (0.1-3295000.0)	40.8 (0.4-514412.0)	0.016	0.048	0.731
AST [IU/L, median (min-max)]	55.0 (12.0-635.0)	68.0 (7.0-3618.0)	76.5 (9.0-580.0)	0.017	0.536	0.448
ALT [IU/L, median (min-max)]	47.0 (1.0-748.0)	60.0 (9.0-860.0)	43.0 (6.0-154.0)	0.200	0.173	0.023
AST/ALT	1.1 (0.1-30.8)	1.3 (0.1-120.6)	1.7 (0.5-6.0)	0.091	0.007	0.052
Serum HBV DNA (log ₁₀ IU/mL, mean \pm SD)	6.5 \pm 1.8	5.1 \pm 1.8	4.5 \pm 2.4	< 0.001	< 0.001	0.146
< 20000 IU/mL, n (%)	6 (8.5)	25 (29.1)	9 (40.9)	0.001	< 0.001	0.286
\geq 20000 IU/mL, n (%)	65 (91.5)	61 (70.9)	13 (59.1)			
Clinical status						
CH, n (%)	36 (50.7)	22 (25.6)	6 (27.3)	0.001	0.054	0.871
LC, n (%)	19 (26.8)	40 (46.5)	6 (27.3)	0.011	0.963	0.104
HCC, n (%)	16 (22.5)	24 (27.9)	10 (45.4)	0.442	0.036	0.114
Genotype						
B, n (%)	50 (70.4)	66 (76.7)	16 (72.7)	0.369	0.835	0.694
C, n (%)	21 (29.6)	20 (23.3)	6 (27.3)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α -fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

converted and non-expressing groups ($P = 0.146$) (Table 3), suggesting that HBeAg expression did not affect HBV replication. The percentage of HBeAg(+) samples was high in CH (50.7%) and was less in LC (26.8%) and HCC (22.5%).

Precore/core promoter mutations and HBeAg status

The prevalence of mutations in the precore and core promoter regions was compared between HBeAg(+) and HBeAg(-) patients (Table 4). As expected, the precore A1896 mutation, which is associated with HBeAg expression, was absent in HBeAg(+) patients, but was found in 70.7% of HBeAg(-) patients. Interestingly, the A1896

mutation was frequently found not only in HBeAg non-expressing patients (87.7%), but also in HBeAg seroconverted patients (65.1%). The A1899 precore mutation was found to be more common in HBeAg(-) than in HBeAg(+) patients (34.5% *vs* 10.4%, $P = 0.004$), but there was no significant difference between the HBeAg seroconverted group and the HBeAg non-expressing group (37.2% *vs* 26.7%, $P = 0.116$), suggesting that this mutation contributes to the expression of HBeAg. On the other hand, the T1762/A1764 core promoter mutation was found both in HBeAg(+) (40.8%) and HBeAg(-) (55.6%) patients, and the prevalence of this mutation was not significantly different between the two groups ($P = 0.054$).

Table 4 Precore and core promoter mutations in patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion *n* (%)

	All	Group 1	Group 2	Group 3	Group 2+3	P-value			
		HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	HBeAg(-)	1 vs 2	1 vs 3	2 vs 3	1 vs (2+3)
T1762/A1764 ¹									
Absent	90 (50.3)	42 (59.2)	38 (44.2)	10 (45.5)	48 (44.4)	0.062	0.258	0.915	0.054
Present	89 (49.7)	29 (40.8)	48 (55.8)	12 (54.5)	60 (55.6)				
A1896 ²									
Absent	65 (61.3)	48 (100.0) ³	15 (34.9)	2 (13.3)	17 (29.3)	< 0.001	< 0.001	0.114	< 0.001
Present	41 (38.7)	0 (0.0)	28 (65.1)	13 (87.7)	41 (70.7)				
A1899 ²									
Absent	81 (76.4)	43 (89.6)	27 (62.8)	11 (73.3)	38 (65.5)	0.003	0.034	0.116	0.004
Present	25 (23.6)	5 (10.4)	16 (37.2)	4 (26.7)	20 (34.5)				
T1762/A1764 and A1896 ²									
Absent	88 (83.0)	48 (100.0)	31 (72.1)	9 (60.0)	40 (68.9)	< 0.001	< 0.001	0.383	< 0.001
Present	18 (17.0)	0 (0.0)	12 (27.9)	6 (40.0)	18 (31.0)				
T1762/A1764 or A1896 ²									
Absent	33 (31.1)	31 (64.6)	2 (4.7)	0 (0.0)	2 (1.9) ⁴	< 0.001	< 0.001	0.395	< 0.001
Present	73 (68.9)	17 (35.4)	41 (95.3)	15 (100.0)	56 (98.1)				

¹Total samples *n* = 179; Hepatitis B e antigen (HBeAg) (+) and hepatitis B e antibody (HBeAb) (-) *n* = 71; HBeAg(-) and HBeAb(+), *n* = 86; HBeAg(-) and HBeAb(-), *n* = 22; ²Total samples *n* = 106; HBeAg(+) and HBeAb(-) *n* = 48; HBeAg(-) and HBeAb(+), *n* = 43; HBeAg(-) and HBeAb(-), *n* = 15; ³Three samples had a mixed population of precore stop codon mutation (A1896) and its wild type (G1896); ⁴Two samples with a mixed population of basal core promoter mutation (T1762/A1764) and its wild type (A1762/G1764).

However, the presence of either the T1762/A1764 mutation or the A1896 mutation was very high in the HBeAg(-) group (98.1%), [including patients that had seroconverted (HBeAb(+)) (95.3%) and HBeAg non-expressing patients (100.0%), compared to the HBeAg(+) group (35.4% of which had the mutations), indicating these mutations are associated with HBeAg status.

We further analyzed the effect of precore and core promoter mutations on HBeAg expression and viral replication in twenty two HBeAg non-expressed samples. Enough DNA was recovered so that the T1762/A1764 core promoter mutation could be analyzed in all samples, however, the A1896 and A1899 precore mutations could only be analyzed in fifteen samples (Table 5). Either core promoter (T1762/A1764) or precore (A1896 or A1899) mutations were found in all samples, with the exception of three samples from which we were unable to obtain the DNA sequence. Almost all samples demonstrated relatively high HBV DNA, although in some samples the HBV DNA was low, but still detectable. Taken together, these results suggest that precore or core promoter mutations were associated with reduced HBeAg expression, but did not affect HBV replication in groups that did not express HBeAg.

Table 5 Precore and core promoter mutations in samples with hepatitis B e antigen (-) and hepatitis B e antibody (-)

No.	Sample ID	T1762/ A1764	A1896	A1899	Serum HBV DNA (log ₁₀ IU/mL)
1	07.10.068	Yes	No	No	4.59
2	08.70.091	Yes	No	No	4.87
3	08.100.038	Yes	Yes	No	6.43
4	07.10.121	Yes	Yes	No	7.52
5	08.10.002	Yes	Yes	No	7.18
6	09.41.591	Yes	Yes	Yes	6.53
7	09.40.037	Yes	Yes	Yes	5.62
8	10.80.004	Yes	Yes	No	2.78
9	06.10.062	No	Yes	No	3.32
10	08.10.086	No	Yes	No	4.94
11	09.40.033	No	Yes	No	7.28
12	09.80.040	No	Yes	No	4.05
13	09.41.806	No	Yes	Yes	7.42
14	07.10.070	No	Yes	Yes	4.72
15	09.80.037	No	Yes	Yes	6.78
16	08.10.016	Yes	NA	NA	5.21
17	08.10.020	Yes	NA	NA	-0.37
18	08.10.039	Yes	NA	NA	3.10
19	P.X00.34	Yes	NA	NA	-0.54
20	07.10.117	No	NA	NA	1.73
21	07.10.026	No	NA	NA	1.27
22	07.10.173	No	NA	NA	3.48

HBV: Hepatitis B virus.

Precore/core promoter mutations and HBV viral load and ALT

Precore and core promoter mutations correlated with different levels of HBV DNA in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, the T1762/A1764 core promoter mutation correlated with lower HBV DNA levels ($P < 0.001$) (Table 6). The A1899 mutation was not associated with HBV DNA level ($P = 0.609$) and, as expected, no A1896 mutations were detected in the samples. The

presence of either the T1762/A1764 or A1896 mutations also correlated with lower HBV DNA levels ($P = 0.011$). On the other hand, in HBeAg(-) patients, T1762/A1764 core promoter, as well as A1896 and A1899 precore mutations were not individually correlated with higher or lower HBV DNA level ($P = 0.095, 0.231, 0.382$, respectively), however, the presence of either the T1762/A1764 muta-

Table 6 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (+) patients

	Serum HBV DNA (log ₁₀ IU/mL) (mean ± SD) (n)		P-value	ALT (IU/L) [median (min-max)] (n)		P-value
	Absent	Present		Absent	Present	
T1762/ A1764 ¹	7.14 ± 1.46 (42)	5.60 ± 1.99 (29)	< 0.001	43.5 (13.0-748.0) (42)	48.5 (1.0-215.0) (29)	0.806
A1896 ²	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
A1899 ²	7.01 ± 1.25 (43)	6.68 ± 1.53 (5)	0.609	45.5 (1.0-748.0) (43)	55.0 (36.0-92.0) (5)	0.627
T1762/ A1764 and A1896 ²	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
T1762/ A1764 or A1896 ²	7.33 ± 1.23 (31)	6.33 ± 1.08 (17)	0.011	50.0 (15.0-748.0) (31)	41.5 (1.0-117.0) (17)	0.459

¹Total samples hepatitis B e antigen (HBeAg) (+) *n* = 71; ²Total samples HBeAg(+) *n* = 48. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Table 7 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (-) patients

	Serum HBV DNA (log ₁₀ IU/mL) (mean ± SD) (n)		P-value	ALT (IU/L) [median (min-max)] (n)		P-value
	Absent	Present		Absent	Present	
T1762/ A1764 ¹	4.64 ± 2.00 (48)	5.23 ± 1.79 (60)	0.095	57.0 (6.0-302) (48)	51.5 (6.0-860.0) (60)	0.885
A1896 ²	5.44 ± 1.66 (17)	5.91 ± 1.35 (41)	0.231	46.0 (15.0-860.0) (17)	63.5 (14.0-302.0) (41)	0.321
A1899 ²	5.80 ± 1.29 (38)	5.72 ± 1.76 (20)	0.382	52.0 (14.0-860.0) (38)	58.5 (17.0-302.0) (20)	0.693
T1762/ A1764 and A1896 ²	5.59 ± 1.51 (40)	6.17 ± 1.27 (18)	0.138	65.0 (14.0-860.0) (40)	51.0 (17.0-174.0) (18)	0.543
T1762/ A1764 or A1896 ²	2.77 ± 4.31 (2)	5.88 ± 1.23 (56)	< 0.001	59.5 (23.0-96.0) (2)	52.0 (42.0-860.0) (56)	0.847

¹Total samples hepatitis B e antigen (HBeAg) (-) *n* = 108; ²Total samples HBeAg(-) *n* = 58. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

tion or the A1896 mutation was associated with increased HBV DNA levels (*P* < 0.001) (Table 7). In addition, no correlations between precore and core mutations and serum ALT levels were observed in either the HBeAg(+) or HBeAg(-) patients (Tables 6 and 7).

DISCUSSION

The present study was an epidemiological investigation of the precore and core promoter mutations and their relationship to HBeAg expression levels in Indonesian patients. The majority of patients enrolled in this study were infected with HBV genotype B (73.7%), while the rest were genotype C (26.3%) (Table 1), which is consistent with previous reports^[17,19-21]. The prevalence of HBeAg(-) chronic hepatitis B patients was 60.3%, which is similar to other Asian countries^[16]. Among the HBeAg(-) patients, 79.6% were HBeAb(+), which meant that they were HBeAg seroconverted (Table 3). Studies in Europe, Asia, and the United States have all reported an increased prevalence of HBeAg(-) chronic hepatitis among HBeAg(+) patients^[16]. Our results support previous studies which reported that HBeAg(-) chronic hepatitis B is the most common form of chronic HBV infection in Asia. However, in our study, most of the HBeAg(-) patients had been seroconverted, and the percentage of HBeAg(-) due to abolition of HBeAg synthesis was relatively low (20.4%).

Analysis of the A1896 mutation by direct sequencing demonstrated that all HBeAg(+) samples were wild type (i.e. did not bear the A1896 mutation) (Table 4), however, in our initial sequencing the A1896 mutation was found in three HBeAg(+) samples. The PCR fragments from

these samples were cloned into pBluescript II SK(+), and sequence analysis of ten clones showed that wild type virus was also present in some isolates. Thus, the presence of these wild type viruses was presumably responsible for HBeAg synthesis. On the other hand, a high percentage of the HBeAg(-) patients bore the A1896 precore mutation. As expected, in HBeAg(-) patients, this mutation was more prevalent in HBeAg non-expressing patients (87.7%) compared to seroconverted patients (65.1%) (Table 4). Nevertheless, the percentage of this mutation in seroconverted patients was relatively high. Since the A1896 mutation creates a premature stop codon which results in abolition of HBeAg synthesis^[8-10], seroconverted patients cannot have always had virus with the A1896 mutation. It is believed that precore mutants emerge as a result of selection under immune pressure during the process of HBeAg seroconversion^[22-24]. Therefore, at the early stage of infection, the virus might be a wild-type and the A1896 mutation occurs during the process of HBeAg seroconversion. In this study, because the mutation analysis was carried out after HBeAg seroconversion, it is also possible that the A1896 mutation detected in the samples occurred during the process of HBeAg seroconversion. In addition, the prevalence of the A1899 precore mutation was significantly higher in HBeAg(-) than in HBeAg(+) patients (*P* = 0.004), and was also higher in samples before HBeAg seroconversion compared to that after HBeAg seroconversion (*P* = 0.003) (Table 4). These results suggest that the A1899 mutation is associated with expression and seroconversion of HBeAg, which is in accordance with previous studies in Taiwanese patients^[25]. Another study from Korea also reported that the A1899 mutation

was frequently found in HBeAg(-) patients, however, the authors found that the A1899 mutation was always accompanied by A1896 mutation^[26], which was different to our results.

The frequency of the T1762/A1764 mutation was relatively high both in HBeAg(+) and HBeAg(-) patients, and there was no significant difference between the two groups or between patients before and after HBeAg seroconversion. These results suggest that there is no independent association between the T1762/A1764 mutation on HBeAg expression and seroconversion. The presence of both the T1762/A1764 and A1896 mutations correlated with HBeAg expression and seroconversion, but was likely due to the effect of A1896 mutation alone. Although previous studies demonstrated that the core promoter region regulates transcription of the pregenomic and precore RNA and T1762/A1764 mutation suppression, it does not abolish the synthesis of HBeAg, leading to a reduction in HBeAg expression^[12,27-29], and we did not observe this phenomenon in our study. Moreover, by measuring HBeAg titer quantitatively, a recent study also reported that the T1762/A1764 mutation reduced the expression of HBeAg^[30]. In this study, however, HBeAg analysis was performed qualitatively, which perhaps explains the different results from those of previous studies.

Further analysis of precore and core promoter mutations in HBeAg non-expressed patients revealed that the A1896 mutation alone did not abolish HBeAg synthesis. Theoretically, A1896 mutation creates a premature stop codon which results in abolishment of HBeAg synthesis, however, A1896 was not found in all HBeAg non-expressed patients. Among twenty-two patients with HBeAg(-) and HBeAb(-), we identified the A1896 mutation in fifteen patients of which two did not show the A1896 mutation, but showed the T1762/A1764 mutation (Table 5). To confirm the A1896 mutation in these two samples, we cloned the amplified fragments into the plasmid, and sequenced eighteen clones, and no mutations were found in any of the clones. Furthermore, the A1899 mutation was not found in these samples. These results suggest that the expression of HBeAg might not be affected by the A1896 mutation alone, however, further study is needed to investigate other factors involved in HBeAg expression.

In addition to precore and core promoter mutation, HBV variants with point mutations around the Kozak sequence (nucleotides 1809-1812) were also analyzed. Mutations at the Kozak sequence were less common in our samples and were not associated with HBeAg expression (data not shown), which is similar to the results from Korea^[31]. Some studies have found that mutations in the Kozak sequence are correlated with HBeAg expression^[32-34]. Our study demonstrated that these mutations do not play an important role in the clinical outcome of chronic hepatitis B patients. The differences may be attributable to genetic differences.

Another interesting finding of this study is that the effect of precore and core promoter mutation on HBV viral

load was different in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, T1762/A1764 mutation was associated with lower viral load. On the other hand, this mutation was associated with higher viral load in HBeAg(-) patients, including HBeAg seroconverted patients. These results are in accordance with a previous study in Chinese patients^[35,36]. It may be postulated that, among individuals who are HBeAg(+), those with both wild-type and mutant viruses have them in different phases of the infection, the former being in the first phase and the latter, at the end of the second phase. These patients experience different immune pressures, resulting in different levels of virus replication. However, those who are HBeAg(-), regardless of core promoter sequence, are in the same phase (the third phase) and experience similar immune pressures, resulting in similar levels of virus replication. If this is the case, it is not difficult to understand why T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but have no effect in HBeAg(-) patients.

In conclusion, the percentage of HBV infected HBeAg(-) patients is relatively high in Indonesia. Most of the HBeAg(-) patients had been seroconverted and the remaining patients did not express HBeAg. A1896 mutation in the precore region was the major cause of the loss of HBeAg expression. T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but are associated with higher viral loads in HBeAg(-) patients.

ACKNOWLEDGMENTS

The authors appreciate the assistance of Rama Dhenni, Griskalia Christine and Shinta Soraya in sample collection. The authors also appreciate Dr. David Vaux (La Trobe University, Australia) for critical reading.

COMMENTS

Background

Seroconversion from hepatitis B e antigen (HBeAg)-positive to hepatitis B e antibody (HBeAb)-positive correlates with reduced hepatitis B virus (HBV) replication in the liver and low infectivity during the natural course of infection. However, the immune pressure associated with HBeAg seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage and is known as HBeAg-negative chronic hepatitis B. The aims of the study were to identify the prevalence of HBeAg-negative patients and to assess the association between HBV core promoter mutations and viral load in Indonesian patients.

Research frontiers

To date, there have been no reports on the prevalence of HBeAg-negative chronic hepatitis B in Indonesia. Therefore, it is important to obtain information on the HBeAg status of liver disease patients in Indonesia, and its association with precore and core promoter mutations. In addition, the correlation between precore and core promoter mutations and HBV replication is crucial.

Innovations and breakthroughs

The present study showed that the prevalence of HBeAg-negative chronic hepatitis B in Indonesia is high and most patients had seroconverted. The A1896 mutation was most likely to be the major cause of HBeAg loss. A1899 mutation is also associated with HBeAg-negative and is not always accompa-

nied by A1896 mutation. Furthermore, A1896 was not found in all HBeAg non-expressing patients, two HBeAg(-) and HBeAb(-) patients did not show either the A1896 or A1899 mutation, but had the T1762/A1764 mutation, suggesting that the expression of HBeAg might not be affected by the A1896 mutation alone. Interestingly, the T1762/A1764 mutation was associated with lower viral loads in HBeAg-positive, but not in HBeAg-negative patients.

Applications

The A1896 mutation can be used to predict HBeAg-negative variants in patients who achieve HBeAg seroconversion during disease progression or anti-viral therapy. In addition, the A1899 and T1762/A1764 mutations can be used to predict HBeAg-negative chronic hepatitis B, although there is no A1896 mutation.

Terminology

Core promoter: Part of the HBx gene that regulate the HBe and core gene expression. HBeAg seroconversion: The clearance of HBeAg by the production of HBeAb.

Peer review

The reported work is very intriguing and represents a large undertaking.

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Glutamine depletion induces murine neonatal melena with increased apoptosis of the intestinal epithelium

Takayuki Motoki, Yoshio Naomoto, Junji Hoshiba, Yasuhiro Shirakawa, Tomoki Yamatsuji, Junji Matsuoka, Munenori Takaoka, Yasuko Tomono, Yasuhiro Fujiwara, Hiroshi Tsuchita, Mehmet Gunduz, Hitoshi Nagatsuka, Noriaki Tanaka, Toshiyoshi Fujiwara

Takayuki Motoki, Yoshio Naomoto, Yasuhiro Shirakawa, Tomoki Yamatsuji, Junji Matsuoka, Munenori Takaoka, Yasuhiro Fujiwara, Noriaki Tanaka, Toshiyoshi Fujiwara, Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, 2-5-1, Shikata-cho, Okayama, 700-8558, Japan

Yoshio Naomoto, Department of General Surgery, Kawasaki Medical School Kawasaki Hospital, 2-1-80, Nakasange, Kita-ku, Okayama, 700-8505, Japan

Junji Hoshiba, Department of Animal Resources, Advanced Science Research Center, Okayama University, 2-5-1, Shikata-cho, Okayama, 700-8558, Japan

Yasuko Tomono, Division of Molecular and Cell Biology, Shigei Medical Research Institute, 2117 Yamada, Okayama, 701-0202, Japan

Hiroshi Tsuchita, Food Technology Research Institute, Meiji Dairies Corporation, 540 Naruda, Odawara, Kanagawa, 250-0862, Japan

Mehmet Gunduz, Hitoshi Nagatsuka, Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, 2-5-1, Shikata-cho, Okayama, 700-8558, Japan

Author contributions: Motoki T, Naomoto Y, Matsuoka J, Yamatsuji T, Shirakawa Y, Takaoka M and Tanaka N conceived and designed the study; Motoki T, Naomoto Y, Nagatsuka H and Fujiwara T conducted the study; Hoshiba J produced the feeding bottles; Tsuchita H synthesized the milk; Tomono Y performed the Western blotting; Motoki T and Fujiwara Y statistically analyzed the data; Motoki T, Naomoto Y, Matsuoka J, Yamatsuji T, Shirakawa Y, Takaoka M and Gunduz M wrote the manuscript; all authors were responsible for data interpretation.

Supported by The trust accounts of the Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University

Correspondence to: Yoshio Naomoto, MD, PhD, Professor of Department of General Surgery, Kawasaki Medical School Kawasaki Hospital, 2-1-80, Nakasange, Kita-ku, Okayama, 700-8505, Japan. ynaomoto@med.kawasaki-m.ac.jp
 Telephone: +81-86-2252111 Fax: +81-86-2328343

Received: June 20, 2010 Revised: September 17, 2010

Accepted: September 24, 2010

Published online: February 14, 2011

Abstract

AIM: To investigate the possible biological outcome and effect of glutamine depletion in neonatal mice and rodent intestinal epithelial cells.

METHODS: We developed three kinds of artificial milk with different amounts of glutamine; Complete amino acid milk (CAM), which is based on maternal mouse milk, glutamine-depleted milk (GDM), and glutamine-rich milk (GRM). GRM contains three-fold more glutamine than CAM. Eighty-seven newborn mice were divided into three groups and were fed with either of CAM, GDM, or GRM *via* a recently improved nipple-bottle system for seven days. After the feeding period, the mice were subjected to macroscopic and microscopic observations by immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) and Ki-67 as markers of cell proliferation, and for cleaved-caspase-3 as a marker of apoptosis. Moreover, IEC6 rat intestinal epithelial cells were cultured in different concentrations of glutamine and were subject to a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate cell proliferation assay, flow cytometry, and western blotting to examine the biological effect of glutamine on cell growth and apoptosis.

RESULTS: During the feeding period, we found colonic hemorrhage in six of 28 GDM-fed mice (21.4%), but not in the GRM-fed mice, with no differences in body weight gain between each group. Microscopic examination showed destruction of microvilli and the disappearance of glycocalyx of the intestinal wall in the colon epithelial tissues taken from GDM-fed mice. Intake of GDM reduced BrdU incorporation (the average percentage of BrdU-positive staining; GRM: 13.8%, CAM: 10.7%, GDM: 1.14%, GRM *vs* GDM: $P < 0.001$, CAM *vs* GDM: $P < 0.001$) and Ki-67 labeling index (the average percentage of Ki-67-positive staining; GRM: 24.5%, CAM: 22.4% GDM: 19.4%, GRM *vs* GDM: $P = 0.001$, CAM *vs* GDM: $P =$

0.049), suggesting that glutamine depletion inhibited cell proliferation of intestinal epithelial cells. Glutamine deprivation further caused the deformation of the nuclear membrane and the plasma membrane, accompanied by chromatin degeneration and an absence of fat droplets from the colonic epithelia, indicating that the cells underwent apoptosis. Moreover, immunohistochemical analysis revealed the appearance of cleaved caspase-3 in colonic epithelial cells of GDM-fed mice. Finally, when IEC6 rat intestinal epithelial cells were cultured without glutamine, cell proliferation was significantly suppressed after 24 h (relative cell growth; 4 mmol/L: 100.0% \pm 36.1%, 0 mmol/L: 25.3% \pm 25.0%, $P < 0.05$), with severe cellular damage. The cells underwent apoptosis, accompanied by increased cell population in sub-G0 phase (4 mmol/L: 1.68%, 0.4 mmol/L: 1.35%, 0 mmol/L: 5.21%), where dying cells are supposed to accumulate.

CONCLUSION: Glutamine is an important alimentary component for the maintenance of intestinal mucosa. Glutamine deprivation can cause instability of the intestinal epithelial alignment by increased apoptosis.

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Key words: Glutamine; Newborn mice; Artificial milk; Melena; Intestinal epithelial cells; Apoptosis

Peer reviewer: Julio Mayol, MD, PhD, Department of Digestive surgery, Hospital Clinico San Carlos, MARTIN-LAGOS S/n, Madrid, 28040, Spain

Motoki T, Naomoto Y, Hoshiba J, Shirakawa Y, Yamatsuji T, Matsuoka J, Takaoka M, Tomono Y, Fujiwara Y, Tsuchita H, Gunduz M, Nagatsuka H, Tanaka N, Fujiwara T. Glutamine depletion induces murine neonatal melena with increased apoptosis of the intestinal epithelium. *World J Gastroenterol* 2011; 17(6): 717-726 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/717.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.717>

INTRODUCTION

Amino acids have various roles in the living body; however, their cytobiological functions associated with cell proliferation or immune responses have not been fully elucidated. Among them, glutamine, which is the most abundant amino acid in the human body, has been recognized as a conditional essential amino acid in critical illness, stress, and injury^[1-4]. Glutamine also has a key role in intermediary metabolism for rapidly dividing cells, such as enterocytes and cells of the immune system^[1,5]. Indeed, the small intestine accounts for the largest uptake of glutamine of any organ, absorbing this amino acid from the lumen of the gut, as well as from the bloodstream^[6]. Illness or injury can lead to a significant decrease in plasma levels of glutamine, and when this decrease is severe, it correlates with increased mortality^[7,8]. Several studies have demonstrated the efficacy of either enteral or parenteral

glutamine in adults and infants with a variety of conditions, such as bone marrow transplantation, critical illness, burns, trauma, surgically treated patients, and very low birth weight infants^[9,10]. Thus, glutamine appears especially important for susceptible individuals who are in high stress conditions.

In the past, it was difficult to identify the roles played by these amino acids *in vivo*. Past studies involved the evaluation of immunological effects of amino acids by the administration of a diet rich or deficient in each amino acid (glutamine, arginine, *etc.*) by means of total parenteral nutrition or stomach tubing^[11]. However, no such study in newborn mice has yet been reported. The lack of such a study in newborn mice is attributable to the fact that it is quite difficult to develop a method of alimentation or to prepare a diet for neonatal mice. To overcome these issues, Yajima *et al.*^[12] have recently developed artificial milk for mice with a composition very close to that of mouse maternal milk. Subsequently, artificial milk for mice with all of the proteins broken down into amino acids was developed. We expected that the use of this milk would make it possible for us to feed newborn mice with milk either deficient or enriched in certain amino acids. In addition, Hoshiba established and reported a new system for the artificial alimentation of rats and mice immediately after birth^[13]. With this system, it is possible to administer certain nutrients orally to newborn mice and rats and to evaluate the effects of these nutrients directly.

In this study, we utilized the above improved special milk and feeding device to achieve the following scientific aims: (1) to explore what happens when newborn mice are fed with glutamine-devoid milk; and (2) to dissect the physiological mechanism of glutamine deprivation-oriented events.

MATERIALS AND METHODS

Bottle-nipple system

The nursing bottle and nipple, which were made by Hoshiba^[13], were used to feed the newborn mice. The nursing bottle has three tubes (a filling tube, an outlet tube, and a ventilation tube) and the nipples are made of a silicone rubber. The nipple consists of inner and outer parts, and a stopper devised to control the pressure as well as to avoid milk leakage.

Animals and study design

Specific-pathogen-free Jcl:ICR pregnant mice were purchased from Charles River Laboratories (Yokohama, Japan). They were maintained under the following environmental conditions: lighting, 12:12-h light:dark cycle; temperature, 22 to 25°C; air changes, 12 to 14 times per hour; and humidity, 40% to 50%. Newborn pups were separated from each dam immediately after birth. They were reared with artificial milk from the nursing bottle four times per day (09:00, 12:30, 16:00, and 20:00) for seven days and sacrificed on day eight. Body weights of the pups were measured before and after feeding, and the difference between them was represented the quantity of feeding.

Table 1 Amino acid components of complete amino acid milk

Amino acid	mg/100 mL of milk ¹
Valine	0.60 ± 0.02
Leucine	1.06 ± 0.04
Isoleucine	0.48 ± 0.04
Lysine	0.88 ± 0.06
Threonine	0.47 ± 0.02
Methionine	0.34 ± 0.04
Histidine	0.25 ± 0.03
Phenylalanine	0.51 ± 0.16
Tryptophan	0.19 ± 0.01
Alanine	0.40 ± 0.00
Arginine	0.32 ± 0.01
Glutamine	2.09 ± 0.20
Proline	0.74 ± 0.15
Cystine	0.08 ± 0.00
Tyrosine	0.56 ± 0.05
Asparagine	0.91 ± 0.07
Glycine	0.19 ± 0.00
Serine	0.53 ± 0.01

¹The amount of each amino acid is presented as average ± SE (mg/100 mL of milk).

Milk formula

Artificial milk was purchased from Meiji Dairies Corporation (Odawara, Japan). The amino acid composition of the milk is shown in Table 1. The composition of the milk was made as similar as possible to mouse maternal milk. Table 2 shows the composition of mouse milk and artificial amino acid milk. The artificial amino acid milk showed an extremely high osmotic pressure compared to that of mouse maternal milk, because the proteins in the milk used in this study were in the form of amino acids with a low amount of fat. Thus, 10% lipid microsphere Intralipid (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) was added to this artificial amino acid milk at a ratio of 1:1 to yield the complete amino acid milk, thus the amount of amino acid contained in this milk was 50% of the initial amount with 16% fat, similar with that of mouse milk (22%). We named this milk complete amino acid milk (CAM). Furthermore, glutamine rich milk (GRM) had three-fold more glutamine than CAM, whereas glutamine-depleted milk (GDM) contained no glutamine.

Histological study

Resected tissues from the mice were fixed in 10% formalin, embedded in paraffin, and cut at a thickness of 5 µm. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. On day eight, the animals were perfused with 2% glutaraldehyde (5 mL) and fixed, followed by the removal of each organ for subsequent thin slicing and observation under an electron microscope.

Immunohistochemistry

To detect cells synthesizing DNA, 5-bromo-2'-deoxyuridine (BrdU) was injected (100 mg/kg i.p.) 1 h before sacrificing the animals. The organs were then placed in 4% paraformaldehyde for 48 h prior to paraffin embedding. BrdU incorporation in colon sections was determined by immunohistochemical staining. Briefly, sections were

deparaffinized with xylene (Mallinckrodt Baker, Paris, Kentucky) and taken through a graded series of alcohol/water mixtures to rehydrate the tissue. To retrieve the antigen, sections were incubated with 2 mol/L HCl for 30 min and then incubated with 0.1 mol/L Na₂VO₄O₇ at room temperature for 30 s. Sections were exposed to rat anti-BrdU monoclonal antibodies (OBT, Oxford, UK) for 60 min at room temperature. Peroxidase-conjugated anti-mouse IgG₁ antibody (DAKO, Carpinteria, CA) was then applied, and 3,3'-diaminobenzidine chromogen was added as the peroxidase substrate. A light counterstain of modified Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan) was then applied so that unlabeled nuclei could be easily identified. Immunostaining for Ki-67 and cleaved caspase-3, the biological markers for cell proliferation and apoptosis, respectively, used a rabbit anti-Ki-67 monoclonal antibody (Ylem, Rome, Italy) and a rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA), respectively.

Cell culture and treatments

There was no commercially available mouse intestinal epithelial cell line; therefore, we used IEC6 rat intestinal epithelial cells, which were obtained from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan), and maintained in dulbecco's modified eagle medium (DMEM) with 10% FBS and 4 mmol/L L-glutamine for 24 h. Cells were incubated in DMEM with 0, 0.4, or 4 mmol/L L-glutamine without FBS for the indicated time periods (0, 3, 6, 12, 24 and 48 h).

Proliferation assay

The number of surviving cells was measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate (WST-1) assay (Roche, Indianapolis, USA) for the indicated time periods after plating.

Immunoblot analysis of caspase-3

Cells were lysed in 1 × sodium dodecyl sulfate (SDS) sample buffer (62.5 mmol/L Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mmol/L dithiothreitol, and 0.01% w/v bromophenol blue or phenol red) and centrifuged at 4°C. The sample viscosity was reduced by pipetting. The samples were then boiled for 5 min and cooled on ice. The samples were separated by SDS-poly-acrylamide gel electrophoresis on 12% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane, immunoblotted with Rabbit anti-caspase-3 polyclonal antibody (Upstate Cell Signaling Solutions, Charlottesville, VA) and Rabbit anti-cleaved caspase-3 polyclonal antibody (Millipore corporation, Bedford, MA) followed by anti-rabbit immunoglobulin G-horseradish peroxidase (GE, Piscataway, NJ). Immuno-reactive proteins were visualized using an Enhanced Chemi-Luminescence kit according to the manufacturer's protocol (GE, Piscataway, NJ).

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) staining. Briefly, IEC6 cells were first seeded into 10-cm

Table 2 Nutrient composition of mouse milk and artificial amino acid milk

	Mouse milk	Artificial amino acid milk	CAM ¹	GDM	GRM
Osmotic pressure (mOsm/kg)	300	1800	1161	ND	ND
Glutamine (g/L)	ND	24.23	12.12	0	36.35
Fat (%)	22	10	16	16	16

¹Emulsified fat was added to artificial amino acid milk at a ratio of 1:1 to yield complete amino acid milk (CAM), because the proteins in the milk used in this study were in the form of amino acids, resulting in a very high osmotic pressure with a low amount of fat. As a result, the amount of amino acid contained in the CAM is 50% of the initial amount. GDM: Glutamine-deleted milk; GRM: Glutamine-rich milk; ND: Not determined.

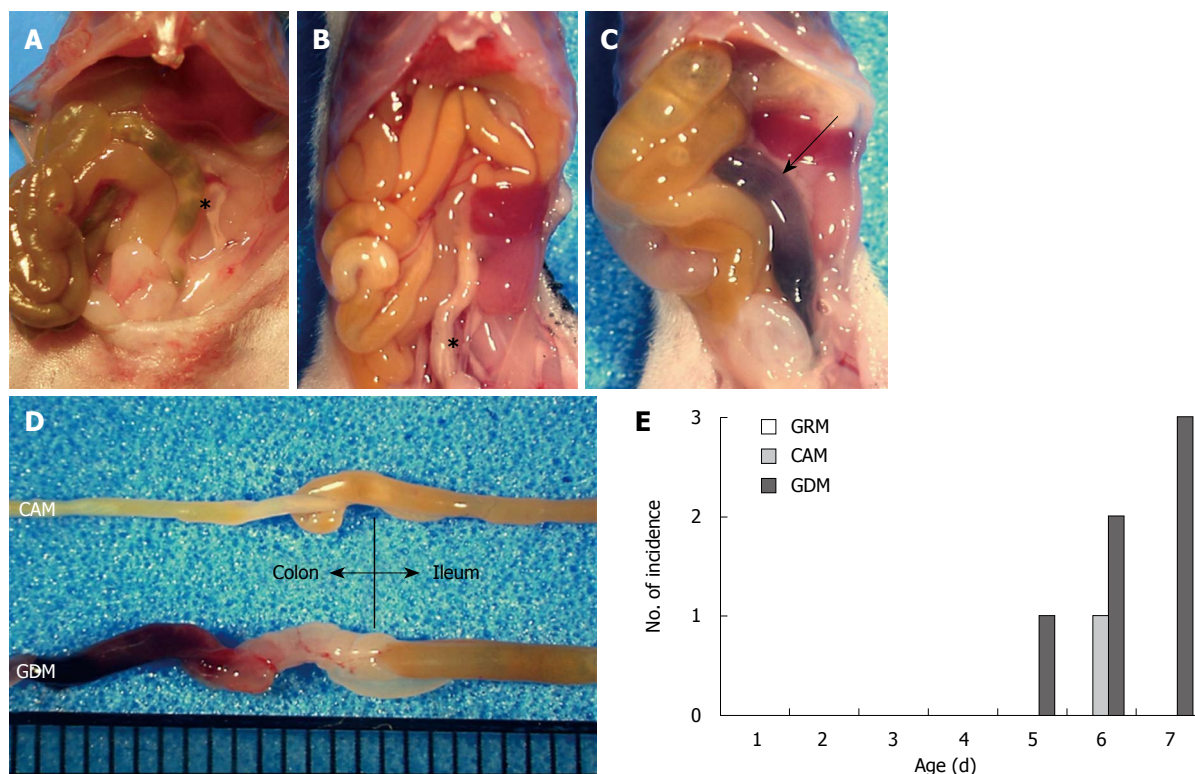


Figure 1 Macroscopic views of milk-fed mice with colonic hemorrhage. Representative macroscopic views of newborn mice that were fed with glutamine-rich milk (GRM) (A), complete amino acid milk (CAM) (B), and glutamine-deleted milk (GDM) (C) are shown. Compared to the colons of GRM-mice and CAM-mice (asterisks), those of the GDM-mice appeared distended and edematous, with a pool of blood (arrow); D: Close-up views of the resected intestines from a CAM-mouse and a GDM-mouse, shown for comparison; E: Bar chart of the number of mice with melena on each day.

dishes at a cell density of 5×10^5 . After further culture for 48 h and 96 h, the cells were trypsinized and harvested in phosphate buffered saline (PBS) followed by resuspension in PBS at $1-2 \times 10^6$ /mL. Finally, the cells were stained with 0.5 mL of PI staining solution (3.8 mmol/L sodium citrate, 50 mg/mL PI in PBS) for 1 h at room temperature to analyze cell cycle distribution by FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) excitation at 488-nm. The DNA-linked red fluorescence (PI) was measured through a 600-nm wavelength filter. This experiment was performed three times.

Statistical analysis

For *in vivo* experiments, the significance of differences between the control and test values was determined by Tukey's test using JMP 6.0.3 software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

Mice fed with GDM display colonic hemorrhage

Newborn mice were assigned to three groups (GRM, CAM, and GDM) and were fed four times a day according to the above-mentioned schedule. During the observation periods, the mice gained weight regardless of the amount of glutamine, and there were no significant differences between the groups (data not shown).

We measured the glutamine concentration in serum taken from each mouse fed with different types of milk. As expected, the GRM-mouse serum contained the highest amount of glutamine (7.53% of total amino acids), while the GDM-mouse serum had the lowest amount of glutamine (4.92% of total amino acids) in circulation. The CAM-mice maintained less glutamine in serum (5.74% of total amino acids) than the dam-reared mice (6.44%).

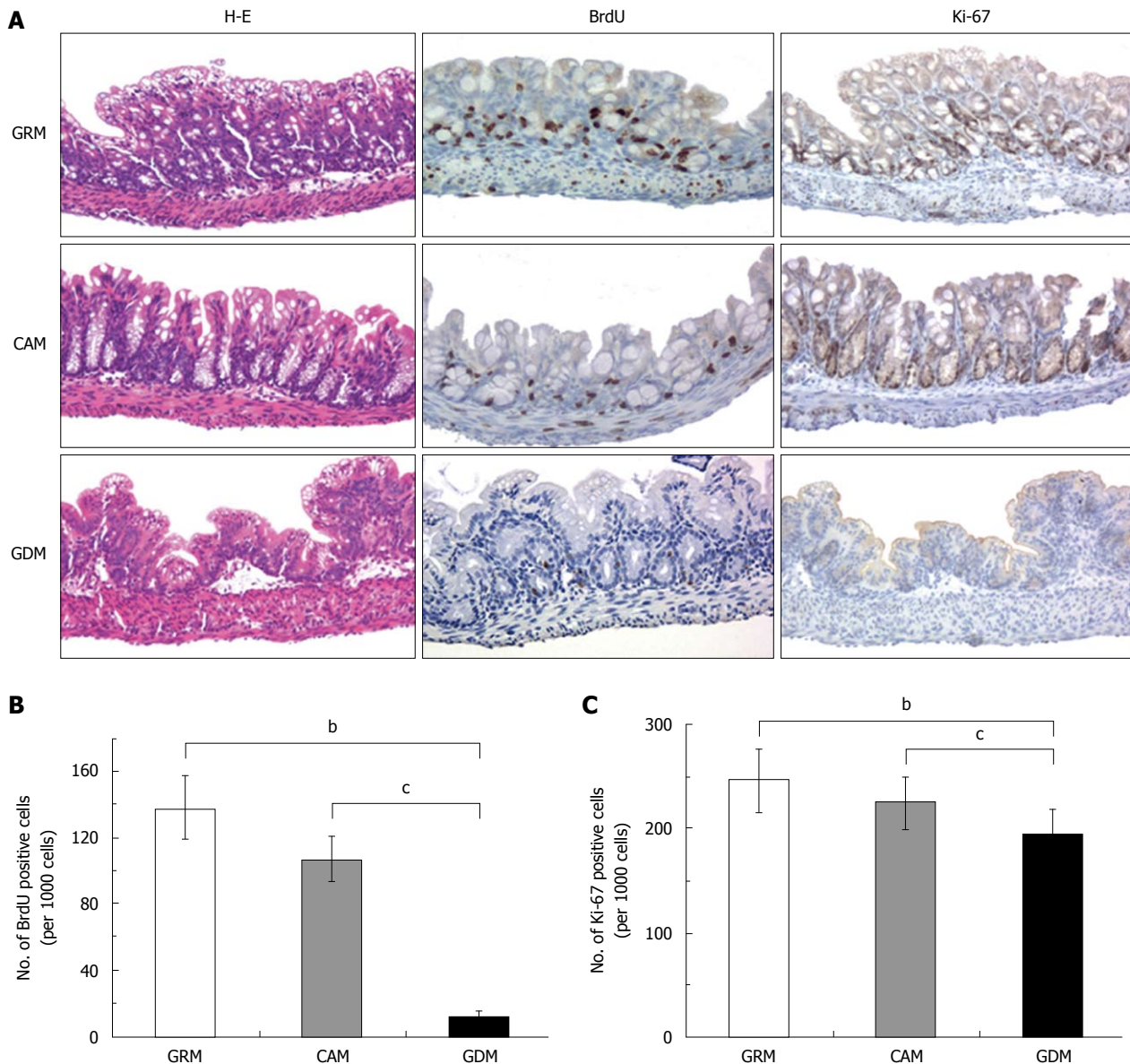


Figure 2 Glutamine depletion induces severe damage of the colonic epithelial structure with reduced epithelial cell growth. A: Representative pictures of H-E staining (left panels) and immunohistochemical staining for BrdU (middle panels) and Ki-67 (right panels). Each colonic epithelium was taken from infant mice fed with glutamine-rich milk (GRM), complete amino acid milk (CAM), or glutamine-deleted milk (GDM). Magnification, $\times 200$. Positive stained epithelial cells with BrdU (B) and Ki-67 (C) were counted and compared with each group in a histogram. $^bP \leq 0.001$; $^cP < 0.05$.

These data suggested that the amount of glutamine in the milk fed to the pups did affect the concentration of glutamine circulating in the animal body.

When the mice were sacrificed on day eight, we found a pool of blood in the colons of the GDM-fed mice accompanied by melena (Figure 1A-C). In addition, the entire bowel of these mice showed a massive edematous change, accompanied by wall thickening, redness, and dilatation of the small intestine (Figure 1D). No macroscopic changes were observed in mice fed with glutamine-containing milk, except for one mouse fed with CAM (Figure 1A-D). These events were observed in six of 28 GDM-fed mice (21.4%) and one CAM-fed mouse (3.3%), and started on days five to seven (one mouse on day five, four mice including the mouse fed with CAM, on day six, and two mice on day seven) (Figure 1E). No mice fed with GRM developed

melena during the observation period. The incidence of melena was significantly higher in the GDM group compared to the other groups (Figure 1E), suggesting that glutamine depletion must affect the maintenance of the neonatal gastrointestinal tract.

Glutamine depletion reduces cell proliferation and increases apoptosis in the colonic epithelium

Histological observation by hematoxylin-eosin staining revealed the destruction of the villous structure, wall thickening with edematous dilatation of the submucosal layer, and inflammatory cell infiltration around the hemorrhage site of the colons in the mice fed without glutamine (Figure 2A). In addition, a reduction of goblet cells was noted in this group, suggesting a disorder of cell maturation. Interestingly, the intestinal mucosa of the GRM-fed mice were

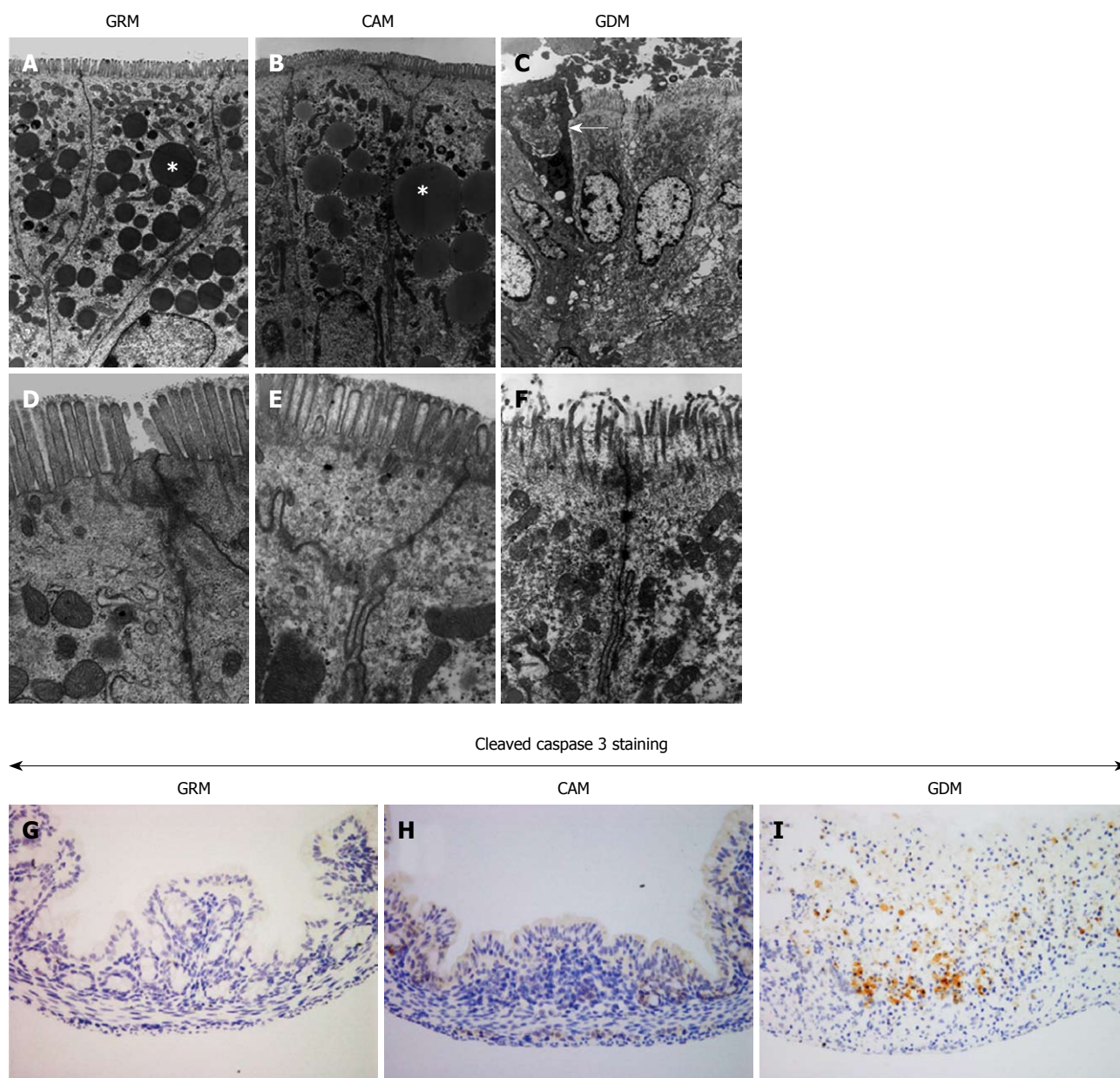


Figure 3 Apoptotic changes observed in the damaged colonic epithelium. Electron micrographs of colonic epithelia obtained from infant mice with glutamine-rich milk (GRM) (A, D), complete amino acid milk (CAM) (B, E), and glutamine-deleted milk (GDM) (C, F) at low magnification (A-C) and high magnification (D-F). Asterisks (*) represent lipid droplets and the arrow shows an apoptotic cell. G-I: Optical microscopic views (magnification, $\times 200$) of immunohistochemistry for cleaved caspase-3 as a marker of apoptosis using resected tissues from the same mice (G: GRM, H: CAM, I: GDM).

higher than those of the CAM-fed mice (Figure 2A).

We then assessed cell proliferation in colonic epithelial cells by immunostaining for BrdU and Ki-67, which are markers for DNA synthesis and cell proliferation, respectively (Figure 2A). BrdU incorporation was significantly decreased in colonic epithelial cells of the GDM-fed mice compared to those of CAM-mice and GRM-mice (average percentage of BrdU-positive staining per 1000 cells; GRM: 13.8%, CAM: 10.7%, GDM: 1.14%, Tukey test; GRM *vs* GDM, $P < 0.001$; CAM *vs* GDM, $P < 0.001$) (Figure 2B). Ki-67-positive staining was also significantly decreased in the colonic epithelia of the GDM-fed mice (average percentage of Ki-67-positive staining; GRM: 24.5%, CAM: 22.4%, GDM: 19.4%, Tukey test; GRM *vs* GDM, $P = 0.001$; CAM *vs* GDM, $P = 0.049$) (Figure 2C). These data indi-

cated that glutamine deprivation strongly diminished cell growth of the intestinal epithelium.

We further examined the damaged colonic mucosa under the electron microscope. Figure 3A-F shows representative pictures of colonic epithelia from each group. Glutamine deprivation caused deformation of the nuclear membrane and the plasma membrane, accompanied by the destruction of microvilli and the disappearance of glycocalyx, resulting in nuclear deformation and chromatin degeneration. Fat droplets, which were seen in the colons of the mice fed with the glutamine-containing milk, were absent in the GDM Group. Furthermore, a partial loss of colonic epithelial cells was noted in the GDM Group, indicating that glutamine deprivation promotes cell death. To confirm glutamine deprivation-induced cell death,

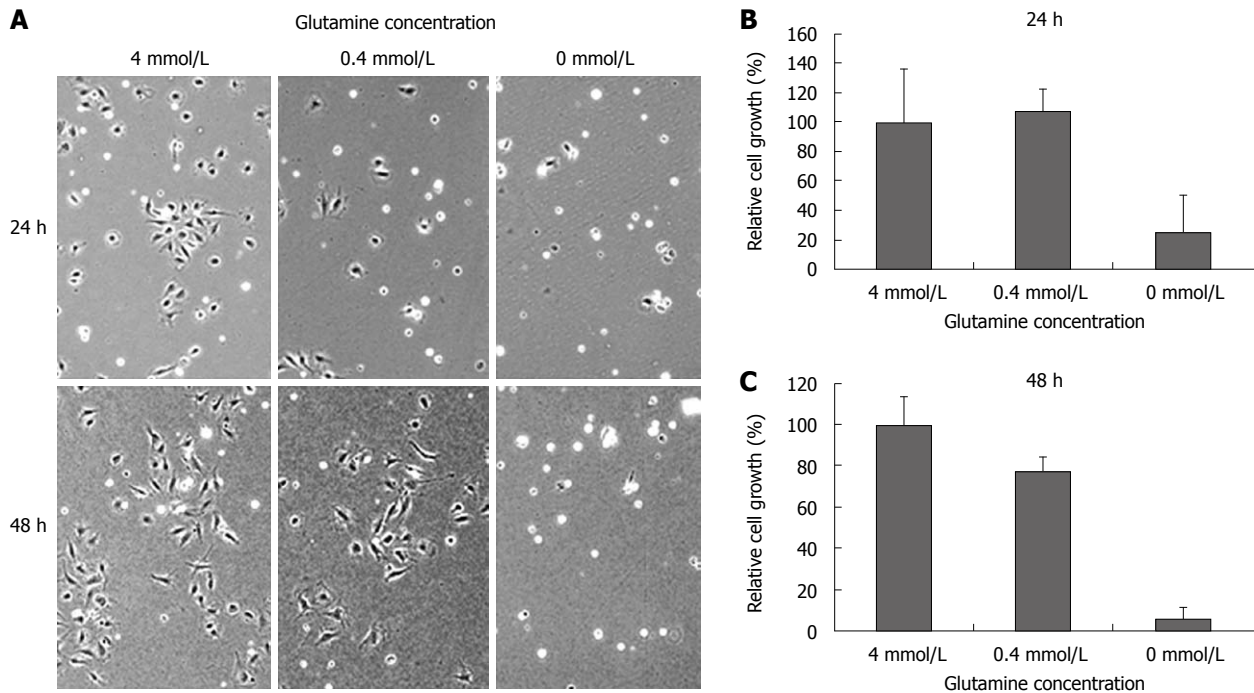


Figure 4 Glutamine depletion suppresses cell proliferation of cultured intestinal epithelial cells. A: IEC6 rat intestinal epithelial cells were treated with media containing different amounts of glutamine (0, 0.4 and 4 mmol/L); Cell morphology and cell number were observed at the indicated time points (B: 24 h, C: 48 h).

cleavage of caspase-3 was assessed by immunohistochemistry. As shown in Figure 3G-I, there was a remarkable number of cleaved caspase-3-positive stained cells in the glutamine-deprived mucosa, suggesting that a lack of glutamine induces colonic epithelial cell death, possibly through the process of apoptosis.

Glutamine depletion reduces cell proliferation in IEC6 rat intestinal cells

To clarify the effect of glutamine depletion on intestinal epithelial cell growth, IEC6 rat intestinal epithelial cells were cultured in the presence of varying concentrations of glutamine. Microscopic observation showed that glutamine depletion damaged cell morphology and reduced cell density (Figure 4A). As shown in Figure 4B and C, glutamine depleted conditions significantly suppressed IEC6 cell growth after 24 h (4 mmol/L: 100.0 ± 36.1 , 0 mmol/L: 25.3 ± 25.0 , $P < 0.05$), while there was no apparent difference between 4 mmol/L and 0.4 mmol/L after 24 h (4 mmol/L: 100.0 ± 36.1 , 0.4 mmol/L: 107.4 ± 15.4 , $P = 0.7614$), or after 48 h (4 mmol/L: 100.0 ± 14.0 , 0.4 mmol/L: 77.4 ± 6.9 , $P = 0.0659$). We further assessed the inhibitory effect on cell proliferation by cell cycle analysis, using flow cytometry (Figure 5A and B). We did not observe significant cell growth arrest at the G1 or G2 phase; however, an increased cell population at the sub-G0 phase was observed within 48 h of glutamine depletion (4 mmol/L: 1.68%, 0.4 mmol/L: 1.35%, 0 mmol/L: 5.21%, Figure 5B), which is consistent with the observation in the animal model that glutamine-depletion seemed to be lethal to intestinal epithelial cells.

Finally, we determined whether glutamine depletion-mediated cell death in cultured IEC6 cells occurs due to

induction of apoptosis. Cells cultured with a complete depletion of glutamine showed a time-dependent decrease in caspase-3 expression, accompanied by cleavage of caspase-3 within 24 h (Figure 5C). These data support our finding in the animal study that a lack of glutamine affects the maintenance of intestinal epithelial cells, suppresses cell growth and induces apoptosis, resulting in melena.

DISCUSSION

Until recently, it was quite difficult to study the physiological and cytobiological effects of amino acids *in vivo*, especially for neonatal animals, because of the lack of methods of alimentation or of preparing a diet for neonatal mice. In this study, using a totally new milk feeding system and amino acid milk, we successfully observed the physiological effects of glutamine depletion in newborn mice. Interestingly, a significantly high incidence of colonic hemorrhage occurred in mice fed with GDM, compared to the CAM or GRM groups. We had one CAM-fed mouse that had a similar colonic hemorrhage on day six. This mouse might have had a genetic/physiological susceptibility to the event. It could also have been caused by the fact that the amount of amino acid contained in the CAM was 50% of the ordinary amount because emulsified fat was added to the artificial amino acid milk at a ratio of 1:1 to yield CAM. The proteins in the milk used in this study were in the form of amino acids, resulting in a very high osmotic pressure with a low amount of fat (Table 2). If the artificial amino acid milk is applied directly to newborn mice without adding fat, which is a major nutrient for them, a fat deficiency might occur. Therefore, the melena that occurred in the CAM-

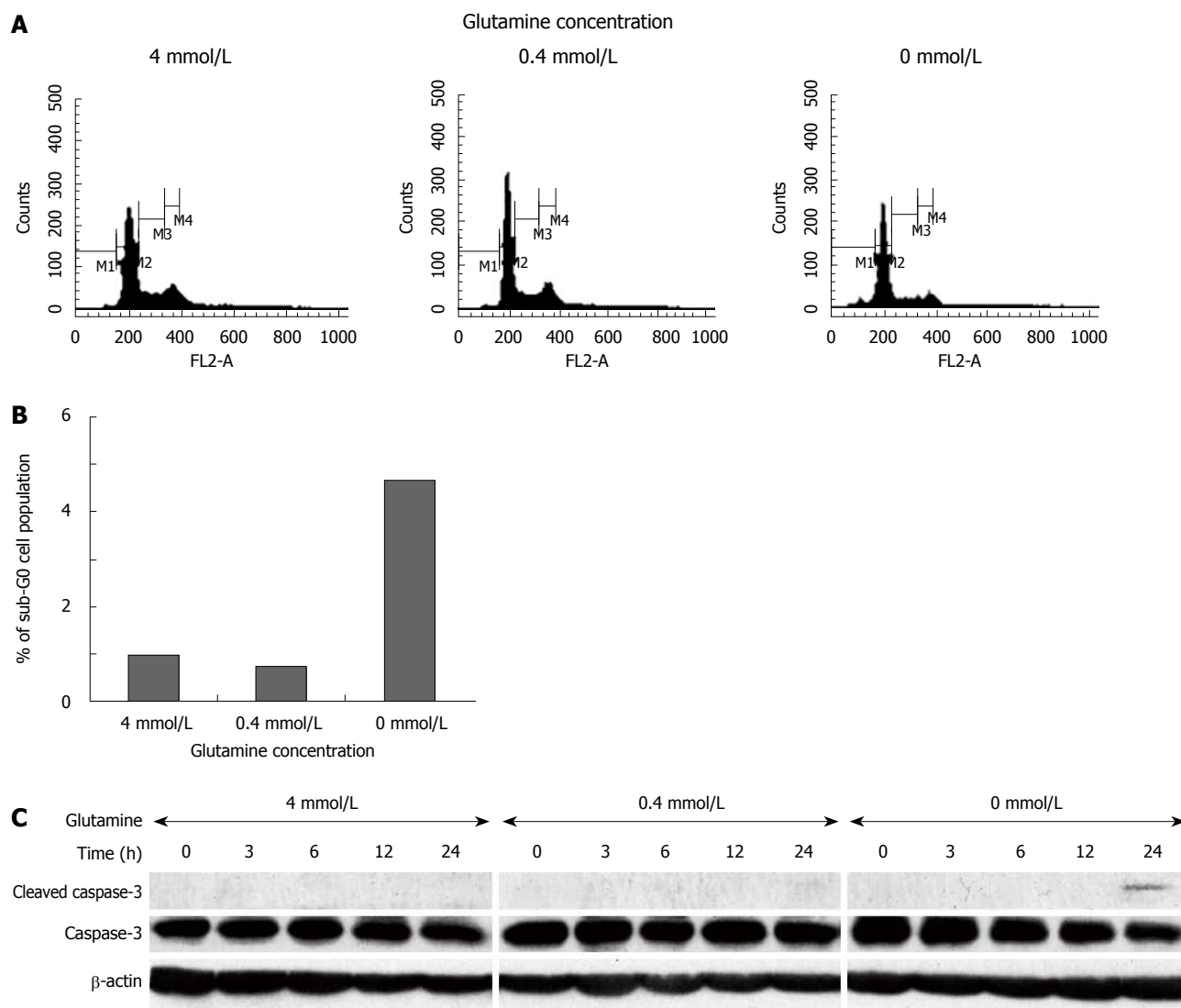


Figure 5 Potent antiproliferative effect of glutamine depletion results in increased cleavage of caspase-3. Cell cycle distribution under culture condition with different concentrations of glutamine was analyzed by flow cytometry (A) and cell populations at the sub-G0 phase were compared with each other (B); C: Immunoblotting for caspase-3 and cleaved caspase-3 revealed the induction of apoptosis in IEC6 cells after 24 h of culture without glutamine. Each experiment was independently repeated three times and the representative data among the similar results are shown.

fed mouse may be explained by an insufficient intake of glutamine. Meanwhile, no animal developed colonic hemorrhage in the GRM Group; thus, we assume that the glutamine level in this milk was high enough to maintain the intestinal epithelium.

Other macroscopic findings in the hemorrhagic intestines of the GDM-fed mice were the apparent inflammatory changes of the entire intestine, with intestinal wall thickening by edema. Microscopic observations supported these findings, with infiltration of inflammatory cells in and around destroyed colonic mucosa at the site of the hemorrhage. On the other hand, the height of the colonic mucosa of GRM-fed mice was well conserved and was higher than that of the CAM mice. In addition, as more glutamine was administered, more positive-staining cells for BrdU and Ki-67 appeared, suggesting that glutamine is a critical nutrient for the proliferation of intestinal cells. Moreover, both electron microscopic observations and immunohistochemistry for cleaved caspase-3

reflected an increased incidence of apoptosis induced by glutamine depletion. However, no exact intracellular mechanism has been identified for the destruction of the nuclear membrane and microvilli, or the disappearance of glycocalyx, which were induced by GDM. Clarifying the molecular/biophysical mechanism of this glutamine depletion-mediated event will be crucial to understanding the intrinsic functions of glutamine or other amino acids.

Experimental data from cultured IEC6 cells supported the findings of the animal experiments, with the reduced cell proliferation, accumulation of a cell population in the sub-G0 phase, and the cleavage of caspase-3 under glutamine-depleted culture conditions. According to these results, glutamine depletion induces cell death of colonic mucosa, presumably due to an acute induction of apoptosis, followed by the destruction of mucosa maintenance, which eventually leads to colonic hemorrhage.

In the present study, it was remarkable that colonic hemorrhage could be induced simply by removing one

amino acid, glutamine. Glutamine has attracted close attention as an amino acid nutrient and as an immunopotentiating factor for intestinal cells^[14-16]. However, no case of intestinal hemorrhage induced by glutamine deficiency has been reported to date. Although glutamine is a non-essential amino acid and can be produced in the living body, neonatal mice are actively growing and are very dependent on external alimentation due to a heavier consumption of nutrients, including glutamine, compared to adults^[17]. It seems likely that the consumption of glutamine by these neonatal mice might exceed the amount of glutamine pooled and formed in the living body, leading to the emergent status of glutamine deficiency.

In terms of molecular/biological effects of glutamine, there are outstanding several issues that remain to be clarified. One of our interests is to explore the possible involvement of certain signaling pathways. It has recently been unveiled that amino acids are involved in the control of the mTOR signaling pathway^[18]. We have also demonstrated previously that leucine activates the mTOR signaling pathway in a hepatocellular carcinoma cell line^[19]. It has also been shown that leucine and arginine activate the mTOR signaling pathway in a small bowel epithelial cell line derived from rats^[20]. We also found that glutamine regulates the activation of this pathway in the same cell line^[21]. Therefore, future studies should focus on the mechanism for the induction of colonic hemorrhage and how glutamine is involved in the intracellular signaling pathway, such as in mTOR^[22-24].

In conclusion, we found that feeding neonatal mice with GDM induced a high incidence of colonic hemorrhage within a week, and that this was due to an induction of epithelial cell death. Further investigation is necessary to explore the biological mechanism.

ACKNOWLEDGMENTS

We are grateful to Ms. Kazuko Tunashima, Ms. Tae Yamanishi, and Mr. Toru Tanida for technical assistance, Dr. Yuko Ohno (Department of Health Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan) for supervising the statistical analysis, and Drs. Minoru Haisa (Okayama Citizens' Hospital, Okayama, Japan), Keiichi Nishida, Kazuhiro Noma and Shunsuke Tanabe (Okayama University) for useful discussions.

COMMENTS

Background

Glutamine, the most abundant amino acid in the human body, is conditionally essential, especially for susceptible individuals who are in high stress conditions. A lack of glutamine correlates with increased mortality.

Research frontiers

The small intestine accounts for the largest uptake of glutamine of any organ, absorbing this amino acid from the lumen of the gut, as well as from the bloodstream. Past studies involved the evaluation of immunological effects of amino acids by the administration of a diet rich or deficient in each amino acid (glutamine, arginine, etc.) by means of total parenteral nutrition or stomach tubing. However, no such study in newborn mice has yet been reported. The lack of such a study in newborn mice is attributable to the difficulty in developing a method of alimentation or preparing a diet for neonatal mice.

Innovations and breakthroughs

The authors developed three kinds of artificial milk with different amounts of glutamine. Using these amino acid milks and a recently improved nipple-bottle feeding system, they successfully fed the newborn mice with the new amino acid milk and observed colonic hemorrhage in mice fed without glutamine. It was remarkable that colonic hemorrhage could be induced simply by removing one amino acid, glutamine. In addition, glutamine deprivation can cause instability of intestinal epithelial alignment by increased apoptosis.

Applications

No exact intracellular mechanism has been identified for the destruction of the nuclear membrane and microvilli or the disappearance of glycocalyx, which were induced by glutamine depletion. Thus, we must explore the mechanism of the induction of colonic hemorrhage and investigate glutamine's involvement in the intracellular signaling pathway, to better understand the intrinsic functions of glutamine and other amino acids.

Peer review

The authors have described that glutamine depletion induces neonatal mice melena and apoptosis of intestinal epithelium *in vivo* and *in vitro*. The authors obtained good data allowing the conclusion that glutamine depletion induces neonatal mice melena and apoptosis of intestinal epithelium, using appropriate methods. An animal model was established for glutamine deprivation-induced destruction of the intestinal epithelium. It would also be meaningful to study the effects of glutamine-deprivation on human intestinal function.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Lin YP

Prediction of gastric cancer metastasis through urinary metabolomic investigation using GC/MS

Jun-Duo Hu, Hui-Qing Tang, Qiang Zhang, Jing Fan, Jing Hong, Jian-Zhong Gu, Jin-Lian Chen

Jun-Duo Hu, Medical College, Soochow University, Suzhou 215213, Jiangsu Province, China

Jun-Duo Hu, Department of Gastroenterology, Shanghai Sixth People's Hospital, Shanghai 200233, China

Hui-Qing Tang, Jian-Zhong Gu, Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai 201615, China

Qiang Zhang, Jing Fan, Jing Hong, Jin-Lian Chen, Department of Gastroenterology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China

Author contributions: Chen JL designed and conducted the study; Hu JD analyzed the data and prepared the manuscript; Fan J, Hong J, Tang HQ and Gu JZ established the animal model; Hu JD, Zhang Q and Chen JL did the metabolomic analyses; all authors were involved in reviewing, interpreting the results and drafting the manuscript.

Supported by The Key Program of Science and Technology Commission of Shanghai Municipality, Project No. 09JC1411600; and Natural Science Foundation of Shanghai, No. 08ZR1411300

Correspondence to: Jin-Lian Chen, MD, Professor, Department of Gastroenterology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China. wq_021002@163.com

Telephone: +86-21-64369181 Fax: +86-21-64369181

Received: August 14, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: February 14, 2011

selected metabolites were different between normal and cancer groups (non-metastasis and metastasis groups), and seven metabolites were also different between non-metastasis and metastasis groups. Two diagnostic models for gastric cancer and metastasis were constructed respectively by the principal component analysis (PCA). These PCA models were confirmed by corresponding receiver operating characteristic analysis (area under the curve = 1.00).

CONCLUSION: The urinary metabolomic profile is different, and the selected metabolites might be instructive to clinical diagnosis or screening metastasis for gastric cancer.

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Key words: Metabolomic profile; Gastric cancer; Metastasis; Biomarker; Gas chromatography/mass spectrometry

Peer reviewers: Dr. Cuneyt Kayaalp, MD, Professor, Department of General Surgery, Staff Surgeon of Gastrointestinal Surgery, Turgut Ozal Medical Center, Inonu University, Malatya 44315, Turkey; Kevin Michael Reavis, MD, Assistant Clinic Professor, Department of Surgery, Division of Gastrointestinal Surgery, University of California, Irvine Medical Center, 333 City Boulevard West, Suite 850, Orange, CA 92868, United States

Hu JD, Tang HQ, Zhang Q, Fan J, Hong J, Gu JZ, Chen JL. Prediction of gastric cancer metastasis through urinary metabolomic investigation using GC/MS. *World J Gastroenterol* 2011; 17(6): 727-734 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/727.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.727>

Abstract

AIM: To gain new insights into tumor metabolism and to identify possible biomarkers with potential diagnostic values to predict tumor metastasis.

METHODS: Human gastric cancer SGC-7901 cells were implanted into 24 severe combined immune deficiency (SCID) mice, which were randomly divided into metastasis group ($n = 8$), non-metastasis group ($n = 8$), and normal group ($n = 8$). Urinary metabolomic information was obtained by gas chromatography/mass spectrometry (GC/MS).

RESULTS: There were significant metabolic differences among the three groups (t test, $P < 0.05$). Ten

INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries, such as China^[1,2]. Until now, there has been no effective treatment for gastric cancer. Even among patients undergoing gastrectomy,

because of locoregional relapse and distant metastases, the 5-year survival rates remain disappointing^[3]. Early dissemination of the disease through the lymphatic system, blood and peritoneum has limited the therapeutic effects of optimal surgery, except in patients with relatively early-stage tumors^[4]. Therefore, it is significant to establish an accurate early diagnosis of gastric cancer. Currently, the diagnosis or screening of gastric cancer or tumor recurrence mainly depends on endoscopy and pathological examinations. The ratio for identifying early gastric cancer with endoscopy is higher than that with X-ray^[5], and the diagnosis of gastric cancer using endoscopy is more accurate^[6]. Nevertheless, the results of endoscopy are easily affected by artificial factors (e.g. the experience of the endoscopist). Over the past years, epidemiological data have shown that *Helicobacter pylori* (*H. pylori*) infection is strongly associated with the development of gastric cancer^[1], and *H. pylori* eradication may be considered as a strategy to prevent gastric cancer^[7]. In addition, investigation of gastric cancer tissues and some biomarkers have been used for screening gastric cancer^[8-13]. However, compared with tissues and serum, the markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. The urinary metabolic profiling could be used to get urinary metabolites as gastric cancer or tumor recurrence biomarkers.

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems^[14], and offers an analysis of metabolite level changes in biological samples^[15]. In recent years, studies of metabolomics used in various diseases have been conducted, such as stomach cancer^[16], lung cancer^[17], renal cancer^[18,19], brain tumors^[20], and colorectal cancer^[21-24]. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are the most commonly employed techniques for measuring the metabolome^[14]. MS-based techniques, including gas chromatography/mass spectrometry (GC/MS), GC-MS/MS, liquid chromatography/mass spectrometry (LC-MS) and LC-MS/MS, are among the most efficient and versatile for quantitative analysis of endogenous and exogenous substances in biological samples^[25]. Because of its peak resolution, high sensitivity and reproducibility, GC/MS has been well established and widely utilized in metabolomics^[26-28].

In this study, we have established a human gastric cancer non-metastasis model and a metastasis model using severe combined immune deficiency (SCID) mice, and deployed GC/MS following chemical derivatization to profile the mouse model urinary specimens and their matched urine. The metabolic differences among the three groups were characterized by principal components analysis (PCA). On the basis of its results, we expected that the potential metabolic biomarkers could be found in mice for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

MATERIALS AND METHODS

Chemicals and materials

Tetrahydrofuran (THF) and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were obtained from Sigma Chemical

Co. (St Louis, MO, USA). Vacuum dryer was purchased from Shanghai NOTED Technologies. All other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd.

Animal models

Male SCID mice were acquired from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were 6-wk old and weighed 20-25 g. Animal and experimental procedures were performed according to the relative ethical regulations for the care and use of laboratory animals of our university. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Tumors were cut out aseptically. Necrotic tissues were cut and the reserved healthy tumor tissues were scissor minced into pieces (about 3 mm × 4 mm in diameter) in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to be approximately 100 mg. All animals were randomly divided into metastasis group ($n = 8$), non-metastasis group ($n = 8$), and normal group ($n = 8$). Animal models were made using orthotopic implantation of histologically intact tissue of human gastric cancer^[29]. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision of the metastatic group and the normal group was made through the left upper abdominal pararectal line. Then peritoneal cavity was carefully exposed and a part of serosal membrane in the middle of the greater curvature of stomach was mechanically injured by scissors. A tumor piece of 100 mg was fixed on each injured site of the serosal surface of the metastatic group, while normal control mice received no tumor implantation. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed. An incision of the non-metastatic group was made at the left outer. A tumor piece of 100 mg was fixed under the skin. All animals were sent to the breeding room after becoming conscious.

Specimen collection and pathological examination

Six weeks after implantation, all mice were housed in metabolic cages and maintained in an air conditioned room ($24 \pm 2^\circ\text{C}$). They were only allowed free access to water during urine sample collection (8:00 pm that day to 8:00 am the next day). All animal urine was collected in frozen tubes at the sixth week after implantation, and immediately stored at -80°C until processing. The specimens were collected at the same time. Then all mice were killed, tumors growing on the stomach wall were resected and fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. In order to evaluate histologically for liver metastasis or lymph node metastasis or other organ metastasis under microscope, four-micron-thick sections were stained with hematoxylin and eosin, then observed by a blinded pathologist.

Sample pretreatment and derivatization

Each urinary specimen was transferred to a glass cen-

trifuge tube, subsequently centrifuged at $18000 \times g$ for 3 min and 50 μL of the supernatant was collected from each sample into a 1-mL EP tube, respectively. The collected supernatant was evaporated to dryness at 60°C for 24 h, using a vacuum dryer. Then 100 μL THF was added to each of the dried urine extracts and vortex-mixed for 2 min, and 50 μL BSTFA was added to the mixture and vortex-mixed for 2 min. The mixture was incubated at 60°C and derivatized for 30 min. After returning to the ambient temperature, samples were prepared for GC/MS analysis.

GC/MS analysis

Each derivatized sample of 1 μL was injected splitless into an Agilent 6980 GC system equipped with an HP5MS capillary column (30 m \times 0.25 mm i.d., 0.25 μm), electron impact ionization at 70 eV, and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). The column temperature was initially held at 100°C for 3 min, $10^\circ\text{C}/\text{min}$ up to 220°C , then $10^\circ\text{C}/\text{min}$ to 280°C , and remained there for 5 min. The injector temperature was 280°C . Carrier gas flow was helium at a constant flow rate of 1.0 mL/min. The interface temperature and the ion source temperature were set at 200°C . Masses were obtained from 100–600 m/z . GC total ion chromatograms (TICs) and fragmentation patterns were acquired using GC/MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compound identification was performed by comparing the mass spectrum with a standard mass spectrum in the national institute of standards and technology (NIST) mass spectra library. Peaks with similarity index more than 70% were assigned compound names, while those having less than 70% similarity were listed as unknown metabolites^[30]. The chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks, such as peaks due to noise, column bleed and BSTFA derivatization procedure, were excluded from the data set. Integrated peak areas of multiple derivative peaks belonging to the same compound were summed and considered as a single compound. The resulting three dimensional matrix included sample information, peak intensities and peak retention time, and was applied to correlation analysis and pattern recognition.

Data processing and pattern recognition

The relative peak area of each compound would be calculated as the response after the peak areas of compounds were integrated. Each sample was represented by a GC/MS TIC. *t* test was employed for statistical analysis. Data were expressed as mean \pm SD. The differentially expressed compounds with $P < 0.05$ were considered statistically significant. PCA was used to differentiate the samples and performed using the SPSS 16.0 for Windows.

RESULTS

General state of mice and pathological results

The mean weight of mice was 23.81 ± 0.16 g, 23.87 ± 0.19 g and 23.98 ± 0.19 g for normal group, non-metastasis group and metastasis group, respectively ($P > 0.05$).

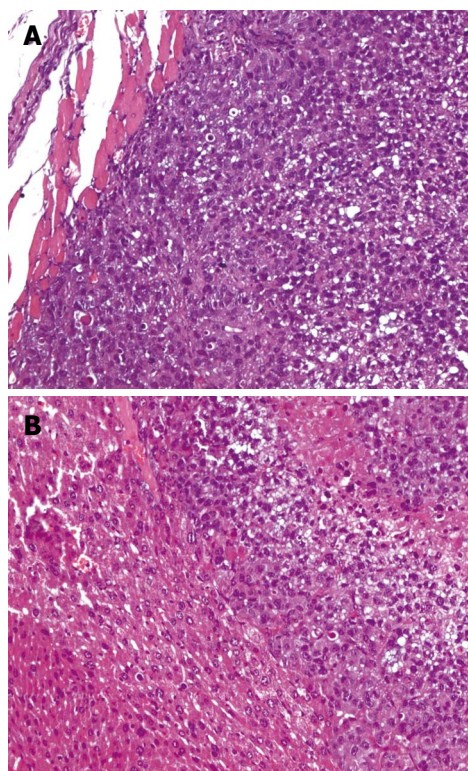


Figure 1 Gastric cancer pathological photographs. A: Gastric cancer cells in mice of the non-metastatic group (HE stain, $\times 200$); B: Gastric cancer metastasis in the liver (HE stain, $\times 200$).

All animals from the three groups were alive at the sixth week. The normal group mice had no tumor and metastasis. The non-metastasis group and metastasis group developed localized tumors at the implanted site, which were poorly-differentiated adenocarcinomas under microscope (Figure 1A). The non-metastasis group tumor tissues (4.28 ± 0.20 g) were located at the left oter, and have no metastasis in regional lymph nodes, liver and other organs. The metastasis group mice had cancer tissues (4.3 ± 0.3 g *vs* non-metastasis group, $P > 0.05$) in the stomach, while metastatic tumors were also found in liver (Figure 1B), regional lymph nodes, and other organs. Six mice developed metastatic tumors in regional lymph nodes, four in liver, and two in other organs.

Metabolomic profiling of samples

GC/MS TIC chromatograms of urine samples derived from the normal group, the non-metastatic group and the metastatic group are presented in Figure 2. In the GC/MS TICs of urinary samples from the three groups, some peaks were identified based on NIST mass spectra library, and several examples of peaks had statistical significance (Figure 2).

With GC/MS, around 120 signals were detected per sample using mass spectral deconvolution software for peak detection. However, many of them were not consistently found in other samples or were of too low abundance or too poor spectral quality to be obviously assigned to unique metabolites. Several choline, amino acids, and fatty acids could not be found, which may be

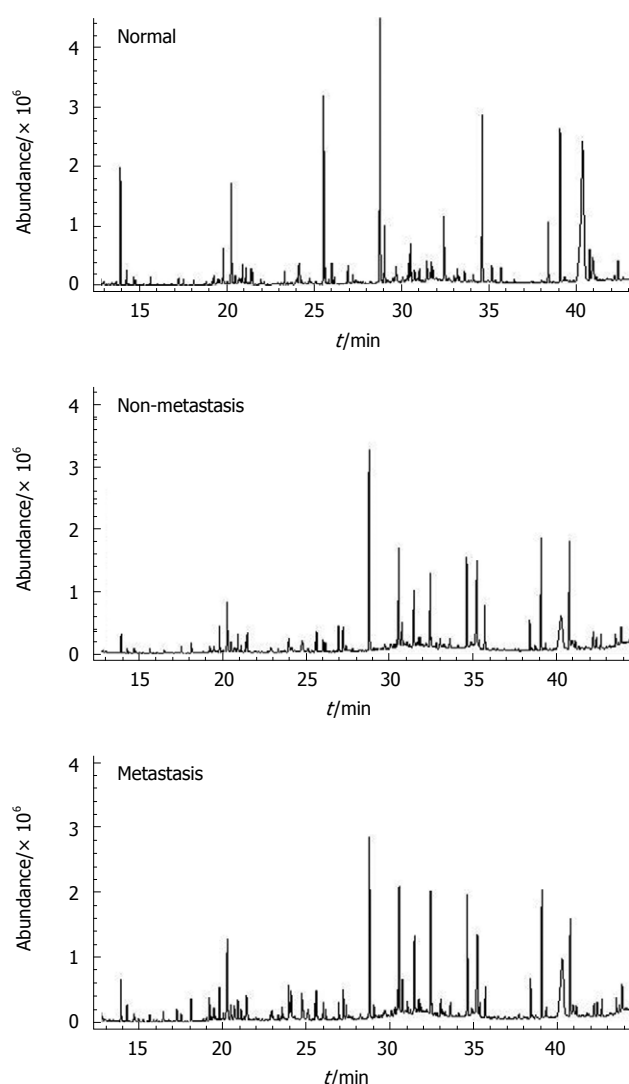


Figure 2 Representative gas chromatography/mass spectrometry total ion chromatograms of the samples from the three groups (normal group, non-metastasis group and metastasis group) after chemical derivatization.

associated with the efficiency of chemical derivatization. Table 1 shows that 46 signals could be auto-identified by the NIST library through comparing with a standard mass chromatogram. The remaining peaks which could not be identified were not listed. In addition, the retention time of metabolites and the match percentage to the NIST library are also listed in Table 1.

Three TIC profiles of consecutively injected samples of the same aliquot are presented in Figure 3, which showed stable retention time with no drift in all of the peaks. The stable TICs reflected the stability of GC/MS analysis and reliability of the metabolomic data.

Urine GC/MS data from the three groups were analyzed. Metabolites selected by *t* test are listed in Tables 2 and 3 after normalization of data. Lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid were found at higher levels in the urine of cancer group (non-metastasis group and metastasis group) than in normal control group (Table 2). Furthermore, the de-

Table 1 Urine metabolites of mice in the three groups (normal, non-metastasis and metastasis)

Peak No.	Retention time	Metabolites	Match percent (%)
1	7.196	Lactic acid	91
2	7.508	Acetic acid	90
3	8.105	Alanine	90
4	8.518	Glycine	91
5	8.936	Pentanoic acid, 4-oxo-	94
6	9.412	Butanoic acid	83
7	11.940	Urea	95
8	12.511	Glycerol	91
9	12.590	Silanol	97
10	13.933	Butanedioic acid	97
11	14.293	Propanoic acid	94
12	14.705	Pyrimidine	93
13	14.774	Triacetin	83
14	15.657	2-Piperidinecarboxylic acid	90
15	16.070	L-threonine	87
16	16.460	N-(1-oxobutyl)-Glycine	90
17	17.270	N-(2-methyl-1-oxopropyl)-Glycine	91
18	17.530	(R*,S*)-3,4-Dihydroxybutanoic acid	94
19	19.301	Malic acid	90
20	19.492	N-(3-methyl-1-oxobutyl)-Glycine	98
21	19.566	2,3,4-oxy-Butanal	90
22	19.814	1,2,3,4-oxy-Butane	90
23	20.497	L-proline	96
24	20.909	L-threonic acid	90
25	21.475	Creatinine	96
26	25.108	Hexanedioic acid	90
27	25.632	Arabitol	91
28	25.843	Nonadecane	83
29	26.018	Xylitol	93
30	26.166	Ribitol	91
31	26.838	4-Pyrimidinecarboxylic acid	96
32	26.938	1-Propene-1,2,3-tricarboxylic acid	91
33	27.208	Phosphoric acid	90
34	28.768	Citric acid	91
35	29.032	Myo-inositol	83
36	30.328	Mannonic acid	95
37	30.540	Hydrazone	96
38	30.730	N-Phenylacetyl glycine	93
39	31.037	Silane	91
40	31.449	L-Gluconic acid	99
41	32.422	D-Gluconic acid	91
42	33.025	Dehydrocholic Acid	92
43	34.612	Hexadecanoic acid	99
44	35.691	Uric acid	98
45	38.388	Retinoic acid, methyl ester	95
46	39.065	Octadecanoic acid	99

Peaks in the total ion chromatograms are numbered according to their retention time. The identification of metabolite is based on national institute of standards and technology mass spectra database according to the match of masses (*m/z*) between the interested peak's fragmentation pattern and that from the standard database.

creased levels of alanine, butanoic acid, glycerol, L-proline and L-threonic acid were found in the metastasis group as compared with the non-metastasis group. However, the levels of butanedioic acid and myo-inositol were significantly higher in the metastasis group than in the non-metastasis group (Table 3).

Pattern recognition and function analysis

A PCA model for gastric cancer was constructed using

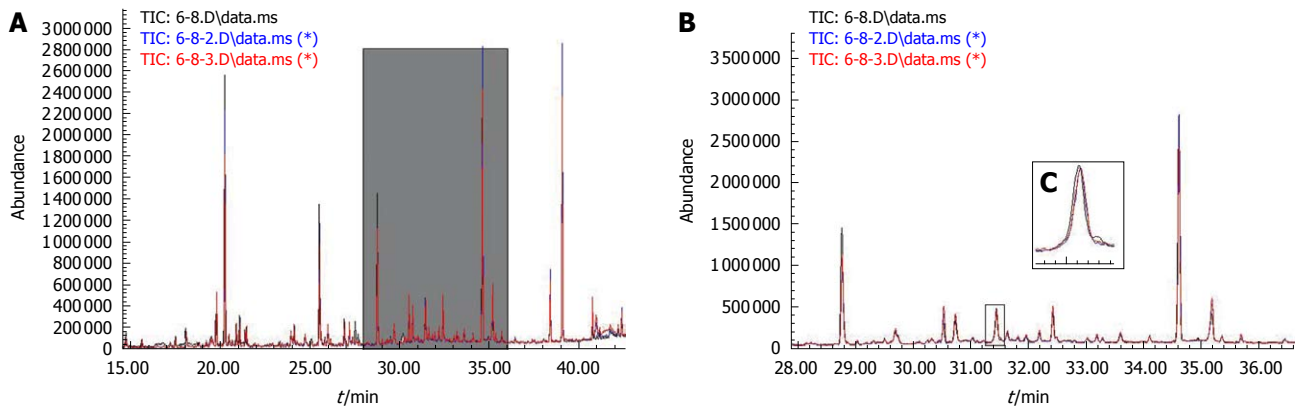


Figure 3 The overlay chromatograms of three parallel samples. A: The total ion chromatograms (TICs) of gas chromatography/mass spectrometry analysis; B: Enlarged part of TIC from 28 to 36 min; C: One peak enlarged.

Table 2 Marker metabolites found in normal and cancer groups

Metabolites	Retention time	P value ¹	A (normal)	B (cancer ²)	R ³
Lactic acid	7.196	2.4×10^{-5}	79.24 ± 6.1	187.04 ± 71.99	1.36
Butanoic acid	9.412	0.000	16.79 ± 0.52	27.33 ± 4.98	0.63
Propanoic acid	14.293	0.000	60.58 ± 9.79	147.77 ± 15.3	1.43
Glycerol	12.511	0.000	147 ± 8.98	269.13 ± 50.31	0.83
Pyrimidine	14.705	0.000	61.68 ± 8.05	163.11 ± 12.23	1.64
Butanedioic acid	13.933	0.1×10^{-5}	161.51 ± 5.85	267.89 ± 54.64	0.66
Malic acid	19.301	0.000	10.7 ± 1.91	32.15 ± 1.16	2.00
Citric acid	28.768	1.4×10^{-4}	1291.89 ± 364.74	2164.74 ± 529.58	0.68
Hexadecanoic acid	34.612	4.17×10^{-4}	1347.84 ± 304.67	2066.57 ± 437.28	0.53
Uric acid	35.691	0.000	172.2 ± 17.03	214.52 ± 7.74	0.25

¹P values were calculated based on Student *t* test (significance at $P < 0.05$); ²Cancer group included the non-metastasis group and the metastasis group; ³R value was calculated from the arithmetic mean values of each group. $R = (B-A)/A$. R with a positive value indicates a relatively higher concentration in cancer group while a negative value means a relatively lower concentration as compared with the normal group.

Table 3 Metabolic differences in the two groups

Metabolites	Retention time	P value ¹	A (non-metastasis)	B (metastasis)	R ²
Alanine	8.105	0.000	173.75 ± 39.59	19.28 ± 10.63	-0.89
Butanoic acid	9.412	0.000	32.09 ± 1.00	22.58 ± 0.72	-0.30
Glycerol	12.511	0.003	303.23 ± 26.16	235.04 ± 45.64	-0.22
Butanedioic acid	13.933	0.1×10^{-5}	216.36 ± 2.63	319.43 ± 17.89	0.48
L-proline	20.497	0.000	184.99 ± 10.26	117.78 ± 7.05	-0.36
L-threonic acid	20.909	2.28×10^{-4}	284.94 ± 46.47	181.48 ± 37.25	-0.36
Myo-inositol	29.032	0.000	33.08 ± 3.58	114.8 ± 2.20	2.47

¹P values were calculated based on Student *t* test (significance at $P < 0.05$); ²R value was calculated from the arithmetic mean values of each group. $R = (B-A)/A$. R with a positive value indicates a relatively higher concentration in metastasis group while a negative value means a relatively lower concentration as compared with the non-metastasis group.

the marker metabolite intensities as variables (lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid). The PCA scores plot showed that the normal group and cancer group (non-metastasis group and metastasis group) samples were scattered into different regions (Figure 4A). ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. These first two components could present the ma-

jority of all significantly different metabolites among the groups (the percentage is 82.7%). Area under the curve (AUC) value of this PCA model was 1.00 (Figure 4B), which demonstrated a good diagnostic value for gastric cancer. In addition, another PCA model for gastric cancer metastasis constructed by seven marker metabolites (alanine, butanoic acid, glycerol, L-threonic acid, L-proline, butanedioic acid and myo-inositol) could differentiate between the non-metastasis group and the metastasis group (Figure 5A). This PCA model was also validated by

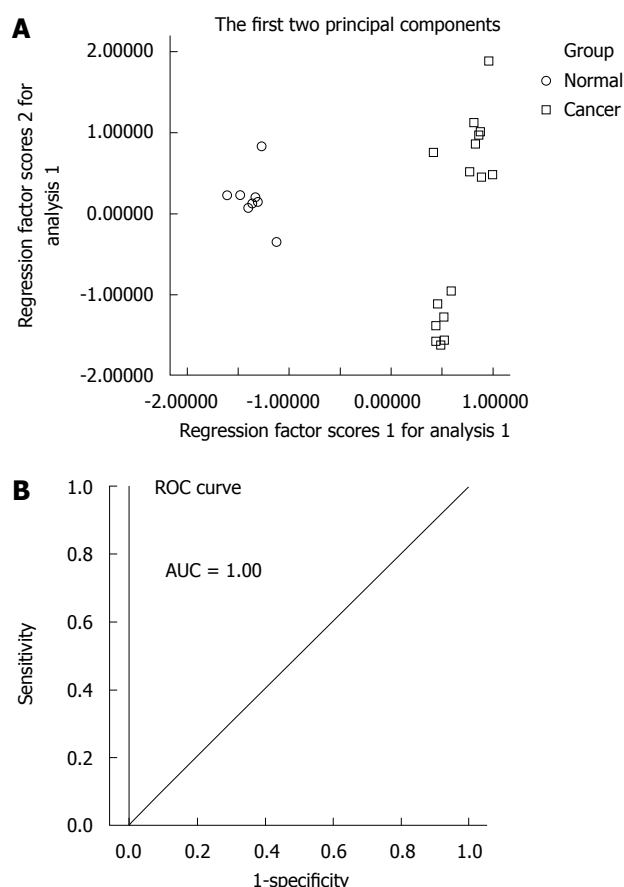


Figure 4 Principal component analysis model and receiver operating characteristic curve for gastric cancer. A: Principal component analysis (PCA) scores plot of gastric tumor specimens from control specimens based on 10 marker metabolites. The PCA scores plot showed different samples (normal group, cancer group including non-metastasis group and metastasis group) were scattered into different regions; B: Receiver operating characteristic (ROC) analysis was performed using the values determined by the first two components. Area under the curve (AUC) = 1.00.

receiver operating characteristic (ROC) analysis (AUC = 1.00, Figure 5B).

DISCUSSION

In this study, we investigated urinary metabolite profiling using GC/MS. This was assessed non-invasively by measuring two voxels (tumor and healthy controls). We have discriminated the gastric cancer model mice from their healthy controls in a PCA analysis of GC-MS urinary metabolite spectra. Moreover, we could also discriminate the gastric cancer metastasis model mice from the non-metastasis model mice by GC-MS and PCA of urinary metabolites. Some marker metabolites were worth investigating in the future. Compared with the normal group, the level of lactic acid was higher in the cancer group urine. It could be explained that glucose is often converted into lactic acid in cancer cells, which is known as the “Warburg effect”, and cancer cells have a higher rate of aerobic glycolysis^[31]. The levels of butanedioic acid, malic acid and citric acid, intermediates of tricarboxylic acid (TCA) cycle, were also found to be higher in the gastric cancer mice. The abnormalities

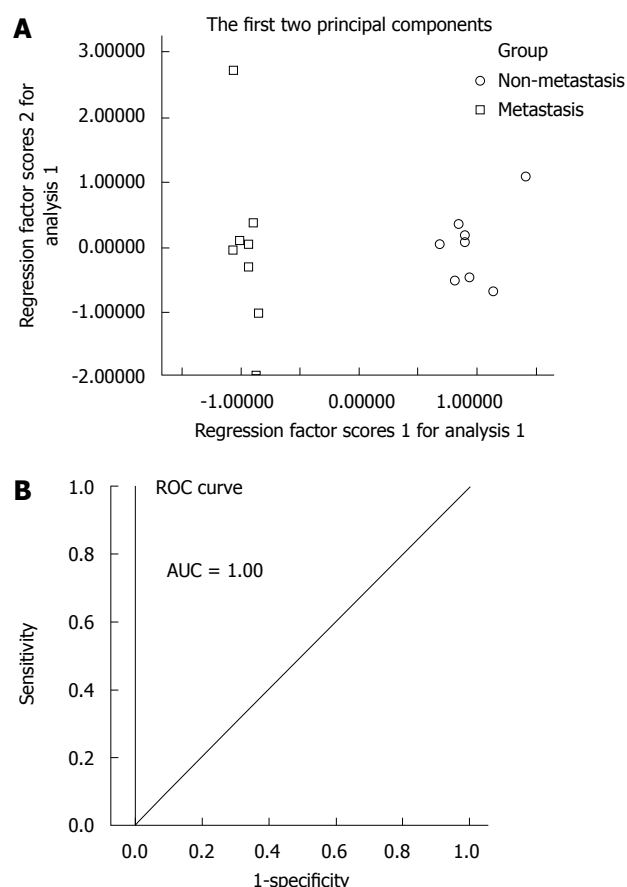


Figure 5 Principal component analysis model and receiver operating characteristic curve for gastric cancer metastasis. A: Principal component analysis (PCA) scores plot of non-metastasis group and metastasis group based on 7 marker metabolites. The PCA scores plot showed the samples from non-metastasis group and metastasis group were scattered into two different regions; B: Receiver operating characteristic (ROC) analysis was performed using the values determined by the first two components. Area under the curve (AUC) = 1.00.

of these metabolite expressions demonstrated a close correlation of TCA cycle with gastric cancer morbidity along with disordered aerobic respiration and mitochondrial functions. The disorder of aerobic respiration (mainly TCA cycle) and the impairment of mitochondrial enzymes have been reported in other malignancies including colorectal cancer, pheochromocytoma and paraganglioma^[22,32,33]. Uric acid, the final metabolite of purines, at enhanced level in cancer mice urine, suggests the abnormalities of purine metabolism in gastric cancer^[34]. In our study, the significantly higher levels of glycerol and hexadecanoic acid in cancer than in normal groups were interpreted as increased adipocyte lipolysis in cancer and enhanced expression and function of adipocyte hormone-sensitive lipase (HSL)^[35].

Cancer metastasis could be considered as an essential prognostic factor^[36]. Figure 5A shows the new constructed tumor metastatic model by seven marker metabolites for the non-metastasis group and the metastasis group. This PCA model was also validated by ROC analysis (AUC = 1.00, Figure 5B). Seven metabolites in this model are capable of predicting the gastric cancer metastasis. Compared with the non-metastasis group, levels of alanine

and glycerol were found to be lower in the metastasis group. Alanine and glycerol could get into the glycolytic pathway through gluconeogenesis, which produced more energy for the tumor progression and metastasis. The decreased level of L-proline in the metastasis group may be interpreted as increased demand for structural proteins synthesis. These proteins, including receptors, membrane channels and enzymes, play an important role in tumor progression and metastasis^[37-39]. Moreover, the higher level of myo-inositol in metastasis group urine, was consistent with the reduction of myo-inositol in lung cancer tissues^[40]. The amount of myo-inositol may be a potential indicator for gastric cancer metastasis, as it has been reported that the Gly:Myo-inositol ratio may be a useful index for brain tumor classification^[41].

What the difference of metabolite changes of butanoic acid and pyrimidine between the normal and the cancer groups, and the decreased levels of butanoic acid and L-threonic acid in the metastasis group indicates remains unclear.

In conclusion, GC/MS revealed detailed information on the metabolic profile of normal and cancer urine and was found to be suitable, in tandem with the PCA model, for the identification of metabolic variations characteristic of the gastric cancer. Furthermore, seven metabolites have been selected, which constructed a diagnostic model for distinguishing the non-metastatic and the metastatic gastric cancer. To our knowledge, this is the first report on urinary metabolomic investigation of gastric cancer metastasis by GC/MS. On the basis of this research, we believe that urinary metabolomic information obtained by GC/MS might play a significant role in the early diagnosis and screening metastasis or recurrence of gastric cancer.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries. Tumor metastasis is one of the leading causes of cancer death. Metabolic alterations play a role in the biology of cancer. The urinary metabolites as gastric cancer or tumor recurrence biomarkers can be obtained by investigating the urinary metabolic profiling.

Research frontiers

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Recently, metabolomic method has shown great potentials in identifying the new diagnostic markers and therapeutic targets for cancers. However, metabolomic studies on cancer metastasis remain scarce.

Innovations and breakthroughs

Recently, metabolomic studies on gastric cancer and colon cancer tissues have been conducted. Compared with tissues and serum, markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. This is the first report on urinary metabolomic investigation in gastric cancer using gas chromatography/mass spectrometry (GC/MS).

Applications

Potential metabolic biomarkers in urine could be used for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

Terminology

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Because of its peak resolu-

tion, high sensitivity and reproducibility, GC/MS has been widely utilized in metabolomics.

Peer review

This manuscript evaluates tumor metabolism with a goal to identify possible biomarkers with potential diagnostic value and the potential for prediction of tumor metastasis. The authors concluded that the urinary metabolomic profiling of each group is different, and the selected metabolites might be instructive to clinical diagnosis or screening metastasis for gastric cancer. This is a relevant randomized control trial using an animal model to evaluate a non-invasive method for surveillance of gastric cancer.

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S- Editor Sun H L- Editor Ma JY E- Editor Ma WH

Non-alcoholic fatty liver disease: An early mediator predicting metabolic syndrome in obese children?

Jun-Fen Fu, Hong-Bo Shi, Li-Rui Liu, Ping Jiang, Li Liang, Chun-Lin Wang, Xi-Yong Liu

Jun-Fen Fu, Hong-Bo Shi, Li-Rui Liu, Ping Jiang, Li Liang, Chun-Lin Wang, Department of Endocrinology, The Children's Hospital of Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, China

Hong-Bo Shi, Department of Pediatrics, Yinzhou People's Hospital, Ningbo 315400, Zhejiang Province, China

Ping Jiang, The First People's Hospital of Hangzhou, Hangzhou 310003, Zhejiang Province, China

Xi-Yong Liu, Clinical and Molecular Pharmacology, City of Hope National Medical Center, Duarte, CA 91009, United States

Author contributions: Fu JF, Shi HB, Liu LR and Jiang P substantially contributed to the conception and design of the study; Fu JF and Shi HB also wrote the manuscript; Wang CL collected and input the data; Liang L interpreted the data; Liu XY performed statistical analysis.

Supported by Zhejiang Provincial Natural Science Foundation, No. Y2080047; funds for Zhejiang Major Medical and Health Science and Technology Program and funds from Ministry of Health, No. WKJ2008-2-026; Major Special Zhejiang Provincial Science and Technology Fund, No. 2008c03002-1; and the National Key Technology R&D Program of China, No. 2009BAI80B01

Correspondence to: Jun-Fen Fu, MD, PhD, Department of Endocrinology, The Children's Hospital of Zhejiang University School of Medicine, 57 Zhugan Avenue, Hangzhou 310003, China. fjf68@yahoo.com.cn

Telephone: +86-571-87061007 Fax: +86-571-87033296

Received: July 20, 2010 Revised: September 6, 2010

Accepted: September 13, 2010

Published online: February 14, 2011

We measured the body mass index, waist and hip circumference, blood pressure, fasting blood glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), whole-body insulin sensitivity index (WBISI), lipid profile and transaminases in all the participants. The risk of developing metabolic syndrome (MS) was assessed according to the degree of liver fatty infiltration based on the B-ultrasound examination.

RESULTS: Among the 861 obese children, 587 (68.18%) were classified as having NAFLD, and 221 (25.67%) as having MS. The prevalence of MS in NAFLD children (groups 1 and 2) was 37.64% (221/587), which was much higher than that in non-NAFLD group (group 0, 12.04%) ($P < 0.01$). There were significantly higher incidences concerning every component of MS in group 2 compared with group 0 ($P < 0.05$). The incidence of NAFLD in MS patients was 84.61% (187/221), which was significantly higher than that of hypertension (57.46%, 127/221) and glucose metabolic anomalies (22.62%, 50/221), and almost equal to the prevalence of dyslipidemia (89.14%, 197/221). Based on the B-ultrasound scales, the presence of moderate and severe liver fatty infiltration carried a high risk of hypertension [odds ratio (OR): 2.18, 95% confidence interval (95% CI): 1.27-3.75], dyslipidemia (OR: 7.99, 95% CI: 4.34-14.73), impaired fasting glucose (OR: 3.65, 95% CI: 1.04-12.85), and whole MS (OR: 3.77; 95% CI: 1.90-7.47, $P < 0.01$). The state of insulin resistance (calculated by HOMA-IR and WBISI) deteriorated as the degree of fatty infiltration increased.

CONCLUSION: NAFLD is not only a liver disease, but also an early mediator that reflects metabolic disorder, and liver B-ultrasound can be a useful tool for MS screening.

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Key words: Childhood obesity; Non-alcoholic fatty liver disease; Metabolic syndrome; Liver B ultrasonography

Abstract

AIM: To investigate if non-alcoholic fatty liver disease (NAFLD) is an early mediator for prediction of metabolic syndrome, and if liver B-ultrasound can be used for its diagnosis.

METHODS: We classified 861 obese children (6-16 years old) into three subgroups: group 0 (normal liver in ultrasound and normal transaminases); group 1 (fatty liver in ultrasound and normal transaminases); and group 2 (fatty liver in ultrasound and elevated transaminases).

Peer reviewer: Michael L Schilsky, MD, Associate Professor of Medicine and Surgery, Yale University School of Medicine, 333 Cedar Street, LMP 1080, New Haven, CT 06520, United States

Fu JF, Shi HB, Liu LR, Jiang P, Liang L, Wang CL, Liu XY. Non-alcoholic fatty liver disease: An early mediator predicting metabolic syndrome in obese children? *World J Gastroenterol* 2011; 17(6): 735-742 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/735.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.735>

INTRODUCTION

There is a growing concern for non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MS) in obese children^[1-3]. NAFLD is a clinicopathological syndrome that ranges from simple steatosis to steatohepatitis, fibrosis or cirrhosis of the liver^[4]. It is associated with dyslipidemia, obesity, and insulin resistance, which are the main features of MS. NAFLD and MS often are seen in the same individual^[5-8], whereas insulin resistance probably is a key event that links them together. The mortality of patients with NAFLD has increased significantly among the general population, and cardiovascular risk competes with liver-related risk in dictating the final outcome^[9,10]. Several prospective studies in adults have demonstrated that NAFLD is associated with an increased incidence of MS and type 2 diabetes mellitus in elderly patients, and independent patients with obesity^[11-14]. Thus, NAFLD may be not only a liver disease, but also an early mediator of type 2 diabetes mellitus and MS in adults. However, the impact of NAFLD on MS among the young population is still not clear. The debate remains as to whether NAFLD should be included as one of the components of the MS. This study aimed to assess idiopathic NAFLD among Chinese obese children, to verify the prevalence of MS in NAFLD patients based on B-ultrasound scan, and to investigate whether NAFLD is associated with MS and MS profile, and whether it is an important risk factor for MS as well.

MATERIALS AND METHODS

Study design and population

A total of 861 obese children and adolescents, aged between 6 and 16 years, who were referred to our endocrinology department with the complaint of obesity from January 2004 to September 2009, were enrolled in this study. Based on an accepted criteria for obesity diagnosis in Chinese children^[15], all the participants had a body mass index (BMI) that was above the 95th percentile for their age and sex, based on the national reference data in 2004^[16]. Exclusion criteria were the known presence of endocrine metabolic or kidney diseases, and the use of medication that altered blood pressure, liver function, and glucose or lipid metabolism. The demographic distribution of subjects is displayed in Table 1. Participants underwent a routine clinical examination, including physical examination, biochemical tests, oral glucose tolerance

Table 1 Characteristics of 861 obese patients

	Girls (n = 263)	Boys (n = 598)
Age (yr)	10.53 ± 2.26	10.81 ± 1.97
Tanner stage (T1/T2-4)	126/137	287/311
BMI (kg/m ²) ^a	27.68 ± 4.24	28.38 ± 3.47
BMI Z-score ^a	4.31 ± 1.58	3.05 ± 1.16
WHR (waist/hip ratio) ^a	0.93 ± 0.07	0.96 ± 0.05
Waist (cm) ^a	86.53 ± 11.83	90.91 ± 10.25
Systolic pressure (mmHg) ^a	114.47 ± 13.50	117.33 ± 12.55
Diastolic pressure (mmHg)	67.85 ± 9.32	68.99 ± 9.00
Cholesterol (mmol/L)	4.48 ± 1.01	4.43 ± 0.93
Triglycerides (mmol/L)	1.79 (0.19-5.17)	1.77 (0.17-10.78)
HDL	1.23 ± 0.28	1.26 ± 0.34
LDL ^a	2.69 ± 0.73	2.51 ± 0.68
AI ^a	2.30 ± 0.83	2.11 ± 0.74
Uric acid (μmol/L)	377.0 (203.2-705.4)	383.0 (198.2-708.5)
Fasting glucose (mmol/L)	4.87 ± 0.58	4.90 ± 0.57
120-min glucose (mmol/L) ^a	6.31 ± 1.52	6.01 ± 1.44
HbA1c (%)	5.74 ± 0.57	5.69 ± 0.52
Fasting insulin (mIU/L) ^a	17.9 (0.3-303.0)	15.3 (0.3-219.0)
HOMA-IR ^a	3.77 (0.07-78.11)	3.33 (0.07-51.59)
WBISI ^a	2.59 (0.39-11.90)	3.37 (0.52-24.19)
ALT (U/L) ^a	37.5 (8.0-283.0)	29.0 (4.0-561.0)

^aP < 0.05 boys vs girls. Data are expressed as mean ± SD or median (range). BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AI: Atherogenic index; HOMA-IR: Homeostasis model assessment of insulin resistance; WBISI: Whole-body insulin sensitivity index; ALT: Alanine aminotransferase.

test (OGTT) and liver ultrasonographic scanning. There was no difference in age and Tanner stages between boys and girls. However, significant differences existed in clinical, anthropometric and laboratory data in relation to sex (Table 1).

The protocol was approved by the Medical Ethics Committee of The Children's Hospital of Zhejiang University School of Medicine. Written informed consent from parents (or guardians) and children (where appropriate) were obtained.

Definition of disease, syndrome and disorders

NAFLD: NAFLD was defined according to the revised definition and treatment guidelines for NAFLD by the Chinese Hepatology Association in February 2006^[17], and was diagnosed by means of a protocol using clinical, laboratory and ultrasound examinations in combination. In this study, NAFLD was diagnosed as a diffusely echogenic change on liver B-ultrasonography (fatty infiltration in liver), with or without elevated serum aminotransferase levels and other factors that can cause liver fatty infiltration or aminotransferase elevation, such as hepatitis virus infection, drug-induced injury, and other metabolic diseases, such as Wilson's disease, were excluded. There was no history of current or past alcohol drinking. Two subgroups were classified in NAFLD obese children: group 1 (fatty liver in ultrasound and normal transaminases) and group 2 (fatty liver in ultrasound and elevated transaminases). Group 0 was diagnosed as obese children without liver disorder (normal liver in ultrasound and normal transaminases).

MS: The diagnosis criteria have been described in our previous study^[18], which followed the suggestions of the Chinese Diabetes Association and the definition modified from the National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATPIII). MS was diagnosed if patients met three or more of the following criteria for age and sex: (1) central obesity; (2) hyperglycemia (fasting glucose ≥ 6.1 mmol/L, or random glucose ≥ 7.8 mmol/L) or impaired glucose tolerance (IGT) or type 2 diabetes; (3) systolic or diastolic blood pressure above the 95th percentile for age and sex; and (4) hypertriglyceridemia (triglyceride concentration > 1.7 mmol/L) or low high-density lipoprotein-cholesterol (HDL-C) (< 1.03 mmol/L). In this study, high blood pressure was based on the percentile data of the 7th edition of Practical Pediatric Text Book by Zhu Fu-Tang^[15]. IGT was defined as a glucose level > 7.8 mmol/L but < 11.1 mmol/L at 2 h. Type 2 diabetes was diagnosed according to the criteria of American Diabetes Association in 1997 and World health Organization in 1999.

Laboratory assessment

Height was measured without socks and shoes, and weight was measured in children who were wearing only under-clothing. Waist was measured at the midpoint between the lower border of the rib cage and the iliac crest. Hip circumference was determined at the widest circle of the bottom. Pubertal development stages were assessed using Tanner stage criteria. Blood pressure was taken twice using the right arm, with the subject in a quiet sitting position, and the average level was recorded.

Subjects underwent routine biochemical evaluation in the morning before 09:00 h after an overnight fast for at least 8 h. Fasting glucose, insulin, lipids total triglyceride (TG), total cholesterol (TCHO), HDL-C and low-density lipoprotein-cholesterol (LDL-C), liver function [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and uric acid were detected. Hepatitis serial serological tests (antibodies for hepatitis A-E) and glucose and insulin levels were also recorded during a standard (75 g) OGTT. Blood glucose was determined using a glucose oxidase method (North Biotechnology Company, Beijing, China) with intra-assay and inter-assay coefficient of variation (CVs) of 2.1% and 4.4%. Insulin serum levels were determined by radioimmunoassay (North Biotechnology Company) with intra-assay and inter-assay CVs of 6.4% and 9.7%, respectively. The serum concentrations of TCHO, TG, HDL-C, LDL-C, ALT, AST and uric acid were measured by routine enzymatic methods in our clinical laboratory.

The homeostasis model assessment of insulin resistance (HOMA-IR), based on serum fasting glucose and insulin levels, was used to measure insulin resistance. The whole body insulin sensitive index (WBISI) and the ratio of early insulin increment to early glucose increment (I30-0/G30-0) following oral glucose loading (75 g) were also obtained. $HOMA-IR = [FIN \text{ (mU/L)} \times FBG \text{ (mg/dL)}] / 22.5$; $WBISI = 10000 / [FIN \text{ (mU/L)} \times FBG \text{ (mg/dL)} \times \text{average insulin (mU/L)} \times \text{average glucose (mg/dL)}]^{1/2}$; $BMI = \text{weight (kg)} / [\text{height (m)}]^2$ (FIN: Free insulin; FBG: Free blood glucose).

Liver ultrasound examination

Liver ultrasound examination was carried out by one specialist who was unaware of the aims of the study and blinded to laboratory values on the same equipment (GE, LOGIC 500), using a convex 3.5-5.0 MHz probe. Sagittal hepatic sections that encompassed longitudinal images of the right liver lobe and the ipsilateral kidney were obtained. Liver-kidney contrast with two other well-known ultrasonographical findings of fatty liver, vascular blurring and deep attenuation, enabled us to grade fatty change semi-quantitatively. Fatty infiltration was graded semi-quantitatively into four classes^[19,20]: no steatosis (class 0), mild steatosis (class 1), moderate steatosis (class 2) and severe steatosis (class 3) (Figure 1).

Statistical analysis

Data were collected using an MS-Excel spreadsheet. Data were analyzed using the JMP Statistical Discovery Software version 7.0 (SAS Institute, Cary, NC, USA). Group comparisons for continuous data were performed using *t* tests for independent means or one-way analysis of variance. A non-parametric test was used to evaluate the significance of abnormally distributed data. For categorical data, we employed the χ^2 test, Fisher's exact test, or binomial test of proportions. Multivariate logistic regression models were used to adjust for covariate effects on the odds ratio (OR). Statistical significance was set at $P < 0.05$.

RESULTS

Prevalence of NAFLD and MS in Chinese obese children

Of 861 obese children, 587 (68.18%) were diagnosed as having NAFLD, and 221 (25.67%) as having MS. The prevalences of hypertension, dyslipidemia, hyperuricacidemia, impaired fasting glucose, IGT and diabetes were 37.28% (321), 42.04% (362), 28.69% (247), 6.38% (55), 8.01% (69) and 1.39% (12), respectively. Moreover, the incidence of NAFLD in MS patients reached 84.61% (187/221), which was significantly higher than that of hypertension (57.46%, 127/221) and glucose metabolic anomalies (22.62%, 50/221), but almost equal to the prevalence of dyslipidemia (89.14%, 197/221).

NAFLD is associated with MS and its components among obese children

To investigate whether liver disorders are associated with MS, participants were divided into three subgroups: group 0 (normal liver in ultrasound and normal transaminases); group 1 (fatty liver in ultrasound and normal transaminases); and group 2 (fatty liver ultrasound and elevated transaminases). Table 2 indicates that sex, age and Tanner stage were comparable among the three subgroups. Nevertheless, the occurrence rates of MS increased with deterioration of liver from 12.04% in group 0 to 29.36% in group 1, and 39.74% in group 2 ($P < 0.05$). The prevalence of hypertension, dyslipidemia, IGT and diabetes in group 2 was significantly higher than that in group 0 ($P < 0.05$). Further investigation indicated that the prevalence of dyslipidemia and IGT increased steadily from group 0 to group 1 and group 2

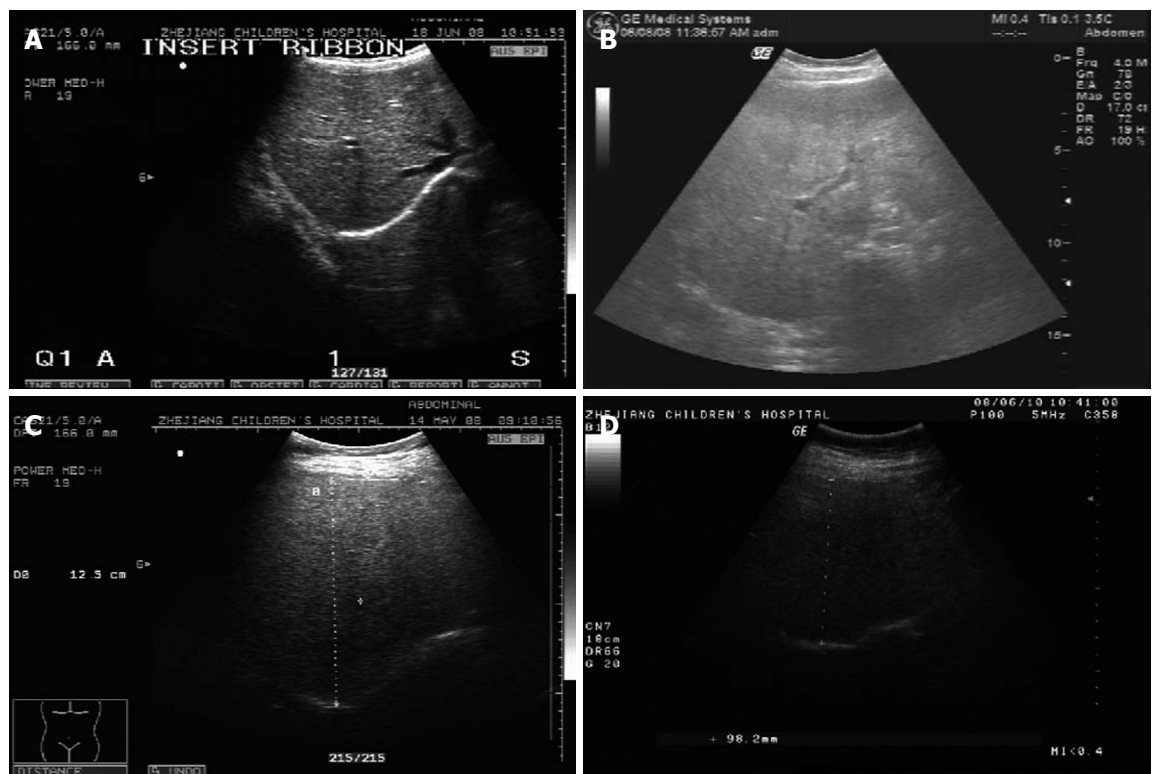


Figure 1 Liver B-ultrasound scans show the panel of four different classes of liver steatosis. A: Class 0: not observed; B: Class 1: mild, liver-kidney contrast without vascular blurring and deep attenuation; C: Class 2: moderate, liver-kidney contrast with vascular blurring, but no deep attenuation; D: Class 3: severe, combination of liver-kidney contrast with vascular blurring and deep attenuation.

Table 2 Comparison of prevalence of metabolic syndrome and its components among three groups			
	Group 0 (n = 274)	NAFLD	
		Group 1 (n = 436)	Group 2 (n = 151)
Age (yr)	10.36 ± 2.15	10.59 ± 1.85	10.40 ± 2.28
Sex (M/F)	187/87	305/131	106/45
Tanner stage (T1/T2-4)	130/144	209/227	74/77
BMI	26.88 ± 3.19	28.78 ± 3.96 ^a	28.85 ± 3.69 ^a
Hypertension	68 (24.82)	180 (41.28) ^a	73 (48.34) ^a
Dyslipidemia	42 (15.32)	204 (46.78) ^a	116 (76.82) ^{a,c}
Impaired fasting glucose	5 (1.82)	30 (6.88)	20 (13.24) ^{a,c}
IGT	13 (4.74)	38 (8.72)	18 (11.92) ^a
Diabetes	0 (0.00)	5 (1.14)	7 (4.63) ^{a,c}
MS	33 (12.04)	128 (29.36) ^a	60 (39.74) ^{a,c}

^a*P* < 0.05 *vs* group 0; ^c*P* < 0.05, group 1 *vs* group 2. Data are expressed as percentage, mean ± SD. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; IGT: Impaired glucose tolerance; MS: Metabolic syndrome.

(15.32% to 46.78% to 76.82%; 1.82% to 6.88% to 13.24%, *P* < 0.05). In addition, the levels of triglycerides, cholesterol, ALT and uric acid in group 2 were significantly higher than those in group 1 and group 0 (*P* < 0.05) (Table 3). It was indicated that the NAFLD was closely associated with progression of MS and its components in these obese children.

NALFD is accompanied by insulin resistance in obese children

Insulin resistance is a key event that causes MS in both

adults and children^[21]. To investigate whether NAFLD is associated with insulin resistance, we performed OGTT and insulin releasing test in all the participants. As for the results of blood glucose, fasting blood glucose and 120-min OGTT glucose levels in group 2 were significantly higher than those in group 0 (*P* < 0.05). The blood level of insulin, including fasting insulin, 30-min OGTT insulin and 120-min OGTT insulin, were significantly increased in groups 1 and 2 as compared with those in group 0 (*P* < 0.05). The HOMA-IR and WBISI reflected insulin resistance and sensitivity respectively. Our analysis indicated that HOMA-IR was elevated significantly in groups 1 and 2, whereas WBISI decreased significantly as compared with that of group 0 (*P* < 0.05) (Table 3). This finding revealed that NAFLD was significantly associated with insulin resistance among these obese children.

MS is associated with liver steatosis found by ultrasound examination

Fatty infiltration is another indicator that reflects liver damage, which can be easily detected by ultrasound examination. Based on B-ultrasound examination, 861 obese children were classified into class 0 (274 cases without steatosis), class 1 (105 cases with mild steatosis), and classes 2-3 (482 cases with moderate and severe steatosis). It was indicated that the relative risk of MS increased to 3.10 [95% confidence interval (95% CI): 1.20-8.00] in class 1 and 3.77 (95% CI: 1.90-7.47) in classes 2-3 (*P* < 0.01) (Table 4). Based on the B-ultrasound scales, the presence of moderate and severe liver fatty infiltration carried a high risk of

Table 3 Clinical and laboratory data of non-alcoholic fatty liver disease compared with obese children without liver disorder

	Group 0 (<i>n</i> = 274)	NAFLD	
		Group 1 (<i>n</i> = 436)	Group 2 (<i>n</i> = 151)
Age (yr)	10.36 ± 2.15	10.59 ± 1.85	10.40 ± 2.28
BMI (kg/m ²)	26.58 ± 3.19	28.85 ± 3.69 ^a	28.78 ± 3.96 ^a
BMI Z-score	3.17 ± 1.26	3.33 ± 1.37	3.93 ± 1.60 ^{a,c}
Waist circumference (cm)	85.63 ± 10.17	91.58 ± 11.24 ^a	92.2 ± 9.31 ^a
Father BMI (kg/m ²)	25.12 ± 3.68	25.68 ± 5.78	25.55 ± 3.75
Mother BMI (kg/m ²)	22.83 ± 3.14	23.46 ± 3.61 ^a	23.64 ± 3.38 ^a
Systolic blood pressure (mmHg)	113.62 ± 11.89	118.30 ± 13.37 ^a	118.03 ± 12.73 ^a
Diastolic blood pressure (mmHg)	67.80 ± 9.02	69.00 ± 9.38	69.43 ± 8.67 ^a
ALT (U/L)	24 (8-74)	29 (4-75) ^a	51.5 (76-561) ^{a,c}
Uric acid (μmol/L)	352.9 (186.5-603.20)	365.9 (168.10-708.50) ^a	412.5 (7.38-771.30) ^{a,c}
Triglycerides (mmol/L)	1.4 (0.19-6.76)	1.42 (0.26-5.90)	1.77 (0.28-8.64) ^{a,c}
Cholesterol (mmol/L)	4.37 ± 0.99	4.34 ± 0.87	4.71 ± 0.99 ^{a,c}
HDL	1.29 ± 0.39	1.23 ± 0.28	1.22 ± 0.28
LDL	2.52 ± 0.66	2.54 ± 0.68	2.68 ± 0.78
AI	2.07 ± 0.73	2.15 ± 0.70	2.31 ± 0.92 ^a
Fasting blood glucose (mmol/L)	4.82 ± 0.52	4.88 ± 0.69	4.95 ± 0.55 ^a
30-min OGTT glucose (mmol/L)	7.54 ± 1.17	7.73 ± 1.24	7.69 ± 1.21
120-min OGTT glucose (mmol/L)	5.66 ± 1.36	6.31 ± 1.42 ^a	6.22 ± 1.63 ^a
HbA1c (%)	5.70 ± 0.50	5.71 ± 0.53	5.65 ± 0.62
Fasting insulin (mIU/L)	12.7 (0.3-187.0)	17.0 (0.3-219.0) ^a	18.2 (2.1-303.0) ^a
30-min OGTT insulin (mIU/L)	85.3 (1.8-400.0)	112.0 (0.5-400.0) ^a	106.2 (1.6-400.0) ^a
120-min OGTT insulin (mIU/L)	36.3 (3.2-400.0)	63.6 (1.7-386.0) ^a	69.8 (7.0-400.0) ^a
HOMA-IR	2.61 (0.07-41.56)	3.71 (0.08-51.59) ^a	3.95 (0.48-78.11) ^a
WBISI	4.0 (0.6-16.4)	2.8 (0.9-24.2) ^a	2.6 (0.4-16.7) ^a

^a*P* < 0.05 *vs* group 0; ^c*P* < 0.05, group 1 *vs* group 2. Data are expressed as mean ± SD or median (range). NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AI: Atherogenic index; OGTT: Oral glucose tolerance test; HOMA-IR: Homeostasis model assessment of insulin resistance; WBISI: Whole-body insulin sensitivity index.

developing hypertension (OR: 2.18, 95% CI: 1.27-3.75), dyslipidemia (OR: 7.99, 95% CI: 4.34-14.73), impaired fasting glucose (OR: 3.65, 95% CI: 1.04-12.85), and hyperuricacidemia (OR: 3.76, 95% CI: 2.03-6.96) (Table 4, Figures 2-4). We also determined whether HOMA-IR and WBISI were associated with the degree of fatty liver. It was revealed that the HOMA-IR increased significantly from 2.64 in class 0 to 3.57 in classes 2-3, but the WBISI decreased from 3.97 in class 0 to 2.67 in classes 2-3 among the obese subjects. All these findings indicated that the scale of fatty infiltration in liver was closely related to MS and insulin resistance among Chinese obese children.

DISCUSSION

The prevalence of both NAFLD and MS was higher in this study at 68.18% and 25.67%, respectively, than that of 9.6% and 4.2% in the general pediatric population^[22]. NAFLD is regarded as an increasing clinical problem in children and adolescents, and accounts for the vast majority of cases with elevated serum liver enzymes^[23]. Moreover, NAFLD is known to be related to the factors that predict the development of coronary heart disease, such as dyslipidemia, central obesity and MS.

Apart from that, NAFLD has been shown increasingly and more convincingly to be an important component of MS. In this study, we demonstrated that the prevalence of MS was three times higher in NAFLD obese children than in those without liver disorders (39.74% *vs* 12.04%, *P* < 0.05). The incidence of each component of MS was also

Table 4 Metabolic syndrome in obese patients diagnosed by B-ultrasound

	Scale (0) (<i>n</i> = 274)	Scale (1) (<i>n</i> = 105)	Scale (2-3) (<i>n</i> = 482)	<i>P</i>
Hypertension				
No	206	54	280	
Yes	68	51	202	< 0.05
OR (95% CI)	1.0	2.87 (1.29-6.37)	2.18 (1.27-3.75)	
Dyslipidemia				
No	232	57	210	
Yes	42	48	272	< 0.01
OR (95% CI)	1.0	5.21 (2.23-12.18)	7.99 (4.34-14.73)	
Impaired fasting glucose				
No	269	102	435	
Yes	5	3	47	< 0.05
OR (95% CI)	1.0	1.0 (0.1-9.94)	3.65 (1.04-12.85)	
Impaired glucose tolerance				
No	261	99	432	
Yes	13	6	50	> 0.05
OR (95% CI)	1.0	1.53 (0.27-8.74)	2.9 (0.95-8.87)	
Hyperuricacidemia				
No	232	81	301	
Yes	42	24	181	< 0.01
OR (95% CI)	1.0	1.83 (0.71-4.74)	3.76 (2.03-6.96)	
MS				
No	241	75	324	
Yes	33	30	158	< 0.01
OR (95% CI)	1.0	3.10 (1.20-8.00)	3.77 (1.90-7.47)	

MS: Metabolic syndrome; OR: Odds ratio; CI: Confidence interval.

significantly higher in NAFLD subjects (*P* < 0.05). Moreover, the presence of moderate and severe liver infil-

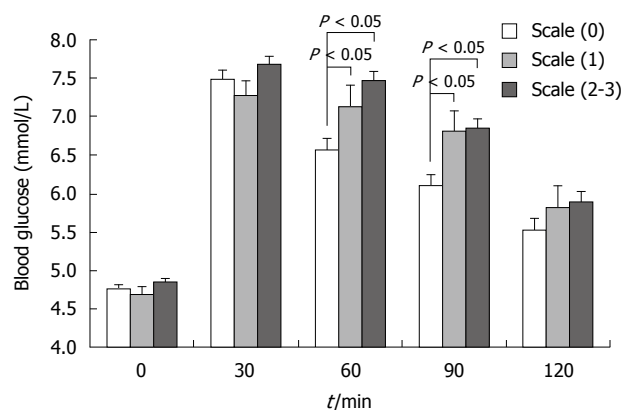


Figure 2 Oral glucose tolerance test in obese patients based on liver B-ultrasound gradings. When patients were stratified according to the presence of liver fatty infiltration based on the B-ultrasound scans, their glucose levels at 60 min and 90 min after tolerance were significantly higher in classes 1-3 ($P < 0.05$) than in class 0, but there was no difference between class 1 and classes 2-3 ($P > 0.05$).

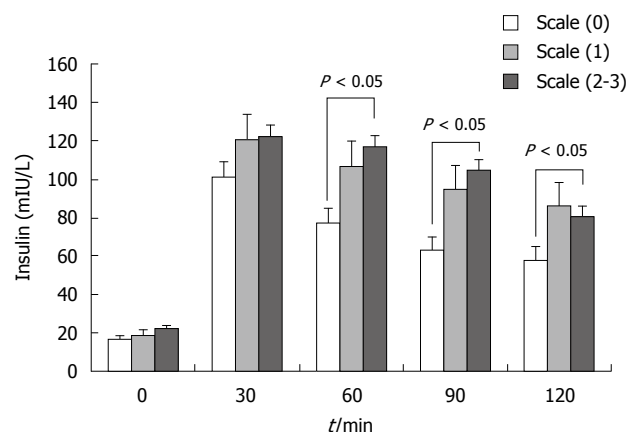


Figure 3 Insulin releasing test in obese patients based on liver B-ultrasound gradings. There was no difference in fasting insulin among the three groups, but 60, 90 and 120 min insulin levels were markedly increased in the class 2-3 groups compared with class 0 ($P < 0.05$).

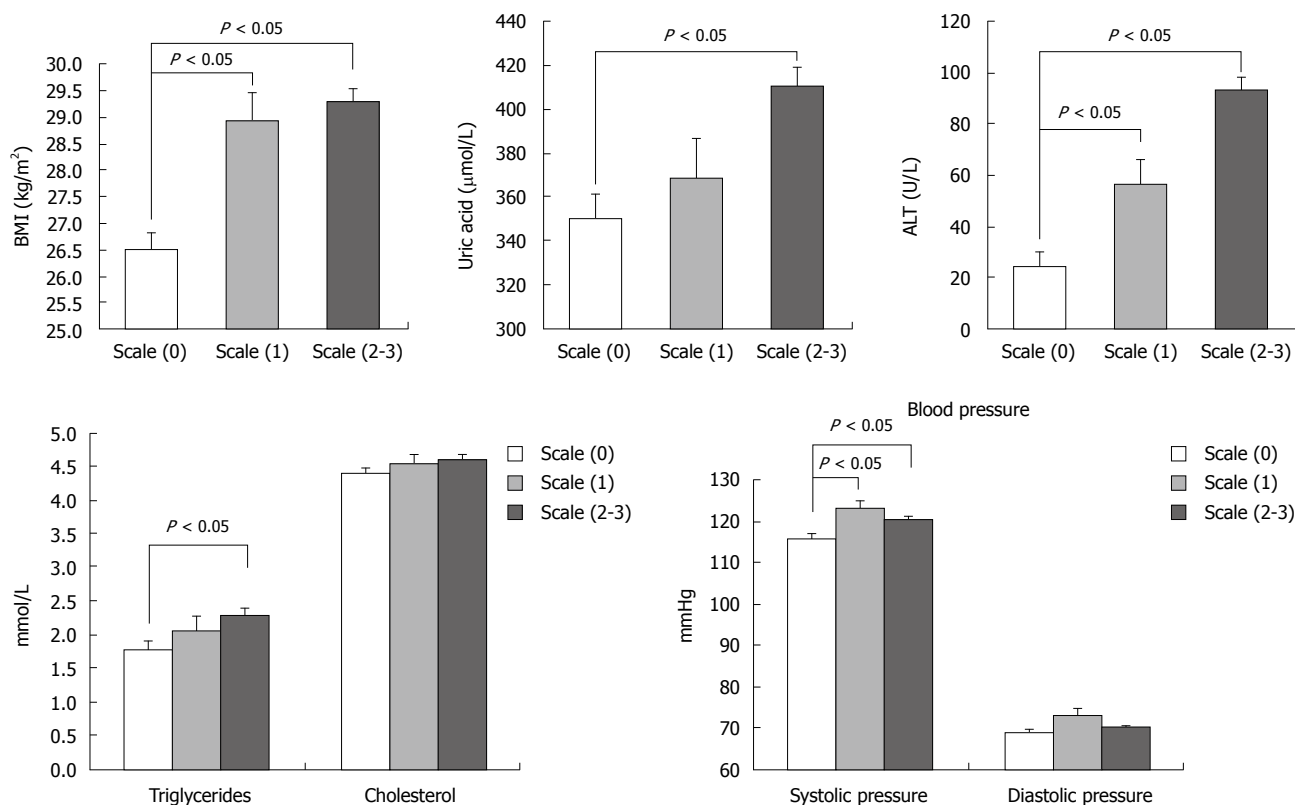


Figure 4 Clinical features of the metabolic syndrome in obese patients based on liver B-ultrasound gradings. Concerning the individual metabolic component, body mass index (BMI), alanine aminotransferase (ALT) and systolic pressure were significantly higher in class 1 and classes 2-3 compared with class 0 ($P < 0.05$), but there was no difference between class 1 and classes 2-3 ($P > 0.05$). The levels of uric acid and triglyceride were only markedly increased in classes 2-3 compared with class 0 ($P < 0.05$). There was no difference in cholesterol levels among the three groups ($P > 0.05$).

tration confirmed by B-ultrasonography carried a high risk of development of MS, which highlights that NAFLD is closely associated with features of MS. In contrast, the incidence of NAFLD in MS patients reached 84.61%, which was significantly higher than that of hypertension (57.46%) and glucose metabolic anomalies (22.62%), and almost equal to the prevalence of dyslipidemia (89.14%). This indicates that NAFLD might be an early stage mediator for prediction of MS.

To date, the biological mechanisms that are involved in the higher risk of developing metabolic disorders in patients with NAFLD are not fully understood. Insulin resistance seems to be the key event. Nevertheless, most obese patients with NAFLD had hyperinsulinemia and higher insulin resistance compared with those without liver disorders, as calculated by fasting insulin, 30 min and 120 min insulin after glucose loading, HOMA-IR and WBISI. In contrast, patients with NAFLD were more

obese and exhibited higher insulin resistance and more marked metabolic complications than those with simple obesity. Fatty liver itself is an insulin resistance status^[24], and because hepatic fat accumulation can lead to hepatic insulin resistance, the latter may occur before any alteration in peripheral insulin action or peripheral insulin resistance. Moreover, insulin resistance may cause abnormalities of lipid storage and lipolysis in insulin-sensitive tissues, which may induce increased fatty acid flux from adipose tissue to the liver and result in steatosis^[25]. Insulin resistance may also cause lipid peroxidation, which in turn, activates inflammatory cytokines and promotes the progression of innocent steatosis to non-alcoholic steatohepatitis and liver fibrosis^[26]. The impairment in fat and glucose metabolism when insulin resistance occurs, can lead to similar biochemical and clinical abnormalities in patients with NAFLD, and sooner or later it will inevitably develop to systemic MS.

An accurate fatty liver diagnosis and staging of non-alcoholic steatohepatitis requires liver biopsy. However, liver biopsy is not performed often in patients, especially in children, with no significant or trivial liver diseases. Most of the patients with liver steatosis can be well-managed without a need for liver biopsy. In our study, steatosis was assessed by liver ultrasonography with a sensitivity of 83% and a specificity of 100% in comparison with the gold standard of histological diagnosis. Therefore, liver ultrasonography is being strongly suggested as a non-invasive study of NAFLD^[18,27,28]. It was particularly interesting to find that both the prevalence of MS and every component of MS increased as the liver ultrasonographic grade deteriorated. Moreover, liver fat is highly significantly associated with all components of MS. Compared with abdominal and overall obesity, fatty liver has the highest frequency of clustering, greater specificity, higher positive predictive value and the most attributable risk for detecting risk factors of cardiovascular disease, type 2 diabetes and MS^[29,30]. Thus, fatty liver seems to be an early mediator for prediction of other metabolic disorders. Since B ultrasound scan can be easily and widely applied in hospitals, this simple and effective scanning technique may provide a new method of MS screening in the future.

All findings in our study have stimulated interest in the possible role of NAFLD in the development of metabolic complications. Their coexistence in the same individual increases the likelihood of having more severe dysmetabolic status. Several studies in adults also have demonstrated that NAFLD is the primary hepatic complication of obesity and insulin resistance, and may be considered the early hepatic manifestation of MS. Early treatment, such as lifestyle or diet modification, aerobic exercise, or medication (metformin or vitamin E), for NAFLD may not only improve the prognosis of liver disease, but also reduce the occurrence of underlying metabolic and vascular complications. Furthermore, it is also implied that earlier adjustment to mobilizing fat out of the liver might reduce the risks of MS.

ACKNOWLEDGMENTS

We are grateful to Dr. Li-Qin Chen and Zhong-Sheng Yu for their assay expertise. We sincerely thank the parents and children for participating in these studies. We thank the nursing staff of our department for their dedicated care of these young patients during the collection and evaluation of blood samples.

COMMENTS

Background

There is a growing concern for non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MS) in obese children. NAFLD and MS often are seen in the same individual, and insulin resistance is a probable key event that links them together. NAFLD may not only be a liver disease, but also an early mediator of type 2 diabetes mellitus and MS in adults. However, the impact of NAFLD on MS among the young population is still not clear.

Research frontiers

NAFLD is now considered a metabolic pathway to advanced liver disease, cirrhosis and hepatocellular carcinoma. Type 2 diabetes mellitus, obesity and dyslipidemia are the principal factors associated with NAFLD, which is now considered the hepatic expression of MS. The risk of liver disease associated with the classical features of MS in children is still unclear. We still need to clarify the mechanisms that are responsible for liver disease progression from pure fatty liver to steatohepatitis and to cirrhosis, and the reasons why only a few NAFLD cases progress to terminal liver failure while others (the majority) will have a cardiovascular outcome.

Innovations and breakthroughs

An accurate fatty liver diagnosis and staging of non-alcoholic steatohepatitis require liver biopsy. However, liver biopsy is not performed often in patients, especially children, with no significant, or trivial liver diseases. Most of the patients with liver steatosis can be well-managed without a need for liver biopsy. In this study, steatosis was assessed by liver ultrasonography, which showed that both the prevalence of MS and every component of MS increased as the liver ultrasonographic grade deteriorated. Liver fat was highly significantly associated with all components of MS.

Applications

Since B ultrasound scan can be easily and widely applied in hospitals, this simple and effective scanning technique may provide a new method of MS screening in the future in the general population.

Peer review

The authors addressed an important subject and described a population of patients referred for obesity with respect to the presence of sonographic evidence for NAFLD, and their metabolic profiles with respect to the insulin axis.

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S- Editor Sun H L- Editor Kerr C E- Editor Lin YP

Perinatal and early life risk factors for inflammatory bowel disease

Stephen E Roberts, Clare J Wotton, John G Williams, Myfanwy Griffith, Michael J Goldacre

Stephen E Roberts, John G Williams, College of Medicine, Swansea University, Swansea SA2 8PP, United Kingdom
 Clare J Wotton, Myfanwy Griffith, Michael J Goldacre, Unit of Health-Care Epidemiology, Department of Public Health, University of Oxford, Oxford OX3 7LF, United Kingdom
 Author contributions: Goldacre MJ and Roberts SE designed the study; Roberts SE and Goldacre MJ reviewed the literature; Wotton CJ and Griffith M undertook the analyses; Roberts SE, Goldacre MJ and Williams JG interpreted the study findings and wrote the manuscript.

Supported by (in part) National Institute for Health Research, England, Grant No. NCCRCD ZRC/002/002/026

Correspondence to: Michael J Goldacre, Professor, Unit of Health-Care Epidemiology, Department of Public Health, University of Oxford, Old Road, Oxford OX3 7LF, United Kingdom. michael.goldacre@dphpc.ox.ac.uk

Telephone: +44-1865-289377 Fax: +44-1865-289379

Received: July 10, 2010 Revised: August 16, 2010

Accepted: August 23, 2010

Published online: February 14, 2011

Abstract

AIM: To investigate associations between perinatal risk factors and subsequent inflammatory bowel disease (IBD) in children and young adults.

METHODS: Record linked abstracts of birth registrations, maternity, day case and inpatient admissions in a defined population of southern England. Investigation of 20 perinatal factors relating to the maternity or the birth: maternal age, Crohn's disease (CD) or ulcerative colitis (UC) in the mother, maternal social class, marital status, smoking in pregnancy, ABO blood group and rhesus status, pre-eclampsia, parity, the infant's presentation at birth, caesarean delivery, forceps delivery, sex, number of babies delivered, gestational age, birthweight, head circumference, breastfeeding and Apgar scores at one and five minutes.

RESULTS: Maternity records were present for 180 children who subsequently developed IBD. Univariate

analysis showed increased risks of CD among children of mothers with CD ($P = 0.011$, based on two cases of CD in both mother and child) and children of mothers who smoked during pregnancy. Multivariate analysis confirmed increased risks of CD among children of mothers who smoked (odds ratio = 2.04, 95% CI = 1.06-3.92) and for older mothers aged 35+ years (4.81, 2.32-9.98). Multivariate analysis showed that there were no significant associations between CD and 17 other perinatal risk factors investigated. It also showed that, for UC, there were no significant associations with the perinatal factors studied.

CONCLUSION: This study shows an association between CD in mother and child; and elevated risks of CD in children of older mothers and of mothers who smoked.

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Key words: Crohn's disease; Ulcerative colitis; Perinatal risk factors; Record linkage

Peer reviewer: Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

Roberts SE, Wotton CJ, Williams JG, Griffith M, Goldacre MJ. Perinatal and early life risk factors for inflammatory bowel disease. *World J Gastroenterol* 2011; 17(6): 743-749 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/743.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.743>

INTRODUCTION

Both Crohn's disease (CD) and ulcerative colitis (UC) are considered to be immune-mediated disorders, although the exact pathogenetic mechanisms are not yet clear. It is thought that a combination of environmental factors in genetically susceptible people lead to disordered im-

munity and chronic inflammation. Over the last 50 years, there have been large increases in the incidence of CD and UC in the UK, in other western countries^[1-3], and more recent increases in Asia^[4], which indicate changes over time in the environmental factors that can lead to inflammatory bowel disease (IBD).

In recent decades, there have been changes in the management of births, including large increases in caesarean deliveries, advances in neonatal medicine and substantial reductions in neonatal mortality. As perinatal risk factors have been associated with some immune-mediated diseases including asthma^[5-7] and type 1 diabetes^[8-10], perinatal risk factors and early life events may be relevant to other immune-mediated diseases, including IBD. One case-control study identified that infectious and non-infectious perinatal health events were linked with 40% of all cases of IBD in the study group^[11]. A systematic review and meta-analysis of 17 (mainly case-control) studies found a small but significant protective effect of breastfeeding against both CD and UC^[12]. However, its authors commented that this finding was far from conclusive, and advocated the need for larger studies. There have been relatively few studies of other perinatal risk factors and IBD.

The aim of this study was to investigate associations between 20 perinatal risk factors and the subsequent development of IBD in children and young adults in a large geographically defined population of South East England. These perinatal risk factors include nine maternal characteristics, such as maternal age, parity, smoking during pregnancy, ABO blood group and social class, and 11 neonatal characteristics, including birthweight, gestational age, head circumference, breastfeeding and Apgar scores.

MATERIALS AND METHODS

Ethical approval for analysis of the record linkage study data was obtained from the Central and South Bristol Multi-Centre Research Ethics Committee (04/Q2006/176).

We used the Oxford record linkage study (ORLS). The ORLS comprises abstracts of records of birth registrations, maternities, day cases and inpatient admissions in a defined geographical region of South East England around Oxford. The maternity data covered all National Health Service (NHS) hospitals in two health districts of the ORLS over the 20-year period from 1970 to 1989. The maternity data are linked to data on all inpatient and day case care for all clinical specialties in the ORLS up to 1999, in two health districts of the ORLS from 1970 to 1999 (population 0.9 million) and a further four adjacent districts (total population 1.9 million) from 1975 to 1999. We used the record linked data to identify cases of subsequent IBD in the children covered by the maternity data. We also used the linked data to identify records of IBD in the mothers, before and after childbirth. The original data comprising the ORLS were abstracted from hospital records by staff who were specifically trained for this purpose by senior clinicians.

We excluded maternity records in the ORLS for 985 abortions, 1560 stillbirths and 1567 early deaths that oc-

curred within 30 d of birth. We also excluded 289 births in which the birthweight was recorded as < 1000 g, because most of these records had implausibly low values and/or substantial missing data for many of the perinatal risk factors that we were investigating. None of these excluded babies were subsequently identified as having IBD. After applying these exclusion criteria, a total of 248 659 births remained in the study.

Cases of CD and UC among offspring and mothers were identified using the following ICD codes on inpatient or day case records: 563.0 and 563.1 for CD and UC, respectively (in the ICD-8th revision), 555 and 556 (ICD-9) and K50 and K51 (ICD-10), when recorded in any diagnostic position on the hospital record. There were 114 and 66 children with both a maternity record and a subsequent admission for CD or UC, respectively. We compared 20 perinatal factors studied in these cases with those in the other 248 479 children without a record of inpatient admission or day case care for CD or UC. Parity was defined as the pregnant woman's number of previous live and still births, as recorded on the ORLS maternity record. The length of "follow-up" for offspring ranged from 30 years for those born in 1970 to 10 years for those born in 1989, with an average follow-up of 18 years.

Statistical methods used included the chi-square test with Yates' correction, odds ratios (ORs) and their 95% CI, and multivariate logistic regression. Statistical significance was accepted at the conventional 5% level. When using logistic regression, all perinatal risk factors that were significant ($P < 0.05$) in the univariate analysis were included in an initial model. Each of the factors that were not significant in the univariate analysis were then re-entered, one at a time, into the regression model. This approach was taken to test whether any perinatal factor that was not significant in the initial univariate analysis, became significant when assessed simultaneously with other significant factors in the multivariate analysis. Cases with missing data for the perinatal risk factors were excluded only for those risk terms that were included in the logistic regression model. Year of birth was routinely included in all of the models, as a potential confounder, because of the different periods of follow-up after different years of birth.

RESULTS

For the 114 and 66 children identified with CD and UC respectively, the age at first admission (mean \pm SD) was 17.5 ± 5.1 years and 17.7 ± 6.0 years. Approximately half of the cases of both CD and UC had a first recorded admission in early adulthood (when aged 18 to 30 years) rather than in childhood (Table 1). A majority of cases were female; 59 (52%) for CD and 37 (56%) for UC.

CD

Considering risk factors relating to the maternity, in univariate analysis there was a significant association ($P =$

Table 1 Age at first day case or inpatient admission for offspring with inflammatory bowel disease *n* (%)

	Age at first hospitalisation (yr) with inflammatory bowel disease						Total
	< 1	1-4	5-9	10-14	15-19	20-29	
Crohn's disease							
Male	1 (2)	1 (2)	4 (7)	11 (20)	18 (33)	20 (36)	55 (100)
Female	0	0	0	11 (19)	19 (32)	29 (49)	59 (100)
Total	1 (1)	1 (1)	4 (4)	22 (19)	37 (32)	49 (43)	114 (100)
Ulcerative colitis							
Male	0	1 (3)	2 (7)	2 (7)	7 (24)	17 (59)	29 (100)
Female	0	1 (3)	3 (8)	6 (16)	11 (30)	16 (43)	37 (100)
Total	0	2 (3)	5 (8)	8 (12)	18 (27)	33 (50)	66 (100)

Table 2 Associations between maternal characteristics and inflammatory bowel disease in the child

Maternal characteristics	No. of births	Crohn's disease			Ulcerative colitis		
		No. of cases	Percent	<i>P</i> -value ¹	No. of cases	Percent	<i>P</i> -value ²
Maternal age (yr)				0.19			0.90
14-24	86 544	41	0.047%		21	0.024%	
25-34	142 939	59	0.041%		40	0.028%	
35-49	18 852	14	0.074%		5	0.027%	
Maternal Crohn's disease or ulcerative colitis				0.011 ^a			1.00
No	248 132	112	0.045%		66	0.027%	
Yes	530	2	0.380%		0		
Maternal social class				0.10			0.12
I & II	68 244	21	0.031%		12	0.018%	
III	86 869	54	0.062%		32	0.037%	
IV & V	35 510	22	0.062%		11	0.031%	
Marital status				0.081			0.042 ^a
Married	224 261	109	0.049%		65	0.029%	
Not married	23 939	5	0.021%		1	0.004%	
Maternal smoking during pregnancy				0.054			0.73
No	110 961	27	0.024%		18	0.016%	
Yes	34 245	16	0.047%		4	0.012%	
Maternal ABO blood group				0.84			0.81
A	101 010	44	0.044%		26	0.026%	
O	105 391	49	0.046%		30	0.028%	
Maternal rhesus Status				0.96			0.97
Negative	39 805	18	0.045%		10	0.025%	
Positive	196 652	87	0.044%		53	0.027%	
Pre-eclampsia				0.29			1.00
No	224 360	99	0.044%		60	0.027%	
Yes	24 250	15	0.062%		6	0.025%	
Parity				0.23			0.59
O	104 210	41	0.039%		25	0.024%	
1+	144 214	73	0.051%		41	0.028%	

^{1,2}*P*-values obtained through χ^2 tests, with Yates continuity corrections; ¹Comparing those with Crohn's disease with those without known inflammatory bowel disease (IBD); ²Comparing those with ulcerative colitis with those without known IBD. ^a*P* < 0.05.

0.011) between CD in the mother and CD in the child (OR = 8.36, 95% CI = 2.06-33.9), based on two cases of CD in both (Table 2). There was a borderline significant association (*P* = 0.05) for maternal smoking and CD in the child (OR = 1.92, 95% CI = 1.03-3.56). There was no significant association between CD and mother's age in the age groupings that we originally selected (< 25, 25-34 and 35+ years; Table 2), but there was a non-significantly increased risk among children of older mothers aged 35+ years, when compared with mothers aged under 35 years (OR = 1.70; 0.97-2.08). Accordingly, we recategorised mothers'

age as < 35 years *vs* 35+ years in the multivariate analysis (see below).

We found no significant associations between CD and birth order or with any of the other six maternal risk factors considered, including marital status, ABO blood group, rhesus status and presentation at delivery (Table 2). We found no significant associations between CD and any of the perinatal risk factors relating to the birth, including birthweight, gestational age, caesarean delivery, forceps, Apgar scores and breastfeeding (Table 3).

Using multivariate analysis to assess the independent

Table 3 Associations between characteristics of the births and inflammatory bowel disease in the child

Characteristics of the births	No. of births	Crohn's disease			Ulcerative colitis		
		No. of cases	Percent	<i>P</i> -value ¹	No. of cases	Percent	<i>P</i> -value ²
Presentation at delivery				0.47			1.00
Vertex	158302	52	0.033%		26	0.016%	
Other	8311	1	0.012%		1	0.012%	
Caesarean birth				0.72			0.81
No	223793	104	0.046%		63	0.028%	
Yes	18025	10	0.055%		3	0.017%	
Forceps delivery				1.00			0.95
No	210100	99	0.047%		58	0.028%	
Yes	31718	15	0.047%		8	0.025%	
Sex				0.56			0.28
Male	127829	55	0.043%		29	0.023%	
Female	120823	59	0.049%		37	0.031%	
No. of babies				0.53			0.37
1	243269	113	0.046%		63	0.026%	
2+	5390	1	0.019%		3	0.056%	
Gestational age (wk)				0.72			0.60
24-37	21912	8	0.037%		5	0.023%	
38-41	173868	82	0.047%		51	0.029%	
42-47	20567	12	0.058%		3	0.014%	
Birth weight (g)				0.72			0.46
1000-2999	58553	31	0.053%		14	0.024%	
3000-3499	168149	73	0.043%		43	0.026%	
3500+	21151	10	0.047%		9	0.043%	
Head circumference (cm)				0.34			0.99
< 34	34681	13	0.037%		6	0.017%	
34-35	39128	15	0.038%		6	0.015%	
35-36	38528	6	0.016%		7	0.018%	
36+	51035	14	0.027%		7	0.014%	
Breastfeeding				0.89			0.67
Artificial	50966	17	0.033%		10	0.020%	
Breastfed	117364	36	0.031%		18	0.015%	
Apgar 1 score				0.34			0.99
1-5	21356	14	0.066%		6	0.028%	
6-8	64469	27	0.042%		16	0.025%	
9-10	140267	57	0.041%		37	0.026%	
Apgar 5 score				0.87			0.61
1-5	884	0			1	0.11%	
6-8	4229	2	0.047%		0		
9-10	148835	43	0.029%		23	0.015%	

^{1,2}*P*-values obtained through χ^2 tests, with Yates continuity corrections; ¹Comparing those with Crohn's disease with those without known inflammatory bowel disease (IBD); ²Comparing those with ulcerative colitis with those without known IBD.

significance of perinatal risk factors, there was a significantly increased risk of CD among the offspring of mothers who were aged 35+ years, compared with those aged under 35 years (OR = 4.81, 95% CI = 2.32-9.98), and an increased risk of CD among children of mothers who smoked during pregnancy compared with those who did not (2.04, 1.06-3.92). Numbers were too small to warrant inclusion of maternal CD in the multivariate analysis (Table 4).

UC

For UC, in univariate analysis there was only a (marginal) significantly reduced risk for mothers who were not married (*P* = 0.042), although this was based on only one case of an unmarried mother with a child with UC (Tables 2 and 3). Numbers were too small to warrant inclusion of marital status, i.e. a stratum with just one case, in the multivariate analysis. Using multivariate analysis, there were no

other significant associations between UC and any of the other 19 perinatal risk factors relating to either the mother or the birth (Table 4).

DISCUSSION

A strength of our study is that we have investigated 20 perinatal risk factors for IBD, unlike other studies that have mostly investigated one, two or only a few factors. The study is based on a geographically defined population, covering prospective data collected over 30 years. Another important strength is that information about the perinatal risk factors and the main outcome measure - IBD in offspring - were collected independently of each other. They were subsequently brought together independently through systematic record linkage, such that information collected for each risk factor was not influenced by knowledge of

Table 4 Perinatal factors with significant, independent effect on Crohn's disease in the child

Perinatal risk factor	Odds ratio	95% CI
Maternal age (yr)		
14-34	1.00	Ref.
35-49	4.81	2.32-9.98
Maternal smoking during pregnancy		
No	1.00	Ref.
Yes	2.04	1.06-3.92

the outcome measure. Our study is therefore not subject to potential interviewer and recall bias, e.g. about whether the mother smoked during pregnancy, which can affect studies based on interviews or self-reporting, and which provide much of the evidence about IBD and perinatal risk factors. The Oxford record linkage study has also been used as the basis of previous studies of perinatal risk factors^[7,10,13,14].

The study has several limitations. There was variable follow-up after birth, with shorter durations of follow-up for those born in the more recent years of the study period. However, there was at least 10 years follow-up for all IBD cases among offspring. Maternities during the early years of the study period, and among younger mothers, had fewer years of pre-pregnancy inclusion to ascertain maternal IBD, while data for three of the 20 risk factors (social class, smoking and breastfeeding), were not available for the first four years of the study period. The study would not have identified offspring who were diagnosed with IBD after migrating out of the ORLS region, which would reduce the number of observed cases of IBD.

The identification of cases of IBD in the offspring was restricted to those who were admitted as inpatients or day cases. We will have missed some cases of IBD where the only inpatient or day case admission was for a diagnostic endoscopy and biopsy in patients with suspected IBD, and where the pathology results were not available to create a record of the diagnosis at the time of discharge. We will also have missed people without any day case or inpatient care. Migration over time in the Oxford region population would also have lowered our observed incidence of IBD, particularly among adults. Our age-specific cumulative incidence rates of 1.6 and 0.9 per 100 000 for CD and UC among 0-29 year olds, and 1.0 and 0.5 respectively among 0-19 year olds, are lower than those in some UK studies, but comparable with those in other UK studies^[2,3,15-18].

There were two cases of CD in both the mother and child. Previous studies have identified associations between both maternal CD and maternal UC and IBD in offspring^[19,20], which are part of a well-established genetic association of IBD in families^[21-23]. There was some evidence of increased risks of CD, but not UC, among children whose mothers smoked during pregnancy. Previous studies have reported no association with smoking during pregnancy^[19,24], although one case-control study found modest protection against both CD and UC^[25]. Our data refers to smoking by the mother: in the general literature, there is strong evidence that active smoking increases the

risk and perhaps the severity of CD^[26,27]. Although we did not have information on smoking status of the IBD subjects themselves, it is of some interest that we found an association between maternal smoking and CD.

Some studies have identified slightly increased risks of IBD among children from lower socio-economic groups^[11,20], others have reported reduced risks of CD among children from lower socio-economic backgrounds^[18,28], and some have found no association between social background and IBD^[29,30]. More generally, studies that have investigated the relationship between IBD and socio-economic group have often reported conflicting findings^[31-37]. Overall, this indicates that any possible association between socio-economic background and subsequent IBD in children is probably quite weak. We did not find a significant association between social class and either CD or UC.

A systematic review and meta analysis of (mainly case-control) studies reported a small but significant protective effect of breastfeeding against subsequent IBD in offspring^[12], although it concluded that new larger studies were required. More recent studies have shown little association between breastfeeding and IBD^[19], or even increased risks of CD^[30,38]. We found no association between breastfeeding at the time of discharge from hospital and IBD, although this includes those who subsequently discontinued breastfeeding after discharge, and is therefore an incomplete marker of breastfeeding.

Through the use of multivariate analysis, we found higher risks of CD, but not UC, among children of older mothers (aged 35+ years). This is consistent with a Swedish study that reported an increased risk of paediatric CD among female offspring born to older mothers^[35], although other studies have identified no link between mother's age and IBD in children^[25,39]. Births among older mothers are sometimes associated with increased risks of prenatal medical and obstetric complications, intrapartum complications, perinatal and neonatal morbidity and mortality, as well as increased subsequent risks of various disorders. It is possible that children born to older mothers may be more exposed, or more susceptible, to factors associated with the aetiology of subsequent CD, but not with UC, in their children. It is also possible that our finding on maternal age, though significant, was a chance one, especially as our study investigated 20 perinatal factors. It is worth noting, however, that the finding was highly significant ($P < 0.01$ in multivariate analysis).

We found an indication of reduced risks of IBD, particularly UC, among children of mothers who were unmarried at the time of birth, which is consistent with findings from Sweden^[11] and Australia^[28].

It has been suggested that caesarean section might increase the risk of subsequent IBD in children, because there is less exposure to maternal bacteria than in vaginal delivery^[40]. The reasoning behind this is that, according to the hygiene hypothesis, inadequate exposure to microorganisms in early life might result in higher levels of immune-mediated pathology in later life. Although one study found an increased risk of CD for elective caesarean

sections^[28], another found no association for either CD or UC^[19]. We also found no association for either CD or UC.

We found no association between maternal parity and IBD. Although increased risks of IBD have been reported occasionally for first born^[41,42], or subsequent siblings^[43], most studies have found no association between birth order and IBD^[11,20,25,30,39]. We also found no association between IBD and any of the other perinatal factors studied, including pre-eclampsia, birthweight, gestational age and Apgar score. These perinatal factors have not usually been associated with IBD in previous studies^[11,19,25,28,30].

To summarise, of the 20 perinatal risk factors investigated in this study, we found that maternal CD, smoking during pregnancy and advanced maternal age were associated with increased risks of CD in offspring. For UC, there were no factors associated with increased risks after multivariate adjustment. This, and the fact that the few factors that were associated with CD had quite small effect sizes, suggests that perinatal risk factors have only a minor role in the aetiology of IBD.

ACKNOWLEDGMENTS

The Oxford Record Linkage Study database was funded by the former Oxford Regional Health Authority in the years covered by the maternity data collection and, over many years, it was built by Leicester Gill, Glenys Bettley and Myfanwy Griffith. The views expressed in this paper are those of the authors and not necessarily those of the funding body.

COMMENTS

Background

Both Crohn's disease (CD) and ulcerative colitis (UC) are considered to be immune-mediated disorders, although the exact pathogenetic mechanisms are not yet clear. Perinatal risk factors have been linked with other immune-mediated diseases, including asthma and type 1 diabetes. Other than a suggested, small protective effect of breastfeeding, little has been reported on the role of perinatal factors for either CD or UC.

Research frontiers

This study investigated associations between 20 perinatal risk factors relating to the maternity or the birth and subsequent inflammatory bowel disease (IBD) in offspring in the Oxford region, UK. Risk factors investigated included maternal characteristics such as maternal age, IBD, social class, marital status, smoking in pregnancy, ABO blood group, rhesus status and parity; and characteristics of the birth such as caesarean delivery, number of babies delivered, gestational age, birthweight, breastfeeding and Apgar scores.

Innovations and breakthroughs

The study found increased risks of CD among children of mothers with CD, among children of mothers who smoked during pregnancy, and of older mothers aged 35+ years. There were no significant associations between CD and the 17 other perinatal risk factors investigated, and no associations for UC.

Applications

The findings indicate that these three perinatal risk factors might have some influence on subsequent IBD in children. Overall, however, perinatal factors appear to have a limited role in the aetiology of IBD. This study will help stimulate further research into the influence of perinatal risk factors on IBD. The findings should also provide an important source of information for future systematic reviews and meta analyses of perinatal factors and IBD.

Terminology

Odds ratios were used to assess any increased risks of developing IBD. These denote the chance or odds of developing IBD for a child exposed to a given

perinatal risk factor (e.g. caesarean delivery) as a ratio of the chance or odds for a child not exposed to caesarean delivery. The study used record linkage of maternity exposure data and IBD outcome data, which were collected independently of each other.

Peer review

This is a very well written original article. I would like to congratulate the authors on such a nicely done original paper that contributes a lot of new information about perinatal and early risk factors for IBD.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Ma WH

Probiotic *Lactobacillus rhamnosus* downregulates *FCER1* and *HRH4* expression in human mast cells

Anna Oksaharju, Matti Kankainen, Riina A Kekkonen, Ken A Lindstedt, Petri T Kovanen, Riitta Korpela, Minja Miettinen

Anna Oksaharju, Petri T Kovanen, Wihuri Research Institute, Helsinki 00140, Finland

Matti Kankainen, VTT Technical Research Centre of Finland, Biosystems Modelling, Espoo 02044, Finland

Riina A Kekkonen, Riitta Korpela, Valio Ltd, Research Centre, Helsinki 00370, Finland

Riina A Kekkonen, Riitta Korpela, University of Helsinki, Institute of Biomedicine, Medical Nutrition Physiology, Helsinki 00290, Finland

Ken A Lindstedt, Wihuri Research Institute, Helsinki 00140, Finland and Orion Corporation, Orion Pharma, Nonclinical R&D, Espoo 02601, Finland

Minja Miettinen, National Institute for Health and Welfare, Department of Vaccines and Immune Protection, Helsinki 00580, Finland

Author contributions: Kekkonen RA, Korpela R, Lindstedt KA and Oksaharju A designed the research; Oksaharju A performed the experiments; Oksaharju A, Kankainen M and Miettinen M analyzed the data; Oksaharju A wrote the manuscript; Kankainen M, Kekkonen RA, Lindstedt KA, Kovanen PT, Korpela R and Miettinen M edited the manuscript.

Supported by Valio Research Centre, the Foundation for Nutrition Research, Academy of Finland Research Council for Biosciences and Environment, Grant No. 129954; and Finnish Funding Agency for Technology and Innovation (TEKES) grant No. 2243/31/05. Wihuri Research Institute is maintained by Jenny and Antti Wihuri Foundation

Correspondence to: Anna Oksaharju, MSc, Wihuri Research Institute, Kallioliinantie 4, Helsinki 00140, Finland. anna.oksaharju@helsinki.fi

Telephone: +358-9-681411 Fax: +358-9-637476

Received: May 14, 2010 Revised: August 28, 2010

Accepted: September 5, 2010

Published online: February 14, 2011

cells were stimulated with *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (LGG®), *L. rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12) and their combination for 3 or 24 h, and were subjected to global microarray analysis using an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array. The gene expression differences between unstimulated and bacteria-stimulated samples were further analyzed with GOrilla Gene Enrichment Analysis and Visualization Tool and MeV Multiexperiment Viewer-tool.

RESULTS: LGG and Lc705 were observed to suppress genes that encoded allergy-related high-affinity IgE receptor subunits α and γ (FCER1A and FCER1G, respectively) and histamine H4 receptor. LGG, Lc705 and the combination of four probiotics had the strongest effect on the expression of genes involved in mast cell immune system regulation, and on several genes that encoded proteins with a pro-inflammatory impact, such as interleukin (IL)-8 and tumour necrosis factor alpha. Also genes that encoded proteins with anti-inflammatory functions, such as IL-10, were upregulated.

CONCLUSION: Certain probiotic bacteria might diminish mast cell allergy-related activation by downregulation of the expression of high-affinity IgE and histamine receptor genes, and by inducing a pro-inflammatory response.

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Key words: Probiotic bacteria; Mast cells; Microarray; Allergy; IgE receptor

Peer reviewer: Dr. William R Parker, PhD, Assistant Professor, Department of Surgery, Duke University Medical Center, Box 2605, Durham, NC 27710, United States

Oksaharju A, Kankainen M, Kekkonen RA, Lindstedt KA, Kovanen PT, Korpela R, Miettinen M. Probiotic *Lactobacillus rhamnosus* downregulates *FCER1* and *HRH4* expression in human

Abstract

AIM: To investigate the effects of four probiotic bacteria and their combination on human mast cell gene expression using microarray analysis.

METHODS: Human peripheral-blood-derived mast

mast cells. *World J Gastroenterol* 2011; 17(6): 750-759 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/750.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.750>

INTRODUCTION

Mast cells are multifunctional regulator cells that are located at strategic host-environment interfaces including skin, vascular barriers and gastrointestinal tract, where they encounter antigens and pathogens, as well as commensal microbes. In the healthy intestinal mucosa, mast cells constitute 2%-3% of the cells of the lamina propria^[1]. Mast cells are very heterogeneous cells that are traditionally classified by the content of their specific proteases tryptase and chymase. A mast cell subtype that contains only tryptase (MC_T) is predominant in the lung and intestine, whereas mast cells that contain tryptase and chymase (MC_{TC}) are prevalent in the skin and conjunctiva^[2]. Mast cell subtypes can even vary between different parts of the same organ. Thus, MC_T cells are enriched in the mucosal layer of the intestine but MC_{TC} cells outnumber them in the intestinal submucosa. However, this classification is thought to be interchangeable and can be shaped according to the microenvironment of the cells^[3].

Mast cells participate in a variety of physiological functions, such as epithelial secretion and permeability, blood flow, peristalsis, neuroimmune interactions, and wound healing. One significant task for mast cells is host defence against pathogenic microbes. By secreting several mediators including histamine, proteases, lipid mediators and pro- and anti-inflammatory cytokines, mast cells regulate the immune system and interact with other immune cells^[4]. The multifunctionality of mast cells can explain why they are also involved in the pathogenesis of many inflammatory diseases, such as allergy. The number of mast cells and the amount of mast-cell-derived mediators, such as histamine, which is the key mediator in allergy, are increased at sites of allergic inflammation. The released mediators induce mucus and electrolyte secretion, smooth muscle contraction, nerve-cell activation and other symptoms common in allergic reactions^[5]. The regulation of mast-cell mediators is complex. The best characterized mechanism of mast cell activation is high-affinity IgE receptor (FcεR1)-mediated activation^[6]. IgE-receptor aggregation induces multiple signaling pathways that control the secretion of allergy-related mediators, such as histamine and leukotrienes, and the induction of T helper cell 2 (Th2) type cytokine and tumor necrosis factor (TNF) gene transcription^[7]. The inflammatory effects of the released histamine are mediated by histamine receptors H1-H4^[8]. However, some of the mast cell mediators, including interleukin (IL)-10 and histamine^[9] can have anti-inflammatory effects and decrease inflammation^[10].

Probiotics are defined as live microbes that have beneficial effects on the host's health when administered in adequate amounts^[11]. In clinical intervention studies, certain probiotics have been documented to be effective in the prevention and treatment of various clinical condi-

tions. The most promising results of the health effects of probiotics have been discovered in studies of diarrhea^[12], allergy^[13], irritable bowel syndrome (IBS)^[14,15], and respiratory infections^[16,17]. The effects of probiotics are suggested to be strain-specific, although one strain can have multiple influences^[18]. In the treatment of a complex and heterogeneous condition such as IBS, the use of combinations of different strains of probiotics can have advantages over using a single strain^[14]. The most investigated and used probiotic genera are *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* strains have been effective in beneficially modulating commensal microbes and inhibiting pathogen adhesion to gut mucosa. *Lactobacillus* and *Bifidobacterium* have been shown to produce antimicrobial agents and to alleviate symptoms of allergy^[19]. Dairy *Propionibacterium*, which has been observed to exclude pathogenic microbes from gut mucosa^[20], has also been used as a probiotic.

Evidence from two clinical trials performed with the combination of four probiotic bacteria, i.e. *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (LGG), *L. rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12), suggests that the consumption of such combination alleviates the symptoms in IBS patients^[14,21]. As mast cells are believed to be important in regulating intestinal immunity and perhaps also intestinal sensory functions, we chose to study the effects of the above probiotic combination and each bacterium alone on the global gene expression of primary peripheral-blood-derived human mast cells.

MATERIALS AND METHODS

Cell culture

Freshly collected buffy coats from healthy adult blood donors were provided by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). The health of the subjects for blood donation is strictly controlled. The donors must be 18-65 years of age, free of infections including HIV, hepatitis B and C, and free of allergic symptoms and most chronic illnesses including autoimmune diseases. Mononuclear cells were purified from heparinized blood by Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Mast cell precursor cells were isolated by positive immunomagnetic selection using indirect CD34 MicroBead Kit and MACS® separation columns (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. After selection, the CD34⁺ cells were cultured for 9-11 wk in serum-free Stem Span™ cell culture medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin and streptomycin (GIBCO BRL, Grand Island, NY, USA), human recombinant stem cell factor (SCF; Peprotech, Rocky Hill, NJ, USA), IL-3 (Peprotech), IL-9 (Peprotech), IL-6 (Peprotech) and human low-density lipoprotein, as previously described^[22]. Cultured mast cells were phenotypically and functionally similar to mature MC_{TC} cells as measured by c-kit and IgE receptor (FcεR1) expression, and the presence of chymase, tryptase, heparin and histamine in their granules, as described previously in detail^[23].

Bacterial strains

L. rhamnosus GG (ATCC 53103), *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) and *B. animalis* ssp. *lactis* Bb12 (DSM 15954) were provided by Valio Research Centre (Helsinki, Finland). LGG and Lc705 were grown as previously described^[24]. PJS was grown under optimized aerobic conditions at 30°C in whey broth (Valio) twice for 2 d at a concentration of 2%. Bb12 was grown under anaerobic conditions at 37°C in de Man, Rogosa and Sharpe (MRS) broth enriched with 5 g/L L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany) three times for 17–18 h at a concentration of 2%^[25]. LGG, Lc705, PJS and Bb12 were grown to logarithmic growth phase and the number of bacteria was determined by counting in a Petroff-Hauser counting chamber. *Chlamydia pneumoniae* isolate Kajaani 6 (Cpn) was used as a reference strain, and was obtained from the National Institute for Health and Welfare (Helsinki, Finland) and was propagated as described previously^[22].

Mast-cell stimulation with bacteria

After differentiation, mast cells were collected and re-suspended in fresh Stem SpanTM medium that contained antibiotics and SCF as described above. Single live bacterial strains were added to the cell culture in a bacterium-to-cell ratio of 5:1 based on preliminary experiments (data not shown). When the stimulation was performed with the combination of bacteria, each of the four strains was dosed in a bacterium-to-cell ratio of 1.25:1, thus the total bacterium-to-cell ratio of the combination was 5:1. The cells were incubated with bacteria for 3 or 24 h at 37°C in 5% CO₂. After incubation, mast cells were separated from the medium by centrifugation, and the medium was aliquoted and stored at -20°C. The cells were washed free of bacteria, lysed and homogenized in RLT buffer (Qiagen, Valencia, CA, USA) and stored at -70°C before RNA isolation. All experiments were performed with mast cells obtained from three different blood donors. For analysis, the cells from different donors of each experiment were pooled.

RNA isolation and microarray

Total RNA was isolated from the cell lysates using RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Microarray experiments were performed at Biomedicum Genomics (Helsinki, Finland) using an Affymetrix GeneChip[®] Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). Integrity and purity of the RNA were verified with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA was prepared and hybridized using the two-cycle protocol of the GeneChip[®] Expression Analysis kit (Affymetrix) according to the manufacturer's recommendations. Double-stranded cDNA was synthesized from total RNA. Next, biotin-labeled cRNA was transcribed from the cDNA, and the cRNA was fragmented and hybridized. The hybridization reactions were scanned using a GeneChip Scanner 3000 (Affymetrix).

Microarray analysis

The robust multiarray averaging algorithm^[26] in the Bioconductor simpleaffy package^[27,28] was used to calculate expression estimates from GeneChip signal intensity data. To provide better precision and accuracy and to overcome interpretation problems related to conflicting id gene references^[27], an updated probe set definition was used^[29], based on Ensemble gene information. In contrast to the default Affymetrix chip description file with 54675 probe sets, the used custom chip description file (version 11.0.1) contained 17492 unique Ensemble gene probe sets. The significance of differential expression was assessed using the empirical Bayes moderated paired *t* statistics (eBayes function) in the limma package, followed by intensity-based hierarchical Bayes analysis^[30,31]. In the analysis, a moderated paired *t* test was computed by constructing cell line effects in the linear model. All *P* values were adjusted for multiple hypotheses testing using the bootstrapped *q* value approach in the *q*value package^[32]. Genes with *P* values ≤ 0.05 were identified as significantly differentially expressed.

The GOrilla Gene Enrichment Analysis and Visualization Tool^[33] was used to discover functional categories that were enriched at either end of a gene list sorted by the moderated *t* test score, which was calculated using limma. The input gene set was used as a background. Clustering and visualization of the gene expression differences were done using MeV Multiexperiment Viewer tool and hierarchical clustering with Euclidean as a distance, and average linkage clustering as the linkage method^[34,35].

Quantitative reverse transcriptase-polymerase chain reaction

To validate the microarray data, TaqMan[®] real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described^[22] for selected genes. Total RNA from the same samples used for the microarray experiments was reverse transcribed to cDNA using random hexamers (Invitrogen, Paisley, UK) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). TaqMan[®] Gene Expression assays (Applied Biosystems, Foster City, CA, USA) were chosen for detection of *IL8* (Hs00174103_m1), *CCL2* (Hs00234140_m1), *IL10* (Hs00174086_m1), *HRH4* (Hs00222094_m1), *FCER1A* (Hs00758600_m1) and *FCER1G* (Hs00610227_m1). Transcripts for *TNF-α* were detected by using sense primer 5'-GCTGCACITTTGGAGTGATCG-3', antisense primer 5'-GTTTGCTACAACATGGGCTACAG-3' and probe 5'-FAM-CCCAGGCAGTCAGATCATCTTCTC-GA-BHQ1-3'. Samples were analyzed in triplicate. β-Actin was used as an endogenous normalization control. Relative quantification was determined by standard 2^{-ΔΔCT} calculations^[36].

RESULTS

Gene expression profiling

In order to explore the effects of different probiotic bacteria or their combination on mast cells, transcrip-

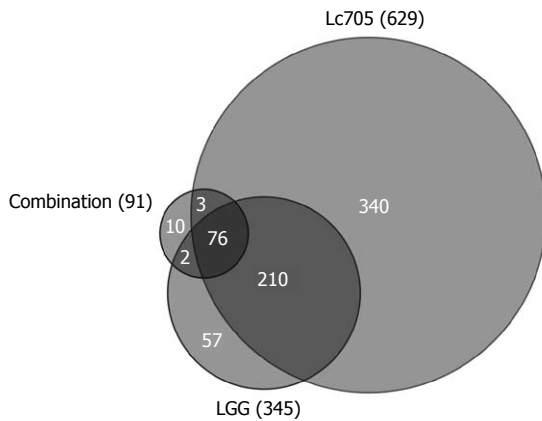


Figure 1 Schematic representation of statistically significant changes ($P < 0.05$) in mast-cell gene expression after 24 h stimulation with *Lactobacillus rhamnosus* Lc705, *Lactobacillus rhamnosus* GG and the combination of four probiotic bacteria. LGG: *Lactobacillus rhamnosus* GG; Lc705: *Lactobacillus rhamnosus* Lc705.

tional changes of the bacteria-stimulated mast cells were studied using a whole genome microarray. A total of 42 Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays were used to analyze gene expression profiles of unstimulated mast cells or those stimulated with live LGG, Lc705, PJS, Bb12, or the combination of these four bacteria. Cpn was included in the analysis as a reference bacterium, which represented a non-probiotic, pathogenic microbe with certain known transcriptional effects on mast cells^[22]. Results are representative of three independent experiments, each performed with cells from three donors at two different time points (3 and 24 h). After 3 h bacterial stimulation, the differences in the levels of mast-cell gene expression were so low that no statistical significance was observed as compared to unstimulated samples (data not shown). At 24 h, however, a statistically significant change was observed in 698 genes. Numbers of differentially expressed genes are illustrated in Figure 1. Lc705, LGG and the combination of the four probiotic strains were the most effective stimulators. Bb12 affected the expression of only one gene, and PJS and Cpn failed to change mast-cell gene expression significantly. Lc705 affected mast-cell gene expression the most by changing the expression of 629 genes significantly. Of these genes, 288 were upregulated and 341 were downregulated. LGG altered the expression of 345 genes (160 upregulated and 185 downregulated), and the combination of the four probiotic strains changed the expression of 91 genes (57 upregulated and 34 downregulated). Raw data of the microarray analysis are available at http://ekhidna.biocenter.helsinki.fi/poxo/download_data.

Gene functional category analysis and hierarchical clustering

To characterize the biological significance of the differentially expressed mast-cell genes, a gene ontology category analysis was performed. GOzilla Gene Enrichment Analysis and Visualization Tool^[33] was used to discover whether some functional categories showed statistically significant,

concordant differences between the stimulated sample and the unstimulated sample. The enrichment analyses were carried out for the whole array gene set, including the genes that did not reach statistical significance in the array analysis, that were ranked based on their moderated t values. For the analysis, the ranked genes were sorted in the order of the highest and lowest t value of each sample. The analysis software used the sorted gene lists to classify the genes of each sample into gene ontology categories^[37] by their biological function. Selected functional groups and examples of genes that represented each category are depicted in Table 1.

Stimulation of mast cells with LGG, Lc705, Bb12, combination of probiotics, or Cpn resulted in upregulation of genes that belong to categories that involve immune system processes, regulation of programmed cell death, and leukocyte activation. Stimulation with LGG, Lc705, Bb12 or the combination of probiotics suppressed mast-cell genes that are involved in general cell activities and metabolism such as cell cycle and lipid biosynthetic processes. Lc705 was found to suppress genes that are related to mast-cell activation.

Representative genes from the functional categorization (Table 1) were selected to compare the expression patterns of different samples. Mast cells have a central role in many inflammatory responses as well as in allergy, therefore, immunologically relevant genes and genes involved in mast cell activation and mediator release were selected. In order to compare the expression of the selected genes, a hierarchical comparison analysis with the MeV two-way hierarchical clustering method was performed. As expected, LGG and Lc705 but also the combination of four probiotics showed similar expression patterns in the clustering analysis, and were therefore considered to alter mast-cell gene expression in a similar manner (Figure 2). Genes that are involved in similar processes were also grouped: genes that encode Toll-like receptor (TLR) 1, nucleotide-binding oligomerization domain containing 2 (NOD2), FCER1A and FCER1G (high-affinity IgE receptor 1 gene subunits α and γ , respectively) grouped together; and genes that encode proteins with inflammatory functions, such as IL-1 β and IL-8 constituted another distinct cluster.

Although there were differences in the intensity of the expression levels, all bacteria except PJS induced upregulation of mast-cell genes included in the functional group of immune system regulation. Examples of upregulated genes in this category were *TLR1*, *TLR6*, *NOD2*, *IL1B*, *IL8*, chemokine (C-C motif) 2 (*CCL2*), *TNF* and *IL10*. Genes that are involved in regulation of programmed cell death, such as caspases 3 and 8 (*CASP3* and *CASP8*) and cyclin-dependent kinase inhibitor 1B (*CDKN1B*), were observed to be upregulated in LGG, Lc705, and the combination-stimulated cells. LGG, Lc705 and the combination also significantly enhanced the expression of the cluster of differentiation 8 A (*CD8A*) and lymphocyte cytosolic protein 2 (*LPC2*) genes, which are involved in leukocyte activation.

Cyclin A2 (*CCNA2*) and mitogen-activated protein kinase 12 (*MAPK12*) genes involved in the regulation of cell

Table 1 Representative subsets of differentially expressed mast-cell genes after bacterial stimulation classified into functional categories

Go class	Description	P value						Gene examples
		LGG	Lc705	PJS	Bb12	Combination	Cpn	
Upregulation								
GO:0002376	Immune system process	3.48E-16	2.95E-13	-	5.25E-7	5.66E-12	1.64E-4	<i>TNF, IL1B, IL8, IL10, CCL2, TLR1, TLR6, NOD2</i>
GO:0043067	Regulation of programmed cell death	2.77E-8	9.63E-8	-	7.18E-4	3.49E-6	-	<i>CASP3, CASP8, CDKN1</i>
GO:0045321	Leukocyte activation	8.97E-9	2.17E-4	-	-	2.23E-4	-	<i>CD8A, LCP2</i>
Downregulation								
GO:0007049	Cell cycle	4.76E-5	9.61E-7	-	-	5.81E-4	-	<i>CCNA2, MAPK12</i>
GO:0033033	Regulation of mast cell activation	-	7.51E-4	-	-	-	-	<i>FCER1A, FCER1G</i>
GO:0008610	Lipid biosynthetic process	-	-	-	2.02E-5	-	-	<i>LPGAT1</i>

The three selected highly upregulated or downregulated functional categories from the enrichment analysis performed with GOrilla Gene Enrichment Analysis and Visualization Tool. *P* value is the enrichment *P* value reported by GOrilla. The resultant categories reflect the gene expression differences of the bacteria-stimulated sample compared to unstimulated sample in 24 h time point. LGG: *Lactobacillus rhamnosus* GG; Lc705: *Lactobacillus rhamnosus* Lc705; PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS; Bb12: *Bifidobacterium animalis* ssp. *lactis* Bb12; Cpn: *Chlamydia pneumoniae* isolate Kajaani 6; TNF: Tumor necrosis factor; IL-1B: Interleukin-1β; IL-8: Interleukin-8; IL-10: Interleukin-10; CCL2: Chemokine (C-C motif) 2; TLR1: Toll-Like receptor 1; TLR6: Toll-Like receptor 6; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; CASP3: Caspase 3; CASP8: Caspase 8; CDKN1: Cyclin-dependent kinase inhibitor 1; CD8A: Cluster of differentiation 8 A (T cell surface glycoprotein); LCP2: Lymphocyte cytosolic protein 2; CCNA2: Cyclin A2; MAPK12: Mitogen-activated protein kinase 12; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; LPGAT1: Lysophosphatidylglycerol acyltransferase 1.

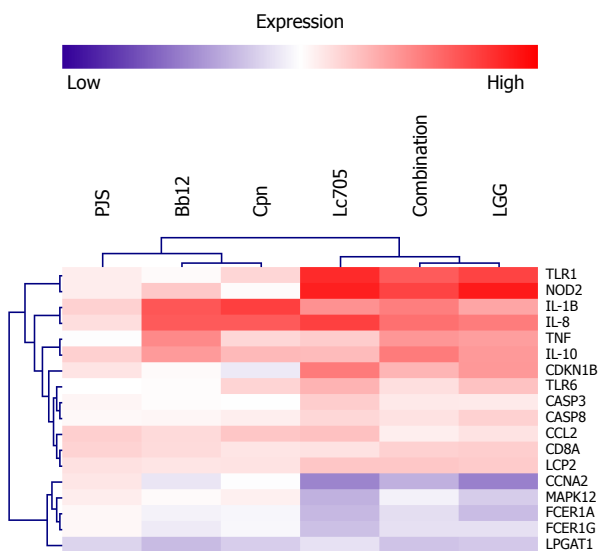


Figure 2 Two-way hierarchical clustering of representative genes selected from the functional category analysis (Table 1). Data are average expression differences between unstimulated and bacteria-stimulated mast cells from three independent experiments at the 24-h time point. Analysis was performed using MeV software. PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS; Bb12: *Bifidobacterium animalis* ssp. *lactis* Bb12; Cpn: *Chlamydia pneumoniae* isolate Kajaani 6; Lc705: *Lactobacillus rhamnosus* Lc705; LGG: *Lactobacillus rhamnosus* GG; TLR1: Toll-Like receptor 1; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; IL-1B: Interleukin-1β; IL-8: Interleukin-8; TNF: Tumor necrosis factor; IL-10: Interleukin-10; CDKN1B: Cyclin-dependent kinase inhibitor 1B; TLR6: Toll-Like receptor 6; CASP3: Caspase 3; CASP8: Caspase 8; CCL2: Chemokine (C-C motif) 2; CD8A: Cluster of differentiation 8 A (T cell surface glycoprotein); LCP2: Lymphocyte cytosolic protein 2; CCNA2: Cyclin A2; MAPK12: Mitogen-activated protein kinase 12; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; LPGAT1: Lysophosphatidylglycerol acyltransferase 1.

cycle, and the latter also in IgE receptor signaling, were observed to be downregulated in cells stimulated with LGG, Lc705, and the combination of four probiotics. Stimulation

of mast cells with Lc705 significantly suppressed the expression of *FCER1A* and *FCER1G* genes. The same trend was also observed after stimulation with LGG and the combination, although these changes in the analysis failed to reach statistical significance. Bb12 was found to suppress the expression of *LPGAT1* (lysophosphatidylglycerol acyltransferase 1), a mast cell gene that is related to lipid biosynthesis, and also the other bacteria studied had a similar impact on the expression of this gene.

Manual screening of the array data

To gain further insight into the probiotic-induced changes in mast-cell activation, the array data set was screened for additional genes that were related to the IgE receptor signaling pathway and mast-cell activation and immunomodulation. The screening was performed manually using the list of genes that reached statistical difference in the array analysis at the 24-h time point (http://ekhidna.biocenter.helsinki.fi/poxo/download_data). The gene that encodes phospholipase C (PLC) that is involved in the mast-cell IgE receptor signaling pathway was observed to be significantly downregulated by LGG and Lc705. The gene that encodes prostaglandin E2 receptor (*PTGER*), which mediates the effects of this inflammatory prostaglandin, was also downregulated by LGG and Lc705. Although only Lc705 was categorized in the functional group as downregulating mast-cell activation, LGG-stimulated cells also reached statistical significance in suppressing *FCER1A* expression in the microarray analysis. Both Lc705 and LGG also suppressed the expression of the gene that encodes mast-cell histamine H4 receptor (*HRH4*). Expression alterations of the genes that encode the end-products of the IgE receptor signaling pathway were also screened, but no changes were observed in the mast-cell expression of leukotrienes, heparin or Th2 type cytokines, such as IL-3, IL-4, IL-5 and IL-15 after bacterial stimulation.

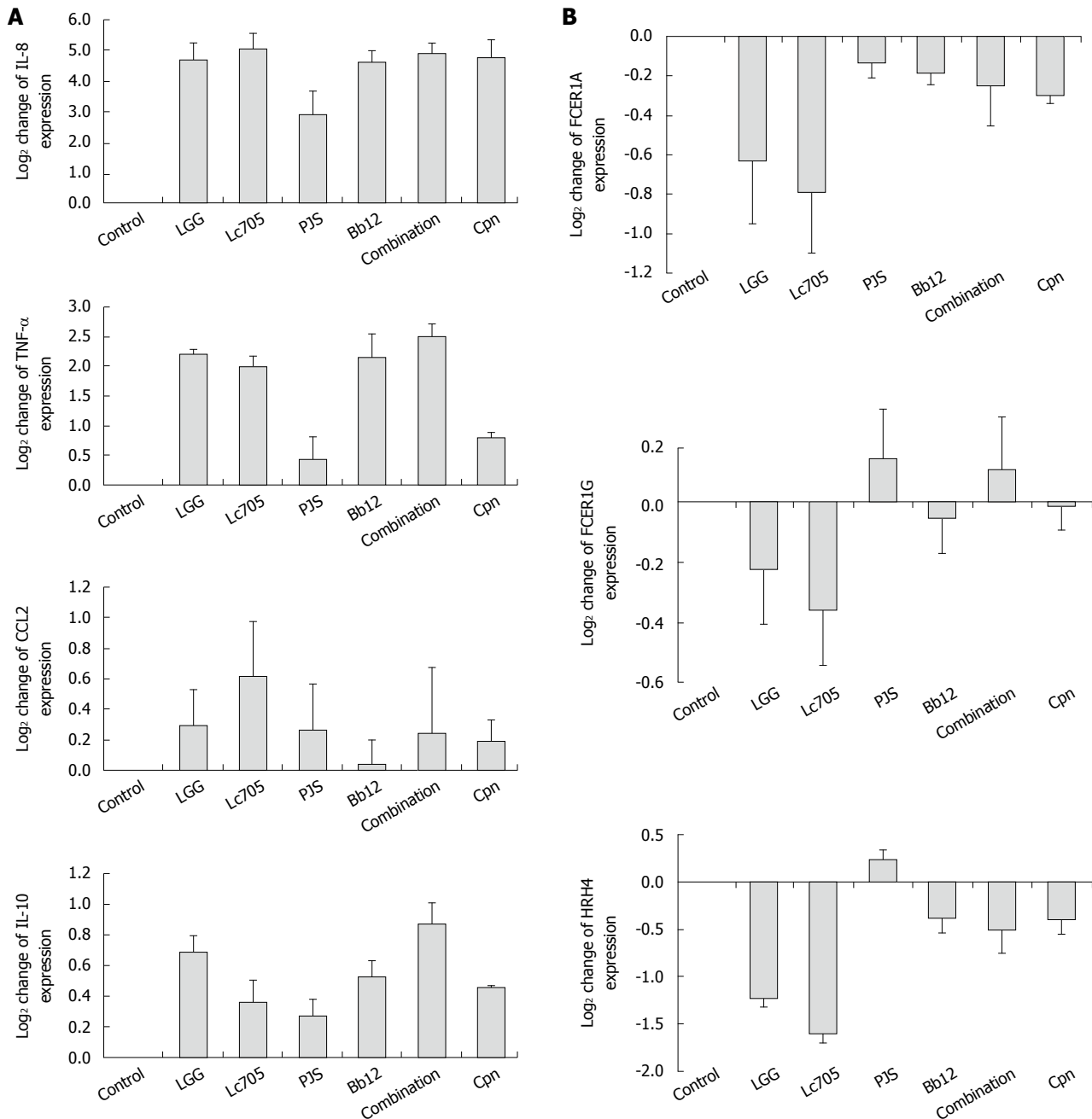


Figure 3 Verification of mast-cell microarray results by quantitative reverse transcriptase-polymerase chain reaction with seven selected genes that are involved in mast-cell immune system regulation (A) and mast-cell activation (B). Gene expression was quantified after 24 h stimulation with four probiotic bacteria; *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS), *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12) and their combination or with *Chlamydia pneumoniae* isolate Kajaani 6 (Cpn). Data are mean values \pm SE of three independent experiments. IL: Interleukin; TNF- α : Tumor necrosis factor- α ; CCL2: Chemokine (C-C motif) 2; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; HRH4: Histamine H4 receptor.

Quantitative RT-PCR

Findings from the microarray analysis were verified by quantitative RT-PCR. Seven genes with statistical significance after 24 h stimulation with at least one of the bacteria in the array analysis or in the functional categorization analysis were selected. Four of the genes encoded proteins that are involved in mast-cell regulation of immunological events: *IL8*, *TNF- α* , *CCL2* and *IL10* (Figure 3A). Three of the genes encoded proteins that are involved in mast-cell activation and in allergy: *FCER1A*, *FCER1G* and *HRH4* (Figure 3B). With minor differences in the levels

of intensities, all of the selected genes followed a similar expression pattern detected in the microarray analysis, and thus confirmed the results of the microarray data.

DISCUSSION

In the present study, microarray analysis was used to gain a broad understanding of interactions between probiotic bacteria and human primary mast cells. We report here, that live probiotic bacteria have species-specific effects on human mast cells. The most significant changes in mast-

cell gene expression were observed in the regulation of genes related to mast-cell activation and mediator release, including *FCER1A*, *FCER1G* and *HRH4*, and immunological responses such as *IL8*, *TNF*, *CCL2* and *IL10*.

In the microarray analysis, lactobacilli affected mast-cell gene expression more than the other bacteria. Stimulation of mast cells with both LGG and Lc705 significantly downregulated the expression of the high-affinity IgE receptor subtype α (*FCER1A*) and *HRH4* (*HRH4*) genes after 24 h stimulation. In addition, Lc705 stimulation downregulated the gene expression of FC ϵ R1 receptor subtype γ (*FCER1G*). PJS, Bb12, the combination, or Cpn did not have an effect on *FCER1* and *HRH4* genes. FC ϵ R1 plays a key role in mediating the allergy-related IgE-dependent activation and degranulation of mast cells^[38] as demonstrated in FC ϵ R1-deficient mice that fail to show allergic reactions after sensitization^[39]. After FC ϵ R1 aggregation, mast cells release inflammatory mediators, such as histamine. Histamine is the key mediator in causing the symptoms of allergy, and it also has a potent role as a modulator of immune responses^[40]. The effects of histamine are mediated through histamine receptors H1-H4 that are expressed on the surface on many cell types, including mast cells and other inflammatory cells as well as epithelial cells^[41]. The most recently discovered *HRH4* has been shown to have mainly immunomodulatory effects^[41]. The expression of histamine receptors is suggested to be influenced by inflammatory stimuli^[40]. In addition, modification of histamine receptor gene expression is suggested to play a role in the pathogenesis of allergy, atherosclerosis and rheumatoid arthritis^[7]. By suppressing the expression of *FCER1* and *HRH4* genes, probiotic lactobacilli could attenuate mast-cell activation and release of allergy-related mediators.

To obtain more evidence of the possible ability of probiotics to suppress mast-cell activation, the array gene set was manually screened for other genes involved in IgE receptor signaling. Expression of the gene that encodes PLC, which is involved in the release of intracellular calcium and in mast-cell degranulation^[39], was suppressed significantly after Lc705 and LGG stimulation. Additionally, the gene that encodes expression of the member of the mitogen-activated protein kinase (MAPK) family, *MAPK12*, that participates in the signaling events that lead to the production of Th2 type cytokines IL-3, IL-4, IL-5 and IL-13, and in the generation of eicosanoids^[38], was also downregulated in Lc705 and LGG-stimulated mast cells. These findings suggest that Lc705 and LGG have an inhibitory effect on mast-cell genes that are involved in the IgE signaling cascade, beyond inhibition of IgE receptor gene expression.

Our results with human primary mast cells are in line with those of two previous studies of the effects of non-pathogenic bacteria on human or mouse mast cells. *Escherichia coli* (*E. coli*) K12 strain has been found to downregulate *FCER1A* in the malignant human mast cell line (LAD3)^[42]. The other study has reported that *E. coli* strain DSM 17252 inhibits mast-cell degranulation in mouse peritoneal mast cells^[43]. Commensal non-pathogenic *E. coli*

and probiotic LGG and Lc705 seem to downregulate mast-cell IgE responses similarly. These results suggest that commensal and probiotic bacteria do not stimulate mast cells but rather diminish their activation. However, it is worth noticing that this effect is not universal response to bacteria, because not all probiotic bacteria or pathogenic Cpn affected the gene expression of high-affinity IgE receptor similarly.

Probiotic bacteria were also observed to alter the expression of genes that have inflammatory functions. In the functional categorization analysis, the expression of a category of immune system process that contains, for example, a gene that encodes an inflammatory mediator that is also regulated by the MAPK pathway, TNF α ^[44], was upregulated in mast cells stimulated with LGG, Lc705, Bb12 and the combination. The categorization analysis also highlighted upregulated functions in known inflammatory genes such as *IL8*, *CCL2*, and *IL1B* in cells stimulated with LGG, Lc705, Bb12 and the combination. The same genes were also upregulated in mast cells stimulated with Cpn, which is in line with our previous study in which Cpn elicited pro-inflammatory effects in mast cells^[22]. However, no upregulation in any of the Th2 type cytokine genes in mast cells after probiotic stimulation in the gene microarray was observed. In the prevention of the symptoms in allergic diseases, probiotics have been suggested to elicit low-grade inflammation and thus shift the immune response away from the allergy-related Th2 type inflammation^[45,46]. In *in vitro* studies, probiotic bacteria have been observed to induce the secretion and expression of Th1 type cytokines in monocytes, macrophages and dendritic cells^[47,49]. Additionally, in a clinical study, low-grade inflammation induced by LGG in allergy-prone children has been proposed to be one mechanism to prevent atopic diseases^[45]. The ability of LGG, Lc705, Bb12 and the combination to induce in mast cells pro-inflammatory rather than Th2 type cytokine expression could have contributed to the observed beneficial low-grade inflammatory response in the above-cited study. Our findings in mast cells support the idea that some probiotic strains shift the mast-cell-mediated immunological response from the Th2 to Th1 type, by inducing expression of pro-inflammatory mediators.

Mast cells stimulated with LGG, Lc705, Bb12, the combination, or with Cpn also induced the expression of a gene that encodes anti-inflammatory IL-10. Mast-cell-derived IL-10 has been observed to be crucial in restriction of chronic skin inflammation in hypersensitivity reactions^[50]. Probiotic *Bifidobacterium* strains have been observed to stimulate IL-10 production in immune cells^[51]. IL-10 induction in mast cells could participate in balancing the inflammatory impact. *In vivo*, the combination of affected genes after bacterial stimulation is likely to be more important than a change in the expression of any single gene. Thus, the downregulation of *FCER1* and *HRH4* genes combined with the upregulation of *IL10* after stimulation with probiotic *Lactobacillus* might be involved in downregulation of inflammatory responses in allergy and other inflammatory diseases in which mast cells are known to play a role, such as atherosclerosis, rheumatoid

arthritis, inflammatory bowel disease and IBS^[5,52-54]. As Cpn upregulated only *IL10* without affecting *FCER1* and *HRH4*, mast cells stimulated with pathogenic Cpn might not have the same clinical effects as those stimulated with probiotic bacteria.

In addition to *FCER1* and *HRH4*, the function of mast cells is regulated through a variety of other receptors, such as TLRs. In our previous study, we have shown that TLR2 is a receptor for LGG, which triggers the nuclear factor- κ B signaling cascade, which leads to expression of different cytokines in human primary macrophages^[55]. In addition, probiotic bacteria have been shown to suppress mast-cell degranulation by interrupting *FCER1*-mediated signaling through TLR2^[56]. The paradox of the ability of probiotic LGG and Lc705 to diminish mast-cell activation, but enhance the mast-cell immune response, could be regulated through the same receptor, TLR2.

PJS failed to change mast-cell gene expression significantly at either time point. Previously, PJS has been observed to induce TNF α expression after 3 h stimulation, and IL-10 expression after 3 and 24 h stimulation in human peripheral blood mononuclear cells (PBMCs)^[24]. It could be that mast cells are unresponsive to PJS unlike the PBMC population. It could also be that the quantitative spectrum of microarray is a limiting factor, especially if the expression differences between unstimulated and stimulated samples are low or if the gene is expressed in low quantities^[57]. In our experiments, the differences in the gene expression levels of the microarrays were relatively low, which is in accordance with other microarray studies performed with probiotic bacteria^[58].

Crosstalk between probiotic bacteria and intestinal mast cells is likely to occur mainly through gut epithelial cells^[59]. Probiotic bacteria or their products could incidentally translocate to the lamina propria through intestinal M cells and make direct contact with immunological cells such as mast cells. Additionally, mast cells are suggested to have the possibility to be in direct contact with bacteria or bacterial fragments in atherosclerotic plaques^[53,60]. Fragments of Firmicutes and Proteobacteria, which are probably of intestinal origin, as well as Cpn have been detected from atherosclerotic plaques^[60]. The intestinal barrier is believed to leak also in healthy humans, which would allow probiotic bacteria to access spaces that contain macrophages, dendritic cells and mast cells. Even though *in vivo* evidence of direct contact between mast cells and probiotic bacteria in the human intestine is missing, *in vitro* interaction studies performed with direct contact between bacteria and host cells, such as mast cells, are required for better understanding of the molecular basis of the immunomodulative properties of probiotic bacteria.

The mast cells differentiated by the peripheral-blood-derived isolation method comprise mainly the MC_{Tc} phenotype^[23], whereas the gut contains both MC_{Tc} and MC_T phenotypes; the latter being the predominant mucosal mast-cell phenotype^[2]. However, the MC_T and MC_{Tc} phenotypes both express high-affinity IgE receptors and produce a variety of inflammatory mediators. The responses observed in this study could be further evaluated in a

more physiological context, e.g. in *ex vivo* culture models using mast cells derived from human intestinal tissues, or in *in vivo* animal or clinical studies.

The present study is believed to be the first to describe the effects of probiotic bacteria on human mast cells. Our data suggest that especially probiotic *L. rhamnosus* Lc705 and *L. rhamnosus* GG could diminish mast-cell activation and the effects of allergy-related mediators by down-regulating expression of the high-affinity IgE and *HRH4* receptors, and by stimulating mast-cell immune responses. Mast cells are important mediators of allergic responses on host surfaces including the intestine, therefore, we propose that mast cells participate in regulating the beneficial immunological responses to probiotic bacteria.

ACKNOWLEDGMENTS

Professor Heikki Vapaatalo is acknowledged for his advice and critical comments on the study and the manuscript. The authors thank Mirja Puolakkainen in the National Institute for Health and Welfare for kindly providing Cpn for this study. The authors acknowledge the excellent technical assistance of Anna-Maija Ahonen in growing bacteria, and Mari Jokinen and Maija Atuegwu for their help with culturing mast cells.

COMMENTS

Background

Probiotic bacteria are widely used to prevent or relieve symptoms of various clinical conditions, such as intestinal disorders and allergy. However, it is not fully understood what make probiotics effective.

Research frontiers

Mast cells are important immunological cells that have many functions. Mast cells also participate in the pathogenesis of many inflammatory diseases, with allergy being the best-known example. In the prevention of the symptoms in allergic diseases, probiotics have been suggested to elicit low-grade inflammation. In the present study, the authors explored for the first time the role of human mast cells in contributing to the beneficial effects of probiotic bacteria.

Innovations and breakthroughs

In the present study, the authors found that probiotic lactobacilli strains *Lactobacillus rhamnosus* (*L. rhamnosus*) GG and *L. rhamnosus* Lc705, but not propionibacteria or bifidobacteria, downregulated expression of high-affinity IgE and histamine H4 receptors, and enhanced mast-cell immune activity, and thus, might diminish the impact of the allergenic response.

Applications

Understanding the mechanisms by which probiotic bacteria elicit their health effects is crucial when designing and using different probiotic strains for specific preventive or therapeutic purposes.

Terminology

Probiotic bacteria are defined as live microorganisms that have beneficial effects on human health. High-affinity IgE receptor mediates mast-cell activation and histamine production in allergy. Histamine H4 receptor is a protein on mast cells that mediates the effects of histamine.

Peer review

In this well-designed study, the authors explored the action of four probiotic strains and their combination on human mast-cell global gene expression. The analysis revealed changes in genes involved in immune responses and mast-cell activation, which are especially involved in the pathogenesis of inflammatory diseases, such as allergy. The authors suggest that lactobacilli are able to diminish the impact of allergenic responses by downregulating expression of high-affinity IgE and histamine H4 receptors, and by enhancing mast-cell immune activity. The study addresses an interesting and important question.

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S- Editor Sun H L- Editor Kerr C E- Editor Lin YP

N-Acetyltransferase 2 genetic polymorphisms and risk of colorectal cancer

Tiago Donizetti da Silva, Aledson Vitor Felipe, Jacqueline Miranda de Lima, Celina Tizuko Fujiyama Oshima, Nora Manoukian Forones

Tiago Donizetti da Silva, Aledson Vitor Felipe, Jacqueline Miranda de Lima, Nora Manoukian Forones, Oncology Group - Gastroenterology Division, Universidade Federal de São Paulo, 04023-900 São Paulo, Brazil

Celina Tizuko Fujiyama Oshima, Pathology Department, Universidade Federal de São Paulo, 04023-900 São Paulo, Brazil

Author contributions: Silva TD performed the majority of experiments; Felipe AV and de Lima JM were involved in editing the manuscript; Oshima CTF coordinated this work; Forones NM designed the study and ordinate this work.

Supported by The São Paulo Research Foundation (FAPESP), Oncology Group - Gastroenterology Division, Universidade Federal de São Paulo, São Paulo, Brazil

Correspondence to: Dr. Nora Manoukian Forones, Gastroenterology Division, Universidade Federal de São Paulo, R Botucatu 740 - 2º andar, 04023-900 São Paulo, Brazil. nora@gastro.epm.br

Telephone: +55-11-50841743 Fax: +55-11-50841743

Received: September 14, 2010 Revised: November 2, 2010

Accepted: November 9, 2010

Published online: February 14, 2011

62 years. More than half the subjects (59.8% in the case group and 51.9% in the control group) were NAT2 slow acetylators. The odds ratio for colorectal cancer was 1.38 (95% CI: 0.90-2.12) in slow acetylators. Although the number of women was small ($n = 76$ in the case group), the cancer risk was found to be lower in intermediate (W/Mx) acetylators [odds ratio (OR): 0.55, 95% confidence interval (95% CI): 0.29-1.02]. This difference was not observed in men (OR: 0.56, 95% CI: 0.16-2.00). Among NAT2 fast acetylators (W/W or W/Mx), meat consumption more than 3 times a week increased the risk of colorectal cancer (OR: 2.05, 95% CI: 1.01-4.16). In contrast, cigarette smoking increased the risk of CRC among slow acetylators (OR: 1.97, 95% CI: 1.02-3.79).

CONCLUSION: The risk of CRC was higher among fast acetylators who reported a higher meat intake. Slow NAT2 acetylation was associated with an increased risk of CRC.

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Key words: N-acetyltransferase 2; Polymorphism; Colorectal cancer

Peer reviewer: Qin Su, Professor, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Silva TD, Felipe AV, de Lima JM, Oshima CTF, Forones NM. N-Acetyltransferase 2 genetic polymorphisms and risk of colorectal cancer. *World J Gastroenterol* 2011; 17(6): 760-765 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/760.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.760>

Abstract

AIM: To investigate the possible association between meat intake, cigarette smoking and N-acetyltransferase 2 (NAT2) genetic polymorphisms on colorectal cancer (CRC) risk.

METHODS: Patients with CRC were matched for gender and age to healthy controls. Meat intake and cigarette smoking were assessed using a specific frequency questionnaire. DNA was extracted from peripheral blood and the genotypes of the polymorphism were assessed by polymerase chain reaction-restriction fragment length polymorphism. Five NAT2 alleles were studied (WT, M1, M2, M3 and M4) using specific digestion enzymes.

RESULTS: A total of 147 patients with colorectal cancer (76 women and 90 men with colon cancer) and 212 controls were studied. The mean age of the two groups was

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the world. In Brazil, 13 310 new cases in men and

14800 in women are estimated to occur in 2010^[1].

N-acetyltransferase 2 (NAT2) is an enzyme found in a large number of organs such as the lungs, colon, breast, prostate, and liver. The expression of this enzyme suggests that it plays a key role in the protection against reactive molecules resulting from environmental insults not only in the liver but in all target tissues^[2]. The *NAT2* gene is located in the chromosome 8p22 region and has no introns. The gene contains an 870-bp open reading frame and encodes a protein of 290 amino acids^[3]. It is an important phase II enzyme that catalyzes the acetylation of aromatic and heterocyclic amines and hydrazines present in carcinogenic compounds and medicines. Individuals can be divided into three different phenotypes based on the acetylation activity of NAT2: fast, intermediate, and slow. These phenotypes are determined by single nucleotide polymorphisms in *NAT2*^[4].

Some NAT2 polymorphisms have been consistently associated with a reduction in acetylation activity (e.g. T³⁴¹C). The functional state of the phenotype is due to the impairment of protein translation or stability. No changes in mRNA levels are detected. For several polymorphisms, the classification as “fast” or “slow” is not final^[5].

The probability of developing cancer depends on the natural response of each organism to different aggressive agents. Humans present different susceptibilities to carcinogens^[6,7]. This difference in susceptibility to various environmental aggressors is related to genetic polymorphisms^[8].

Studies have associated meat consumption with an increased risk of CRC^[9]. Red meat, especially meat that is well done, is a source of chemical carcinogens such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, and other products. The fast acetylation genotype is probably related to larger amounts of metabolic activators of heterocyclic aromatic amines when compared to the slow NAT2 acetylation genotype. Metabolic activators are transported to colorectal tissues through the bloodstream, causing DNA damage and mutations in tumor suppressor genes involved in the carcinogenesis of CRC^[10].

The association between genetic polymorphisms in the *NAT2* gene and CRC has been studied extensively; however, the results are not conclusive, possibly because of ethnic differences and differences in the lifestyle and number of patients studied. The proportion of fast and slow acetylation phenotypes varies markedly depending on ethnicity and geographic origin^[11]. Thus, there is an urgent need for studies investigating the distribution of NAT2 genotypes in different countries.

The aim of the present study was to investigate NAT2 polymorphisms in Brazilian patients from São Paulo.

MATERIALS AND METHODS

A case-control study involving 147 patients with CRC and 212 healthy subjects was carried out between March 2008 and December 2009. All patients were born in Brazil and were treated at the Oncology Division, Department of Gastroenterology, University Hospital, Universidade Fed-

eral de São Paulo (UNIFESP). The study was approved by the Ethics Committee of UNIFESP and all patients signed an informed consent form.

The patients answered a questionnaire regarding food habits and food frequency, whether they were current or former cigarette smokers, and their pattern of alcohol consumption.

Peripheral blood was collected for genomic DNA extraction. The *NAT2* gene polymorphisms were investigated by the polymerase chain reaction (PCR)-restriction fragment length polymorphism genotyping technique.

DNA extraction

Leukocyte DNA was extracted from peripheral venous blood collected with ethylenediaminetetraacetic acid using the Invisorb® Spin Blood Mini Kit.

Analysis of NAT2 genetic polymorphisms

The genotypes of the NAT2 polymorphism were analyzed as described previously^[9]. Genomic DNA was amplified using the following primers: 5'-GGAACAAATTG-GACTTGG-3' and 5'-TCTAGCATGAATCACTCTGC-3'. After amplification, the PCR product was digested with KpnI (M1 allele), BamHI (M3 allele) and MspI/AluI (M4 allele), and with TaqI (M2 allele). The digestion products were separated on agarose gels stained with ethidium bromide, and were then visualized under UV light^[15]. The W/W and W/MX genotypes were classified as conferring the fast acetylation phenotype and the Mx/MX genotype as conferring the slow acetylation phenotype.

Statistical analysis

The Student *t*-test was used for the comparison of age between groups. Differences in the polymorphisms between the two groups were determined by the χ^2 test. This test was also used to compare clinical variables between NAT2 genotypes and alleles in the group of cancer patients. The association between the risk of developing cancer and these variables was assessed by calculating the odds ratio (OR) and 95% confidence interval (95% CI). A *P* value < 0.05 was considered to be statistically significant and a *P* value of 0.05 to 0.10 was considered to be marginally significant.

RESULTS

A case-control study including 147 patients with CRC and 212 healthy controls was conducted to determine whether NAT2 genetic polymorphisms are associated with the development of this disease. The characteristics of the cancer patients and controls are shown in Table 1. No difference in age or gender was observed between groups. Among the 147 patients with cancer, 90 (61.2%) had colon cancer and 57 (38.8%) had rectal cancer. According to the TNM classification, most patients were stage II (44.2%) or stage III (26.5%).

Four different NAT2 alleles were found, including the wild type (WT) and the M1, M2 and M3 polymorphisms. The M4 allele was not detected. Among healthy control

Table 1 Characteristics of the patients in both groups *n* (%)

Parameters	Patients	Control	<i>P</i>
Age (yr, \pm DP)	61.9 (13.6)	62.0 (13.4)	0.96 ^a
≤ 50	30 (20.4)	31 (14.6)	0.196 ^b
> 50	17 (79.5)	181 (85.4)	
Gender			0.15 ^b
Male	71 (48.3)	85 (40.1)	
Female	76 (51.7)	127 (59.9)	
Tumor site			
Colon	90 (61.2)		
Rectum	57 (38.8)		
Stage			
I	23 (15.6)		
II	65 (44.2)		
III	39 (26.5)		
IV	20 (13.6)		

^a*t* test; ^b χ^2 test.

subjects, the observed genotype frequency of the NAT2 polymorphisms were consistent with the expected frequency of the Hardy-Weinberg equilibrium ($P = 0.56$), suggesting that the distribution of NAT2 genotypes is adequate in the cancer-free population.

The slow acetylation phenotype predominated in the two groups (59.8% in the case group and 51.9% in the control group). No significant differences in the frequency of the NAT2 polymorphisms were observed between groups (Table 2).

The odds ratio for CRC was 1.38 (95% CI: 0.90-2.12) in slow acetylators. The M1 allele was the most frequent allele in the two groups, with a frequency of 45% in the control group and of 44.5% in the case group, followed by the WT allele (28% in the control group and 25.1% in the case group).

No significant association was observed between NAT2 polymorphism and acetylation phenotype or tumor site. Comparison of patients in TNM stage I or II *vs* stage III or IV showed a higher frequency of the slow acetylation phenotype in stage I and II patients (60.1%).

Analysis of red meat intake showed that half of the subjects consumed red meat more than 3 times a week. Subjects with the fast acetylation phenotype who consumed meat more than 3 times a week presented an increased risk of CRC (OR: 2.05, 95% CI: 1.01-4.16) (Table 3). With respect to cigarette smoking, the number of ex-smokers was marginally higher in the cancer group. Cigarette smoking increased the risk of CRC among slow acetylators (Mx/Mx) (OR: 1.97, 95% CI: 1.02-3.79) (Table 4).

DISCUSSION

CRC is one of the most common cancers. Almost 70% of CRC patients are diagnosed at age 65 years or older^[12]. Most of the subjects studied here were women ($n = 76$, 51.7%), and the mean age was 61.9 years. These findings agree with data published by the Brazilian National Cancer Institute^[11].

According to the Annual Report to the Nation on the Status of Cancer, prostate cancer is the most frequent cancer among men, followed by lung, colon and rectal

cancer, except for Latin America where the incidence of CRC is slightly higher than that of lung cancer. Among women, the most frequent cancer is breast cancer, followed by lung cancer and CRC^[13].

Variations in the frequency of NAT2 genotypes/phenotypes among different populations and ethnic groups have been reported in several studies carried out in different regions around the world. In this respect, a high frequency of the slow acetylator phenotype is observed in populations of European and African descent. Other populations are characterized by a high frequency of fast acetylation phenotypes, such as Japanese, Chinese and Amerindians^[14-16].

In the present study, the slow acetylation phenotype was slightly more frequent in the two groups, although the difference was not statistically significant. However, when divided by gender, the fast acetylation phenotype tended to be more common among women. The M1 allele was the most frequent allele in the two groups (45% in the control group and 44.5% in the case group), followed by the WT allele (28% in the control group and 25.1% in the case group). The M4 allele was not detected. These data are consistent with the literature, which indicates a difference in the frequency of the WT, M1 and M4 alleles between Caucasians and Africans, whereas the frequency of the M2 and M3 alleles is similar. The M4 allele is detected at a rate of less than 1% in Caucasians, whereas its frequency is 18% in the African population^[9,17,18].

No significant difference in NAT2 polymorphism or acetylation phenotype was observed between tumor sites. Analysis according to TNM stage showed that the slow acetylation phenotype was more frequent among stage I or II patients (60.1%) compared to stage III or IV. This finding might be explained by the fact that the NAT2 fast acetylation phenotype activates carcinogens and produces mutations more quickly, resulting in aggressive tumors. However, these findings should be analyzed carefully because of the small number of patients participating in the present study.

For a long time, genetic susceptibility to cancer has been attributed to xenobiotic exposure. This view was mainly due to the fact that the molecular mechanisms involved in carcinogenesis were not known. However, this view has changed over recent years with the advances in molecular biology. It is now known that exposure to xenobiotics and the development of cancer vary among individuals because of variations that occur at the molecular level which, in turn, are under genetic control^[19]. In recent studies, lifestyle habits including alcohol and tobacco use and dietary habits (i.e. adequate protein and fiber intake) have been associated with gene mutations in an attempt to obtain more consistent results regarding cancer risk factors and prognosis. Although currently available data are controversial due to ethnic differences and differences in lifestyle, this has been the best approach to better understand carcinogenesis at the molecular level.

Smoking has been associated with several types of cancer other than lung cancer, including cancer of the oral cavity, pancreas, and kidney^[20]. A recently published meta-

Table 2 Distribution of N-acetyltransferase 2 polymorphism and the risk of cancer

Genetic polymorphism NAT2 ^a	Cancer, <i>n</i> (%)	Control, <i>n</i> (%)	<i>P</i>	OR (95% CI)
All	147	212		
Mx/Mx	88 (59.8)	110 (51.9)	0.19	1
W/Mx	44 (30.0)	83 (39.1)		0.66 (0.42-1.05)
W/W	15 (10.2)	19 (8.9)		0.99 (0.47-2.05)
Slow	88 (59.8)	110 (51.9)	0.17	1.38 (0.2-2.12)
Fast	59 (40.1)	102 (48.1)		
Female	76	127		
Mx/Mx	42 (55.2)	58 (45.6)	0.07	1
W/Mx	23 (30.2)	58 (45.6)		0.55 (0.29-1.02)
W/W	11 (14.4)	11 (8.6)		1.38 (0.55-3.48)
Slow	42 (55.2)	58 (45.6)	0.24	1.47 (0.83-2.60)
Fast	34 (44.7)	69 (54.3)		
Male	71	85		
Mx/Mx	46 (64.8)	52 (61.1)	0.67	1
W/Mx	21 (29.5)	25 (29.4)		0.95 (0.47-1.92)
W/W	4 (5.6)	8 (9.4)		0.57 (0.16-2.00)
Slow	46 (64.7)	52 (61.1)	0.76	0.86 (0.45-1.65)
Fast	25 (35.2)	33 (38.9)		

^aHomozygous individuals with genotype W/W, and heterozygote W/Mx, are grouped into fast acetylation phenotype, while homozygous Mx/Mx are grouped in slow acetylators. The percentages of data are in parentheses. NAT2: N-acetyltransferase 2; OR: Odds ratio; 95% CI: Confidence interval.

Table 3 Comparison between meat intake and risk of cancer in rapid acetylator patients *n* (%)

	Cancer	Control	<i>P</i>	OR	Lower ^{95% CI} / upper
All	59	102			
High meat intake ¹	44 (74.5)	60 (58.8)	0.06	2.05	1.01/4.16
Low meat intake ²	15 (25.5)	42 (41.2)			

¹More than 3 time per week; ²Less than 3 times a week. OR: Odds ratio; 95% CI: Confidence interval.

Table 4 Correlation between genotypes and risk for cancer in smokers or ex-smokers

Genetic polymorphism	Cancer, <i>n</i> (%)	Control, <i>n</i> (%)	<i>P</i>	OR (95% CI)
NAT2 ^a				
All	65	87		
Mx/Mx	40 (61.5)	39 (44.8)	0.09	1
W/Mx	22 (33.8)	39 (44.8)		1.82 (0.92-3.60)
W/W	3 (4.6)	9 (10.3)		3.08 (0.77-12.22)
Slow	40 (61.5)	39 (44.8)	0.06	1.97 (1.02-3.79)
Fast	25 (38.5)	48 (61.0)		

^aHomozygous individuals with genotype W/W, and heterozygote W/Mx, are grouped into fast acetylation phenotype, while homozygous Mx/Mx are grouped in slow acetylators. NAT2: N-acetyltransferase 2; OR: Odds ratio; 95% CI: Confidence interval.

analysis reported a strong association between smoking and the development of CRC^[12]. However, smoking is currently not recognized as a risk factor for CRC by the International Agency for Research on Cancer (IARC) or the US Surgeon General^[12]. Sørensen *et al*^[21] studied the association between NAT1 and NAT2 polymorphisms, smoking, meat consumption and CRC risk in 379 cancer patients and 769 healthy subjects. In that study, only the NAT1 polymorphism affected cancer risk. However, the NAT1 and NAT2 fast acetylation phenotype increased the risk of CRC among patients who smoked more cigarettes, suggesting that N-acetylation status affects the relationship between smoking and CRC risk.

In the present study, most subjects in the two groups had never smoked, but the rate of ex-smokers was higher in the case group than in the control group. Once diagnosed with cancer, individuals tend to break old habits that may affect the prognosis and treatment of the disease even if it is not possible to reverse the previous damage. The slow acetylation phenotype was more frequent among smokers of the case group, suggesting an increased risk of cancer (OR: 1.97, 95% CI: 1.02-3.79) in subjects with this phenotype. A higher frequency of the slow acetylation phenotype among patients with lung and bladder cancer has been demonstrated in other studies. Carcinogens

present in tobacco are metabolized by NAT enzymes and activation of these enzymes is reduced in slow acetylators, thus increasing the risk of cancer^[22].

An association between red meat consumption and a higher risk of CRC has been reported in case-control studies, prospective epidemiological studies and in a recent meta-analysis^[23]. The last study suggested that this increased risk is due to the production of polycyclic aromatic hydrocarbons and heterocyclic amines when meat is cooked at high temperatures^[24].

Tamer *et al*^[25], studying 125 patients with CRC and 82 healthy subjects, observed an association between NAT2 polymorphisms and cancer development. In that study, high protein intake was found to be correlated with an increased risk of colon cancer (OR: 1.73, 95% CI: 1.10-3.07). Patients with the NAT2 * 14A (M4 allele) fast acetylation phenotype and high meat intake presented an increased risk of CRC (OR: 3.03, 95% CI: 1.56-5.86). In the present study, the risk of cancer was higher among patients consuming meat more than 3 times per week (OR: 1.65, 95%

CI: 1.05-2.61). The risk of CRC was increased among patients presenting the fast acetylator genotypes (W/W or W/Mx) and a high frequency of meat intake (OR: 2.05, 95% CI: 1.01-4.16).

Heterocyclic amines are formed when meat is cooked by the condensation of creatinine with amino acids. The NAT2 fast acetylation phenotypes are more readily able to convert N-hydroxy heterocyclic amines into carcinogens, a fact predisposing to cancer. Thus, heterocyclic amines require metabolic activation to induce DNA mutations and to initiate carcinogenesis. After N-oxidation, N-hydroxy aromatic and heterocyclic amines are activated (*via* O-acetylation) by NAT to acetoxy intermediates, which react spontaneously with DNA to form adducts^[26,27]. The increased cancer risk observed in patients with the NAT2 fast acetylation phenotype and high meat consumption suggests that heterocyclic amines mediated by metabolic activation of NAT2 fast acetylation might be important carcinogens and increase the risk of cancer.

In conclusion, the proportion of subjects with the slow acetylation phenotype was high in this study. No association was observed between the risk of CRC and NAT2 polymorphisms. However, the slow acetylation phenotype increased the risk of CRC in smokers and the fast acetylation phenotype increased this risk among subjects with high red meat intake.

COMMENTS

Background

Colorectal cancer (CRC) is considered the fourth leading cause of cancer worldwide and is one of the most common malignancies in the West. The probability of developing cancer depends on the natural response of each organism to different exposures from various aggressors. Humans have different susceptibilities to different carcinogens and lifestyle may be a risk factor for cancer. The interaction between diet, alcoholism, cigarette smoking, obesity and physical inactivity can lead to its development.

Research frontiers

The HAAs (present in red meat, tobacco, *etc.*) are bioactivated through N-oxidation by the enzymes CYP1A2 in the liver or by CYP1A1/CYP1B1 in extra hepatic tissues. The products of this oxidation are the N-hydroxy-N, which in turn suffer the bioactivation in the liver by O-acetylation of N-acetyltransferase (NAT) enzymes (mainly NAT2) and sulfotransferase. NAT2 fast acetylation individuals may have larger amounts of metabolic activators of HAA than slow acetylation NAT2 subjects that can be transmitted to the colorectal tissues through the bloodstream, causing DNA damage and mutations.

Innovations and breakthroughs

Previous studies linking meat consumption and colorectal cancer have concluded that diets rich in meat consumption increase the risk of this disease. In the case of red meat, the degree of cooking can be a source of exposure to chemical carcinogens such as heterocyclic amines, nitrosamines and other products. This study aimed to identify the distribution of NAT2 gene polymorphism in a Brazilian population from São Paulo correlating these polymorphisms and the phenotypes of NAT2 acetylation with the consumption of red meat, alcohol and cigarette smoking and the colorectal cancer risk.

Applications

The proportion of people with the phenotype of fast and slow acetylation varies considerably depending on the ethnicity and geographic origin. This variability lead to the investigation into the frequency of NAT2 genotypes and the association of this polymorphism with colorectal cancer in a Brazilian population of São Paulo. The relationship between the mechanism of acetylation by NAT2 and susceptibility to colorectal cancer may possibly screen patients who have a higher risk of developing this disease.

Terminology

The NAT2 is an important phase II enzyme that catalyzes the acetylation of heterocyclic aromatic amines and hydrazines, which include carcinogenic compounds and drugs. Based on the activity of NAT2 acetylation, subjects are divided into three different phenotypes: fast, intermediate and slow. Single nucleotide polymorphisms in NAT2 determine this phenotype.

Peer review

The authors examined the phenotypes and polymorphisms of NAT2 acetylation on a case-control study, involving the eating habits and lifestyle as risk modifiers of colorectal cancer development. The results indicated that cigarette smokers with slow acetylation phenotype had an increased risk of developing colorectal cancer and individuals with fast acetylation phenotype intake of red meat increased the risk of developing the disease. These results are interesting because the study of NAT2 acetylation may identify individuals with a higher risk of developing CRC in relation to environmental factors and diet.

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S- Editor Sun H L- Editor Rutherford A E- Editor Lin YP

Identification of patients at-risk for Lynch syndrome in a hospital-based colorectal surgery clinic

Patrícia Koehler-Santos, Patricia Izetti, Jamile Abud, Carlos Eduardo Pitroski, Silvia Liliana Cossio, Suzi Alves Camey, Cláudio Tarta, Daniel C Damin, Paulo Carvalho Contu, Mario Antonello Rosito, Patricia Ashton-Prolla, João Carlos Prolla

Patrícia Koehler-Santos, Patricia Izetti, Jamile Abud, Carlos Eduardo Pitroski, Silvia Liliana Cossio, Patricia Ashton-Prolla, Genomic Medicine Laboratory, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre - RS, 90035-903, Brazil

Patrícia Koehler-Santos, Carlos Eduardo Pitroski, Patricia Ashton-Prolla, Post-Graduate Program in Medical Sciences, Federal University of Rio Grande do Sul, Porto Alegre - RS, 90035-003, Brazil

Patrícia Koehler-Santos, Silvia Liliana Cossio, Patricia Ashton-Prolla, National Institute for Populational Medical Genetics, Porto Alegre - RS, 90035-903, Brazil

Patricia Izetti, Medical School, Federal University of Rio Grande do Sul, Porto Alegre - RS, 90035-003, Brazil

Jamile Abud, João Carlos Prolla, Post-Graduate Program in Gastroenterological Sciences, Federal University of Rio Grande do Sul, Porto Alegre - RS, 90035-003, Brazil

Suzi Alves Camey, Department of Statistics, Federal University of Rio Grande do Sul, Porto Alegre - RS, 91501-970, Brazil
Suzi Alves Camey, Post-Graduate Program in Epidemiology, Federal University of Rio Grande do Sul, Porto Alegre - RS, 90035-003, Brazil

Suzi Alves Camey, Research and Post-Graduation Group, Hospital de Clínicas de Porto Alegre, Porto Alegre - RS, 90035-903, Brazil

Cláudio Tarta, Daniel C Damin, Paulo Carvalho Contu, Mario Antonello Rosito, Division of Coloproctology, Hospital de Clínicas de Porto Alegre, Porto Alegre - RS, 90035-903, Brazil
Cláudio Tarta, Daniel C Damin, Paulo Carvalho Contu, Mario Antonello Rosito, Department of Surgery, Federal University of Rio Grande do Sul, Porto Alegre - RS, 90035-003, Brazil

Patricia Ashton-Prolla, Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre - RS, 90035-903, Brazil
Patricia Ashton-Prolla, Department of Genetics, Federal University of Rio Grande do Sul, Porto Alegre - RS, 91501-970, Brazil
João Carlos Prolla, Service of Pathology, Hospital de Clínicas de Porto Alegre, Porto Alegre - RS, 90035-903, Brazil

Author contributions: Koehler-Santos P identified and interviewed all consecutive colorectal cancer patients who agreed to participate in the study; Koehler-Santos P and Izetti P designed the database and performed the analysis under the supervision of Camey SA, and were directly involved in writing and editing

the manuscript; Abud J, Pitroski CE and Cossio SL helped with acquisition of the data from patient databases; Tarta C, Damin DC, Contu PC and Rosito MA are the primary physicians of all patients and participated in patient identification; Prolla JC and Ashton-Prolla P were directly involved with the concept and design of the study, evaluation of patients at-risk and editing of the manuscript.

Correspondence to: Patricia Ashton-Prolla, MD, PhD, Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos 2350, Porto Alegre - RS, 90035-903, Brazil. pprolla@hcpa.ufrgs.br

Telephone: +55-51-33598011 Fax: +55-51-33598010

Received: February 8, 2010 Revised: March 23, 2010

Accepted: March 30, 2010

Published online: February 14, 2011

Abstract

AIM: To determine the prevalence of a family history suggestive of Lynch syndrome (LS) among patients with colorectal cancer (CRC) followed in a coloproctology outpatient clinic in Southern Brazil.

METHODS: A consecutive sample of patients with CRC were interviewed regarding personal and family histories of cancer. Clinical data and pathology features of the tumor were obtained from chart review.

RESULTS: Of the 212 CRC patients recruited, 61 (29%) reported a family history of CRC, 45 (21.2%) were diagnosed under age 50 years and 11 (5.2%) had more than one primary CRC. Family histories consistent with Amsterdam and revised Bethesda criteria for LS were identified in 22 (10.4%) and 100 (47.2%) patients, respectively. Twenty percent of the colorectal tumors had features of the high microsatellite instability phenotype, which was associated with younger age at CRC diagnosis and with Bethesda criteria ($P < 0.001$). Only

5.3% of the patients above age 50 years had been previously submitted for CRC screening and only 4% of patients with suspected LS were referred for genetic risk assessment.

CONCLUSION: A significant proportion of patients with CRC were at high risk for LS. Education and training of health care professionals are essential to ensure proper management.

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Key words: Colorectal cancer; Family history; Hereditary cancer; Lynch syndrome; Microsatellite instability phenotype

Peer reviewer: Vamsi R Velchuru, MRCS, FRCSEd, FRCS (Gen Surg), James Paget University Hospital, Great Yarmouth, 6 Pickwick Drive, Off Market Lane, Blundeston, NR32 5BX, United Kingdom

Koehler-Santos P, Izetti P, Abud J, Pitroski CE, Cossio SL, Camey SA, Tarta C, Damin DC, Contu PC, Rosito MA, Ashton-Prolla P, Prolla JC. Identification of patients at-risk for Lynch syndrome in a hospital-based colorectal surgery clinic. *World J Gastroenterol* 2011; 17(6): 766-773 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/766.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.766>

INTRODUCTION

Family history of colorectal cancer (CRC) is a clinically significant risk factor and may be reported by up to 15% of all patients with the disease. Lynch syndrome (LS, OMIM: # 120435), also called hereditary non-polyposis colorectal cancer syndrome (HNPCC), is the most common inherited colon cancer predisposition syndrome. It is an autosomal dominant syndrome caused by germline mutations in the mismatch repair (MMR) genes *hMLH1*, *hMSH2*, *hMSH6* and *PMS2*. The syndrome accounts for 2%-3% of all CRC diagnoses and for 5%-9% of the diagnoses of endometrial cancer in patients under age 50 years^[1-4]. Other extra-colonic tumors including ovarian, upper urologic tract, gastric, small bowel, biliary/pancreatic and brain cancers have been described at an increased frequency in families with LS^[5]. The cumulative lifetime risk of cancer varies depending on geographic/environmental factors and the age-related incidence of each tumor type^[6-8]. Furthermore, the cancer spectrum in families affected with the syndrome varies significantly based upon the DNA MMR gene mutated and the specific mutation^[9,10].

Determining the prevalence of LS among patients with CRC is an important public health issue. Affected patients have an increased risk for second primary cancers and their identification can lead to specific screening and intervention recommendations for patients and their at-risk relatives^[11-13].

Cancer family history is an important tool to identify at-risk patients and families. The Amsterdam criteria, ini-

tially including only CRC and later all tumors of the LS cancer spectrum, define clinical diagnosis of LS and are in themselves an indication for MMR mutation testing. However, even the revised Amsterdam II criteria have a relatively low sensitivity (< 80%) which has turned out to be a major limitation for LS diagnosis. More recently, the Bethesda guidelines were developed to identify a larger proportion of MMR mutation carriers (Table 1)^[14].

Multiple other strategies for identifying individuals with LS have been proposed, including predictive mathematical models to define prior probabilities of carrying a germline MMR mutation, family history instruments and routine testing of CRCs from patients with specific risk factors (e.g. age < 50 years), but the effectiveness of these approaches continues to be debated and has limited applicability in clinical practice^[9,10].

In this study, we aimed to determine the prevalence of a family history suggestive of LS among patients with CRC followed in a coloproctology outpatient clinic of a University Hospital in Southern Brazil, and to identify all potential LS patients who should be referred for genetic counseling. Also, we investigated the frequency of tumors with histopathologic features suggestive of microsatellite instability (MSI) and whether screening recommendations were correctly modified according to the risk identified.

MATERIALS AND METHODS

Ethics

This study was approved at the Institutional Ethics Committee (GPPG-HCPA) under the number 05-257.

Patients

All consecutive patients with a diagnosis of CRC who had an appointment in the outpatient Coloproctology clinic of Hospital de Clínicas de Porto Alegre (HCPA), in Porto Alegre, Southern Brazil, from December 2005 to December 2006, were considered for participation in this study. From a total of 250 patients seen in this period, 212 unrelated patients with adenocarcinoma of the colon and rectum and without a previous diagnosis of inflammatory bowel disease were invited and agreed to participate in the study. After signature of informed consent forms, data regarding personal and family cancer history, pathology reports and additional relevant clinical and/or surgical information were collected. Information collected included types of cancer and age at diagnosis, presence of multiple (synchronous or metachronous) tumors, type and periodicity of colorectal screening, tumor histology, clinical and histological stage of tumors (Dukes and TNM Staging System), family history of cancer (first, second and third degree). Tumor diagnoses in family members were confirmed by medical records and/or death certificates whenever possible.

“Early-onset” colorectal or endometrial cancer was defined as cancer diagnosed before the age of 50 years. All other extra-colonic tumors described in the LS (ovarian, upper urologic tract, gastric, small bowel, biliary/pancre-

Table 1 Clinical criteria for Lynch syndrome

Name	Criteria	Sensitivity ¹	Specificity ¹
Amsterdam	Amsterdam criteria I	61.0%	67.0%
	Three or more relatives with colorectal cancer, one of whom is a first-degree relative of the other two; FAP should be excluded		
	Colorectal cancer involving at least two generations		
	One or more colorectal cancer cases diagnosed before the age of 50		
	Amsterdam criteria II	78.0%	61.0%
	Three or more relatives with histologically verified LS-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis), 1 of whom is a first-degree relative of the other 2; FAP should be excluded		
	Colorectal cancer involving at least two generations		
	One or more cancer cases diagnosed before the age of 50		
Revised Bethesda	At least one of the following features	90.9%	77.1%
	Bethesda 1: Colorectal cancer diagnosed in a patient under the age of 50		
	Bethesda 2: Presence of synchronous or metachronous colorectal cancer, or other LS-associated tumors ² , regardless of age		
	Bethesda 3: Colorectal cancer with the MSI-H histology ³ under the age of 60		
	Bethesda 4: Colorectal cancer in one or more first-degree relatives with an LS-related tumor, with one of the cancers under the age of 50		
	Bethesda 5: Colorectal cancer in two or more first- or second-degree relatives with LS-related tumors, regardless of age		

¹Data on sensitivity and specificity of Amsterdam criteria from Syngal *et al.*^[14] and Revised Bethesda from Piñol *et al.*^[46]; ²Lynch syndrome (LS)-associated tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, brain tumors, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel; ³Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern. FAP: Familial adenomatous polyposis; MSI-H: Microsatellite instability-high.

atic and brain) were considered in the pedigree analyses^[5]. Colorectal surveillance was considered appropriate when colonoscopy was performed by or after age 50 years in patients with no history of CRC in first- or second-degree relatives, and in those with a positive family history, when performed 10 years before the earliest diagnosis of CRC in the family, and every 1-2 years thereafter.

The criteria used to identify patients with LS or at-risk for the syndrome included the Amsterdam I and/or II criteria for clinical diagnosis^[5,15] and the Bethesda Revised Criteria^[16] for a potential diagnosis.

Statistical analysis

SPSS version 16.0 was used for data handling and statistical analyses. For descriptive analysis, categorical variables were described by their absolute and/or relative frequencies and quantitative variables were expressed as mean \pm SD. For analytical statistics, the existence of an association between categorical variables was examined using χ^2 . The Student's *t* test was used to determine the significance between different ages at diagnoses among two independent groups. A difference with a *P* value of less than 0.05 was considered significant.

RESULTS

Clinical information on the 212 patients studied is summarized in Table 2. Mean age of the patients at recruitment was 62.33 years (range: 24-99 years, SD = 12.8 years), and 113 (53.3%) were female. Of the 212 patients, 45 (21.2%) were diagnosed with CRC under the age of 50 years and the mean age at first CRC diagnosis was 59.8 years (range: 20-99 years, SD = 13.1 years). Approximately

Table 2 Sample description by criteria for Lynch syndrome (*n* = 212)

Lynch syndrome criteria	<i>n</i> (%)
Amsterdam	22 (10.4)
Amsterdam I	16 (7.6)
Amsterdam II	6 (2.8)
Bethesda (at least 1 of the 5 criteria)	100 (47.2)
Bethesda (2 or more of the criteria)	41 (19.3)
Bethesda by criteria	
Bethesda 1	45 (21.2)
Bethesda 2	17 (8.0)
Bethesda 3	27 (12.7)
Bethesda 4	23 (10.8)
Bethesda 5	36 (17.0)

5.2% of the patients had a synchronous or metachronous colorectal tumor (*n* = 11) and the mean age at diagnosis in this group was 51.6 years (range: 36-80 years, SD = 13.1 years), lower than the mean age in patients without metachronous colorectal tumor (59.3 years, range: 20-99 years, SD = 12.8 years), as expected (*P* = 0.051). Two patients (0.9% of the sample) were diagnosed with familial adenomatous polyposis.

The age at diagnosis of the first cancer varied from 20 to 86 years (mean = 58.9 years; median = 59 years; SD = 12.9 years). A second primary cancer was present in 33 patients: CRC in 11 (33.3%), endometrial in 2 (6.1%), breast in 2 (6.1%), prostate in 6 (18.2%). The age at diagnosis of the second primary varied from 39 to 99 years (mean = 66.5 years, SD = 13.2 years). One patient was diagnosed with four different primary tumors: two colon cancers at ages 36 and 55 years, endometrial can-

Table 3 Features of the 223 colorectal tumors in the 212 probands¹

Feature	n (%)
Tumor site	
Ascending colon	22 (9.9)
Transverse colon	12 (5.4)
Descending colon	11 (5.0)
Rectosigmoid	158 (71.1)
Other (cecum, unspecified site)	19 (8.6)
Total	222 (100.0)
Missing data	1
Differentiation	
Well differentiated	20 (10.0)
Moderately differentiated	157 (78.1)
Poorly differentiated	24 (11.9)
Total	201 (100.0)
Missing data	22
Mucinous feature	15 (6.7)
Total	223 (100.0)
Missing data	0
MSI-high phenotype ²	42 (21.3)
Total	197 (100.0)
Missing data	26
Dukes stage (n = 195)	
A	6 (3.1)
B	76 (39.0)
C	87 (44.6)
D	26 (13.3)
Total	195 (100.0)
Missing data	28

¹Cases with missing data were excluded from the analysis; ²Cases included all patients with microsatellite instability-high phenotype, independent of age.

cer at 45 years, bladder cancer at 61 years and renal carcinoma at 62 years; all of them confirmed with pathology records. This patient was later found to carry a germline mutation in *hMLH1* (data not shown).

Among the 212 unrelated probands, family history of cancer up to second-degree relatives was observed in 60.4% of patients with early-onset and 52.4% of those with late-onset CRC diagnoses. Twenty-nine percent of the patients reported a family history of CRC and almost 50% fulfilled criteria for LS: 22 (10.4%) fulfilled Amsterdam I and/or II criteria and 100 (47.2%), Revised Bethesda Criteria. In the families of probands with LS criteria, 180 relatives had cancer and the most frequent primary sites were: colon and rectum (43.4%), lung (8.9%), breast (7.8%), stomach (6.7%), endometrium (6.7%), ovaries (3.9%) and prostate (3.4%). As expected, this distribution was different in patients without LS criteria: colon and rectum (30.8%), lung (12.3%), breast (12.3%), stomach (4.6%), uterus (1.5%), ovarian (0%) and prostate (12.3%). About 2.5% of the patients had relatives with multiple primary tumors: one case was diagnosed with uterine and ovarian cancer, and another with colorectal, esophageal and gastric cancer.

Family history of breast cancer was present in 6.4% of the sample with available information (11/171). Among the patients with at least one of the Bethesda criteria, 12% had a family history of breast cancer, compared to 7.1% among patients with none of the criteria ($P = 0.248$).

Table 4 Comparison of different features among patients with and without the microsatellite instability-high phenotype (n = 197¹) n (%)

Features	MSI-high phenotype (n = 42)	Non MSI-high phenotype (n = 155)	P
Age at diagnosis < 50 yr	18 (42.9)	29 (18.7)	0.001
Family history of colorectal cancer	15 (35.7)	45 (29.0)	0.404
Presence of Revised Bethesda criteria ^[16]	42 (100)	56 (36.1)	< 0.001
Presence of Amsterdam II criteria	6 (14.3)	15 (9.7)	0.391
Second primary tumors	3 (7.1)	8 (5.2)	0.620
Early stage at diagnosis	11 (26.2)	64 (41.3)	0.079

¹15 patients were excluded due to missing data on tumor histology. MSI: Microsatellite instability.

In the overall sample, 22 (9.9%) patients had a tumor in the ascending colon and 21.3% had colorectal tumors with histology suggestive of MSI-high (MSI-H) phenotype (Table 3). The clinical features of patients with and without this phenotype are shown in Table 4. As expected, MSI-H histological features were more commonly seen in patients with CRC diagnosed at a younger age (18 patients from a total of 42, $P = 0.001$) and in patients who fulfilled the Bethesda criteria (in all 42 patients, $P < 0.001$). Opposite to what was expected, in a significant number of tumors from individuals fulfilling Amsterdam criteria, histological features suggestive of MSI-H phenotype were not encountered.

Of the 212 charts reviewed, 17% had the family history previously documented. Furthermore, when the previously documented family histories were compared to the pedigrees obtained during patient interview for this study, there was concordance of data in only 56.6% of cases. On the other hand, of the 100 patients with a family history of cancer and fulfilling Bethesda criteria for LS, 57.0% had their family history previously collected and/or reported in the chart by clinicians or surgeons, but a clinical suspicion of LS was not documented in the chart in any of these cases. Only 4% of the patients with clinical criteria for LS were referred for genetic cancer risk evaluation.

Finally, 5.3% of patients with indications for population-based screening by colonoscopy starting at age 50 years had been submitted at least once for colonoscopy before the diagnosis of CRC. Among the 100 patients at risk for LS, only 4.1% had been offered surveillance colonoscopy previously.

DISCUSSION

As in most familial cancer syndromes, early age of onset and multiplicity of cancers have been considered hallmarks of LS. In registry-based series, the mean age at first CRC is about 45 years, compared to 65 years for sporadic CRC, and some LS patients present with CRC in their twenties. Similarly, the mean age of endometrial cancer is about 50 years, which is about 10 years younger than the average age of sporadic endometrial cancer.

As our knowledge of the influences of genetics on cancer risk has increased, so has the need to improve physicians' awareness of the importance of familial cancer history and its proper recording in the medical chart^[17]. Although there is no consensus about the correct method for obtaining information on cancer family history, and using it as a screening tool^[18], general practitioners usually collect the family history data at the time of registration^[19] and different groups have reported screening the adult population for increased genetic risk of cancer using postal questionnaires^[20,21]. Unfortunately, however, health professionals in the first line of patient contact are usually unaware of how and when to contact genetic services. Many specialists, especially coloproctologists and gastroenterologists, have a key role in identifying high-risk patients; the ability to suspect a patient to be at risk for a cancer predisposition syndrome is crucial for a rapid diagnosis and to ensure appropriate care.

Nearly 30% of patients in our study reported a positive family history of CRC, which is much higher than observed positive CRC family history in the general population: approximately 9.0%^[22]. Our findings also indicate that the hereditary CRC phenotype can be easily identified in outpatient coloproctology units, using a systematic approach after proper training of the staff, as reported previously^[20,21].

Surprisingly, a high number of patients fulfilling Amsterdam criteria were found in our sample (22/212, 10.4%), significantly higher than previously reported in the Brazilian population. Viana *et al.*^[23], reviewing 311 medical records of CRC patients from São Paulo, Brazil, found a frequency of 1.3% in families with Amsterdam criteria. The reason for such a high incidence remains to be explained. One potential limitation of this study is the fact that the outpatient coloproctology clinic from which the patients derive is located in a tertiary care university hospital, and reference center for many diseases in the region. Thus, a higher percentage of high-risk patients, including those at risk for hereditary cancer may exist in this setting than in other general hospitals.

An additional point to consider is the fact that this institution has, since 2001, one of the few cancer risk evaluation clinics in Southern Brazil. This fact, however, would ideally be associated with high indices of correct identification and referrals of the hereditary cases, which was not observed in most cases. Most patients evaluated in our study did not have an accurate family history assessment in their charts, (description of family history was present in the charts of only 57.0% of potential LS patients). Moreover, only 4% of patients with LS criteria were referred for genetic counseling, suggesting that even when detailed family cancer history is obtained, it may not be granted the necessary importance. Previous investigations have already reported significant gaps in the documentation of family cancer history in medical charts. Analyzing data from the Direct Observation of Primary Care study, Medalie *et al.*^[24] found that only 40% of 2333 audited charts documented the presence or absence of a family history of breast or colon cancer. Even when family cancer history is obtained, its interpretation seems to

be problematic as referrals often focus on rarer, hereditary cancer syndromes, neglecting cases of average and moderate risk individuals. Tyler and Snyder found a similar deficiency in the referral process among family physicians: from 10 patients considered at moderate or high risk, only 3 had been identified and none had been referred for cancer genetic consultation^[25].

Also, although previous studies have shown a clear benefit for surveillance colonoscopy in patients with suspected or proven LS^[26,27], in our series it was not common practice. Several factors could be contributing to this observation: patient's refusal, physician omission of adequate familial risk assessment and or referral and/or limited access to screening examinations. Our results are in agreement with those previous studies, considering that only 5% of the patients evaluated were undergoing proper screening. The identification of high-risk precursor lesions is considered critically important and colonoscopy screening for individuals with LS is recommended to begin at age 25 years, or 10 years younger than the earliest diagnosis of CRC in the family, whichever comes first, every 1-2 years^[28].

Multiplicity of cancers is a hallmark of LS and according to the literature about 10% of identified Lynch patients have more than one cancer by the time of diagnosis. Although CRC is the most common, other frequent findings include cancers of endometrium, ovary, stomach, small bowel, pancreas, hepatobiliary system, renal pelvis, ureter and glioblastoma. Approximately 20%-40% of patients have been reported to develop metachronous CRC after initial resection if a subtotal colectomy is not performed^[29]. Similarly, clustering of more than one Lynch-associated cancer (colorectal and uterine) in an individual patient should raise suspicion of LS. In our population, among all CRC cases, approximately 15% of patients had a second primary cancer. The most common second primary was CRC (metachronous or synchronous), endometrium, breast and prostate.

The most common extra-colonic tumor in LS is endometrial carcinoma, which develops in up to 70% of women who are mutation gene carriers^[30]. Thus, the presence of endometrial cancer in the family history of an individual with CRC, a feature encountered in 12 (6.7%) of the patients studied here, should always raise a suspicion of LS. When the endometrial cancer is diagnosed under the age of 50 years, the probability of LS is particularly high. This has been confirmed by a high rate of MMR deficiency (up to 40%) in women with early-onset endometrial cancer^[31,32]. Consistent with this, the National Comprehensive Cancer Network (NCCN) has recently revised its guidelines and currently recommends annual surveillance of the endometrium for LS families, as well as MMR deficiency investigation for all women diagnosed with endometrial cancer under age 50 years^[28].

There is some debate with regard to whether prostate or breast cancers might be part of the LS, since some studies have found a high frequency of such tumors among HNPCC families^[33,34]. Oliveira Ferreira *et al.*^[22] found a 26.5% frequency of breast cancer in families fulfilling Amsterdam criteria from São Paulo, Southeastern

Brazil, a higher rate than found in general population-based studies, which could suggest existence of a subset of mutations in this region that are also associated with a higher breast cancer risk. In our study, we found breast cancer at a frequency of approximately 9% in families fulfilling Amsterdam criteria. Although it is common to see LS pedigrees with breast cancer, most genetic and immunohistochemical studies on familial breast cancers have not found any strong relationship with the MMR system deficiency^[35,36]. Recently, a case report of a Lebanese family with LS found a *MSH2* gene defect in a breast cancer tumor of early-onset, suggesting that it could be involved in accelerated breast carcinogenesis^[37]. However, more studies are needed to define such association.

Different studies have shown that CRC in LS differs from typical sporadic CRCs in location, histology, and natural history. Also, it usually displays findings that are suggestive of MSI, such as intense lymphocytic infiltrates, extensive areas with poorly differentiated tissue and mucinous histology^[38]. Overall, 20.3% of the tumors evaluated in this series demonstrated one or more histological features suggestive of MSI-H, similar to previous studies^[39]. In the last few years, it has been suggested that histology could be useful in selecting CRC patients for molecular testing for LS^[40-43], since limiting testing to patients with MSI-H histology could reduce the burden of molecular testing by 60% compared with testing all patients who meet the Revised Bethesda Criteria. More importantly, it could help identify LS among patients with late onset and no cancer family history^[39].

Interestingly, among patients who fulfilled the Amsterdam criteria, only 28.6% presented tumors with features of MSI-H phenotype. This is somewhat surprising and could indicate that in this group of patients, the strong family history of CRC may not be related to abnormalities in the MMR system, and that there could be an increased prevalence of what has been called in the literature the Familial Colorectal Cancer Type X^[44]. Recently, Abdel-Rahman *et al*^[45] analyzed the molecular features of the tumors in CRC patients with MMR germline mutations and in sporadic CRC. They concluded that tumors from the MMR gene-negative group exhibited a novel molecular pattern characterized by a paucity of changes in the common pathways to CRC, which could be associated with non MSI-H histology. Molecular studies with this population are now being carried out in order to test such hypothesis.

Large population-based studies have shown that a family history of CRC in first-degree relatives is associated with an increased risk of CRC. In this study we have shown that a significant proportion of patients diagnosed with CRC and followed in an outpatient clinic of a university hospital in Southern Brazil have either a significant family history of cancer or pathology features suggestive of LS. Our findings underscore the importance of adequate familial risk assessment and, also, of considering MSI-H pathology features in the identification of at-risk patients. Education and training of physicians is essential to ensure that hereditary cancer patients and families are identified and properly referred for genetic counseling and

long-term cancer screening programs. The reason for the high prevalence of patients at risk for LS in our population requires further investigation.

ACKNOWLEDGMENTS

We are indebted to the patients and their family members who agreed to participate in this study and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Programa de Pós-Graduação em Ciências Gastroenterológicas (UFRGS) and Fundação de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE-HCPA) who provided funding for this study. PKS was supported by a postdoctoral fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

COMMENTS

Background

The incidence of colorectal cancer (CRC) is currently rising in Southern Brazil. Patients at-risk for the hereditary forms of the disease are diagnosed at younger ages, and have an increased risk for second colorectal tumors and extra-colonic malignancies. Identification of these patients can lead to specific screening and intervention recommendations.

Research frontiers

In this study, the authors describe high prevalence of patients at-risk for Lynch syndrome (LS) in a coloproctology clinic and results support the principle that education and training of health care professionals are essential to ensure proper management of these individuals.

Innovations and breakthroughs

The study draws attention to the high frequency of potential LS patients in a coloproctology clinic in Southern Brazil. It reinforces the importance of correct identification of these cases and suggests further investigations of the origins for these observations.

Applications

Description of the high frequency of hereditary CRC cases in this setting is the first step to demonstrate that adequate familial risk assessment is fundamental to identify at-risk patients.

Peer review

Study was undertaken very well. It's a well written paper. It highlights the importance of identifying high risk groups in order to carry on with surveillance in CRC.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Ma WH

Single center experience of capsule endoscopy in patients with obscure gastrointestinal bleeding

Mahesh Kumar Goenka, Shounak Majumder, Sanjeev Kumar, Pradeepta Kumar Sethy, Usha Goenka

Mahesh Kumar Goenka, Shounak Majumder, Sanjeev Kumar, Pradeepta Kumar Sethy, Usha Goenka, Institute of Gastroenterology, Apollo Gleneagles Hospital, 58 Canal Circular Road, Kolkata-700054, India

Author contributions: Goenka MK and Majumder S were the major contributors to this work; Goenka MK and Majumder S designed the research; Goenka MK interpreted all capsule endoscopy images; Majumder S was responsible for tabulating and analyzing the data, drafting the manuscript, and processing the images; Goenka MK and Majumder S wrote the article; Kumar S, Sethy PK and Goenka U performed a critical scientific review of the article and provided valuable input for modifying parts of the article prior to submission.

Correspondence to: Dr. Mahesh Kumar Goenka, Institute of Gastroenterology, Apollo Gleneagles Hospital, 58 Canal Circular Road, Kolkata-700054, India. mkgkolkata@gmail.com
Telephone: +91-33-22820636 Fax: +91-33-23205218

Received: August 5, 2010 Revised: September 13, 2010

Accepted: September 20, 2010

Published online: February 14, 2011

Abstract

AIM: To identify optimum timing to maximize diagnostic yield by capsule endoscopy (CE) in patients with obscure gastrointestinal bleeding (OGIB).

METHODS: We identified patients who underwent CE at our institution from August 2003 to December 2009. Patient medical records were reviewed to determine type of OGIB (occult, overt), CE results and complications, and timing of CE with respect to onset of bleeding.

RESULTS: Out of 385 patients investigated for OGIB, 284 (74%) had some lesion detected by CE. In 222 patients (58%), definite lesions were detected that could unequivocally explain OGIB. Small bowel ulcer/erosions secondary to Crohn's disease, tuberculosis or non-steroidal anti-inflammatory agent use were the commonest lesions detected. Patients with overt GI bleeding for < 48 h before CE had the highest diagnostic yield (87%). This was significantly greater ($P < 0.05$) compared to

that in patients with overt bleeding prior to 48 h (68%), as well as those with occult OGIB (59%).

CONCLUSION: We established the importance of early CE in management of OGIB. CE within 48 h of overt bleeding has the greatest potential for lesion detection.

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Key words: Capsule endoscopy; Gastrointestinal bleeding

Peer reviewer: Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

Goenka MK, Majumder S, Kumar S, Sethy PK, Goenka U. Single center experience of capsule endoscopy in patients with obscure gastrointestinal bleeding. *World J Gastroenterol* 2011; 17(6): 774-778 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/774.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.774>

INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is responsible for about 5% of all gastrointestinal (GI) bleeding^[1]. Although it represents a small proportion of patients with GI bleeding, OGIB continues to be a challenge because of delay in diagnosis and consequent morbidity and mortality. In recent times, capsule endoscopy (CE) and device-assisted enteroscopy have established their position in the management algorithm for OGIB, and have had a significant impact on the outcome. CE is superior to push enteroscopy^[2,3], small bowel follow-through^[4] and computed tomography (CT)^[5] for detection of the bleeding source in the small bowel. There is however concern about sensitivity of CE in the setting of ongoing GI bleeding, due to possible visualization of blood limiting the interpretation.

Most published reports on CE in OGIB are limited to small groups of patients^[6-8]. Although most differentiate between occult and overt GI bleeding when analyzing diagnostic yield, they do not identify the optimum time for performing CE in the overt GI bleeding group. We evaluated the diagnostic yield of CE in identifying the source of bleeding in OGIB. We further analyzed our patients to answer the question regarding proper timing of performing CE in overt OGIB, to maximize diagnostic yield. To the best of our knowledge, the present study of 385 patients with OGIB is the largest single-center experience of CE in OGIB.

MATERIALS AND METHODS

Patient selection

Patients who presented with evidence of GI bleeding at the clinic or emergency department were enrolled in the present study after negative upper GI endoscopy and full-length colonoscopy. Between August 2003 and December 2009, 505 patients underwent CE at our center. 345 patients underwent the procedure as inpatients, whereas 160 were outpatients. Of these, 385 (76.2%) had CE for OGIB (Figure 1). Patients with OGIB were further classified into three categories: (1) persistent overt bleeding, i.e. bleeding documented within 48 h at the time of first evaluation; (2) recent overt bleeding, i.e. last episode of bleeding > 48 h prior to the first evaluation; and (3) obscure occult bleeding, i.e. anemia associated with positive fecal occult blood without overt bleeding.

CE procedure

The GIVEN Video Capsule system (Given Imaging, Yoqneam, Israel) was used with M2A/SB capsules. The reader system was updated during the study period from Rapid 3 to Rapid 5. The real time viewer (Given Imaging) was used during the final 6 mo of the study. Patients were allowed a light diet on the previous evening and were prepared by using an oral purge at night (2 L polyethylene-glycol-based solution or 90 mL sodium phosphate mixed with 350 mL lime-based drink followed by 1 L water). Patients swallowed the capsule between 09:00 and 11:00 h, and were maintained on nil by mouth for the next 4 h. Six patients had swallowing difficulty and had their capsule delivered into the stomach using an endoscope with the help of an AdvanCE device. Patients with known diabetes mellitus and history of vomiting that was suggestive of gastroparesis were given two doses of intravenous metoclopramide (10 mg) during the study. Intravenous metoclopramide was also given to three of 40 patients who were found to have their capsule in the stomach on real time study, even at 2.5 h after capsule ingestion. The recorder of CE was disconnected only after the battery stopped blinking at 8-11 h after capsule ingestion. Only one procedure had technical difficulty with the capsule not becoming active after removal from the container, and had to be replaced with another capsule. All other patients had a smooth examination.

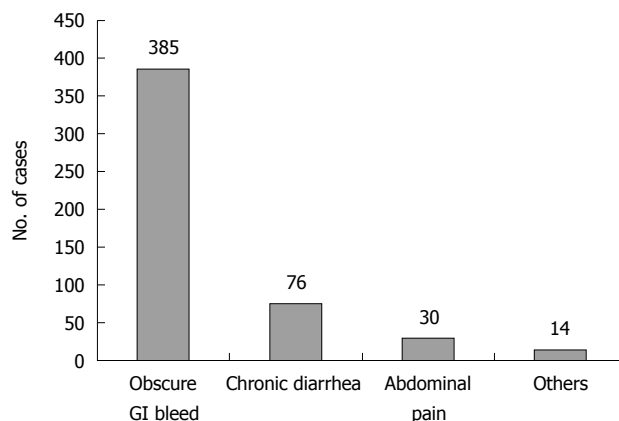


Figure 1 Indication for capsule endoscopy (n = 505).

Image interpretation

The interpretation of images was done by a single gastroenterologist (MKG) after initial detailed evaluation by a trained technician who had been involved in > 50 000 GI endoscopic procedures. Findings were categorized as definite, suspicious or negative as follows: (1) definite: lesions with definite bleeding potential that clearly explained the clinical situation; (2) suspicious: mucosal lesions identified, but bleeding could not be conclusively attributed to them, or blood was seen in the small intestine without any definite lesion being identified; and (3) negative: no lesion or bleeding identified, or incomplete study.

Follow-up

Patients were asked to note evacuation of the capsule, and those who were uncertain or concerned, as well as those who were suspected to have retained the capsule, as suggested by capsule image interpretation, were followed by serial X-ray/fluoroscopic screening at weekly intervals. Patients were also followed up with medical therapy (such as treatment of Crohn's disease, institution of antitubercular therapy, or antihelminthic therapy), surgical therapy (for tumors or bleeding ulcers) or enteroscopic evaluation (ulcers, polyps, or bleeding angiodysplasia), depending on the CE results. Those with negative CE were followed up with expectant treatment or surgery with preoperative enteroscopy. The study was approved by our institutional review board.

Statistical analysis

Statistical methods included χ^2 analysis for comparison of the positive diagnostic yield of CE between the three different categories of OGIB. $P < 0.05$ was considered to be statistically significant.

RESULTS

OGIB was the commonest (76.2%) indication for CE during the study period (Figure 1). Out of the 385 patients with OGIB, 275 (71%) were male with age ranging from 12 to 80 years. One hundred and one patients (26.2%) had a negative examination, either because no obvious lesion was found until the small intestine ($n = 93$) or because

Table 1 Positive/suspicious lesions detected by capsule endoscopy in patients with obscure gastrointestinal bleeding ($n = 284$)

	Definite ($n = 222$)	Suspicious ($n = 62$)	Total ($n = 284$)
Ulcers/erosions	156	32	188
Tumor	48	0	48
AVM	18	7	25
Worms	0	8	8
Only blood	0	15	15

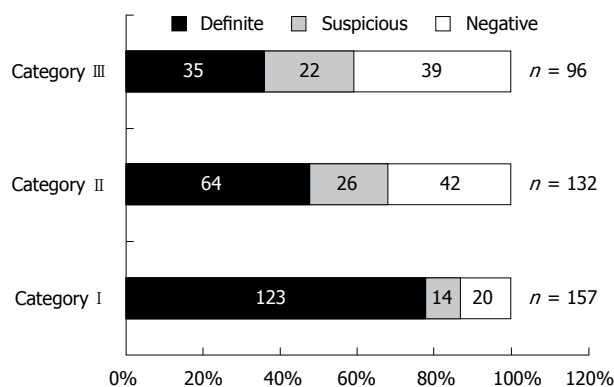
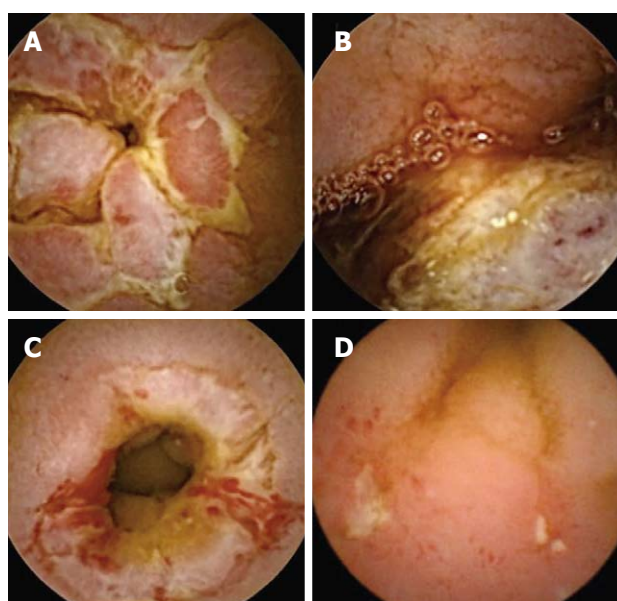
101 patients had negative capsule endoscopy. AVM: Arteriovenous malformation.

the progress was slow ($n = 8$). Of the eight patients with slow progress of the capsule, six had diabetes mellitus. Of the 101 patients with negative CE, nine underwent laparotomy because of recurrent/persistent bleeding, and in eight, some lesion was found at preoperative enteroscopy (Meckel's diverticulum, 1; small-intestinal ulcers, 3; and angiodysplasia, 4). One patient had negative laparotomy. Of the remaining 92 patients in this group, only 52 were available for a follow-up of 1 year and none had any significant bleeding.

Two hundred and eighty-four patients (73.8%) had some lesion detected at CE. Although 272 of these lesions were located in the small intestine, 12 (ulcers/erosions, 8; angiodysplasia, 3; and gastric fundal tumor, 1) had findings in the stomach/duodenum that were missed at pre-CE gastroscopy. Two hundred and twenty-two patients (57.7%) were considered to have definite lesions that could explain OGIB, whereas another 62 (16.1%) had lesions that were suspicious but bleeding could not be completely attributed to these findings. The latter included 32 patients with small ulcers and erosions, seven with doubtful angiodysplasia, eight with worms (3 roundworm, 3 whipworm, and 2 hookworm) and 15 patients with blood in the jejunum or ileum, without any underlying lesion being identified. Four of these patients with evidence of bleeding but no underlying lesions underwent mesenteric angiography that also detected bleeding, but no obvious pathology was found and bleeding stopped with supportive treatment alone.

As demonstrated in Table 1, the 222 patients with definite lesions at CE included ulcers/erosions in 156, tumors in 48, and angiodysplasia in 18. Among those with tumors, two had multiple polyps that were suggestive of Peutz-Jeghers syndrome. It was difficult to characterize ulcers/erosions, but at least 12 were considered to be tubercular (based on abdominal CT scan \pm fine needle aspiration cytology and follow-up), 42 patients were considered to have Crohn's disease (based on fissuring serpiginous ulcers with cobble-stone appearance, or histology from tissue obtained at enteroscopy or surgery), and 12 were nonsteroidal anti-inflammatory drug (NSAID)-induced. Of the 18 patients with arteriovenous malformation (AVM), four underwent double-balloon enteroscopy (DBE), three had successful treatment with argon plasma coagulation, and the others were put on hormonal therapy/tranexamic acid.

Figure 2 shows the distribution of patients according

**Figure 2** Diagnostic yield of capsule endoscopy according to category of bleeding ($n = 385$)**Figure 3** Capsule endoscopy images. A: Cobble-stone appearance characteristic of Crohn's disease; B: An ileal tumor; C: Circumferential ulcer with narrowing and minor ongoing bleeding in a patient with Crohn's disease; D: Small intestinal ulcers in a patient with tuberculosis.

to category of bleeding. In patients with ongoing bleeding (Category I), positive findings were seen in 87.2% (definite in 78.3%), whereas in patients with previous overt bleeding (Category II), it was 68.2% (definite in 48.5%), and in the occult OGIB group (Category III), only 59.3% (definite in 36.4%). The ability of CE to identify a definite bleeding source was significantly higher for Category I than Category II and III patients ($P < 0.05$), but there was no significant difference in the diagnostic yield when comparing Category II and III patients (Figure 3).

Capsule retention was noted in six of 385 patients (1.6%). All these patients had strictures in the small bowel either due to tuberculosis or Crohn's disease, which were not suspected or identified prior to CE. Three of these patients underwent surgery, two were lost to follow-up, and one refused surgery and continues to have capsule retention, but has been asymptomatic during follow-up of 9 mo.

DISCUSSION

CE has gained widespread clinical acceptance in the diagnostic algorithm of OGIB^[9,10]. As in our study, OGIB is now the leading indication for CE in most centers around the world. Prior to the introduction of CE, barium examination, push enteroscopy and angiography were the principle diagnostic tools for OGIB. The diagnostic yield of these tests has been shown to be unequivocally inferior to CE in several studies. Recently, DBE has been used in several centers for diagnosis of OGIB. However, diagnostic yield of CE has been found to be significantly higher compared to a single DBE examination done via the oral or anal route (137/219 *vs* 110/219, OR: 1.67, 95% CI: 1.14-2.44, $P < 0.01$)^[11].

The reported yield of CE in OGIB varies widely. Previous studies have shown that detection rates for the source of bleeding varies from 38% to 93%, and is in the higher range for those with overt OGIB^[9,10]. This is further influenced by subjective interpretation of positive findings. To address this issue in our study, we divided positive findings into definite and suspicious groups. Although the overall diagnostic yield in our study cohort was 73.8%, a definite lesion that could explain OGIB was obtained in only 57.7%. A recently published study by Hindryckx *et al.*^[8] which considered CE to be positive only when lesions with sufficient bleeding potential were detected, reported a similar diagnostic yield of 59.8%.

Recent studies have indicated that the optimum timing of CE in OGIB is within the first few days, with acceptable maximum duration of 2 wk^[13-17]. In a recently reported series of 260 patients with OGIB, the yield was 87% in patients with ongoing overt OGIB and 46% in those with occult OGIB^[10]. In our patients, a definite lesion could be detected in 64.7% of patients with overt OGIB compared to 36.4% in patients with occult OGIB ($P < 0.01$). Moreover, the diagnostic yield of CE was significantly higher in patients who had evidence of bleeding within 48 h of CE (Category I) compared to those who had remote overt bleeding (Category II) [123/157 (78.3%) *vs* 64/132 (48.5%) OR: 3.84, 95% CI: 2.31-6.41, $P < 0.01$, respectively]. The diagnostic yield of CE was not significantly different when comparing patients with remote overt OGIB (> 48 h before CE) (Category II) and those with occult OGIB (Category III). This highlights the importance of using CE early in the diagnosis of OGIB. Pennazio *et al.* also have found the highest yield in patients with ongoing GI bleeding, and therefore have recommended ordering CE earlier in the setting of overt OGIB. There have been concerns in the past regarding the possibility of blood obscuring proper visualization of the mucosa in patients who are actively bleeding. A recent study that has compared massively bleeding patients with chronic overt OGIB has found a similar positive yield in both groups [59.18% (29/49) and 52.69% (137/260), respectively]^[18]. These results demonstrate that, for optimum diagnostic efficacy, CE should be done within 48 h of bleeding in patients with OGIB.

The definition of a positive finding on CE continues

to be ambiguous. For the purpose of this study, nonspecific mucosal changes such as red spots, focal erythema and fold thickening, were not considered to be clinically significant. Ulcers and erosions were included as positive findings in this series if they could completely or partially account for the GI bleeding. Moreover, active bleeding without definite lesions was described as a suspicious finding in this study. The commonest lesion detected in our patients was small-bowel ulcers and erosions, followed by tumors and AVM. A previous study from India also has documented small-bowel ulcers to be the commonest lesion detected by CE in OGIB^[19]. A definite underlying etiology could be established in 66 (42.3%) out of the 156 patients with ulcers/erosions, and nearly two-thirds (42/66) of them were considered to be due to Crohn's disease.

The current study has several limitations. In the first place it is a retrospective single-center study. However data was obtained from forms filled at the time of CE, thereby minimizing data collection bias. Secondly this study does not offer long-term follow-up of the patients and hence makes it impossible to draw a strong conclusion as to the fate of CE-negative OGIB. Moreover a large proportion of ulcers/erosions could not be characterized due to inherent difficulty of obtaining small bowel mucosal biopsies. However, this study enabled us to analyze positivity rates, nature of lesions and optimum timing of CE in a relatively large cohort of subjects comprising of a heterogeneous population of patients with OGIB. Although the data demonstrates the diagnostic utility of early CE in OGIB it does not reflect whether an early diagnostic intervention and unequivocal identification of a bleeding source affects clinical outcome in this group of patients.

In summary, high diagnostic yield, relative safety and tolerability have established CE as an important diagnostic tool for OGIB. In this large cohort of OGIB patients, we demonstrate that small bowel ulcer/erosions secondary to Crohn's disease, tuberculosis or NSAID-use are the commonest lesions responsible for OGIB in this part of the world. Moreover, the diagnostic yield is significantly affected by the timing of CE and studies done within 48 h of an episode of overt bleed have the greatest potential for detecting a definite lesion.

ACKNOWLEDGMENTS

We would like to acknowledge the services of our capsule endoscopy nurse Ms. Sonali Chattopadhyay who assisted us throughout the duration of the study.

COMMENTS

Background

The advent of video capsule endoscopy (CE) has resulted in a paradigm shift in the approach to the diagnosis and management of patients with obscure gastrointestinal bleed (OGIB). With increasing global availability of this diagnostic tool, it has now become an integral part of the diagnostic algorithm for OGIB in most parts of the world. However there is scant data on optimum timing of CE for maximizing diagnostic yield. OGIB continues to be a challenge because of delay in diagnosis and consequent morbidity and mortality.

Research frontiers

Previous studies have shown that capsule endoscopy detection rates for the source of bleeding varies from 38% to 93%, being in the higher range for those with overt OGIB. Results in most studies are further influenced by subjective interpretation of "positive findings". The authors classified our patients depending on time since last episode of bleed and looked at diagnostic yield in the different groups with the aim to identify a time-frame to guide clinical decision-making on when to do a capsule endoscopy in this cohort of patients.

Innovations and breakthroughs

Diagnostic yield is significantly affected by the timing of CE and studies done within 48 h of an episode of overt bleed have the greatest potential for detecting a definite lesion. The diagnostic yield of CE was not significantly different when comparing patients with overt OGIB prior to 48 h of CE and those with occult OGIB. This highlights the importance of obtaining a CE early in the diagnosis of OGIB.

Applications

This article suggests a potential benefit of doing a capsule endoscopy within 48 h of an episode of bleed in patients with OGIB in terms of increasing chances of detecting a bleeding source. However, further studies are needed to determine if early detection of lesion translates into better patient outcome.

Peer review

The paper provides well-collected information about early detection of small intestinal lesions by CE in obscure GI bleed. Research should be aimed at finding if early detection results in improved patient outcome.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Serum magnesium concentration in children with functional constipation treated with magnesium oxide

Maiko Tatsuki, Reiko Miyazawa, Takeshi Tomomasa, Takashi Ishige, Tomoko Nakazawa, Hirokazu Arakawa

Maiko Tatsuki, Reiko Miyazawa, Takashi Ishige, Tomoko Nakazawa, Hirokazu Arakawa, Department of Pediatrics, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

Takeshi Tomomasa, PAL Clinic, 694 Tanakajima-machi, Isesaki, Gunma 372-0802, Japan

Author contributions: Tatsuki M and Miyazawa R performed the majority of the study and wrote the manuscript; Tomomasa T and Arakawa H designed the study; Ishige T and Nakazawa T collected and provided subjects' data.

Correspondence to: Dr. Reiko Miyazawa, Department of Pediatrics, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. rmiyazaw@gunma-u.ac.jp

Telephone: +81-27-2208200 Fax: +81-27-2208215

Received: September 26, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: February 14, 2011

of the control group [2.2 (2.0-2.2) mg/dL] ($P < 0.001$). The highest value was 3.2 mg/dL. Renal magnesium clearance was significantly increased in the constipation group. Serum magnesium concentration in the constipation group decreased significantly with age ($P < 0.01$). There was no significant correlation between the serum level of magnesium and the duration of treatment with magnesium oxide or the daily dose. None of the patients had side effects associated with hypermagnesemia.

CONCLUSION: Serum magnesium concentration increased significantly, but not critically, after daily treatment with magnesium oxide in constipated children with normal renal function.

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Key words: Children; Constipation; Hypermagnesemia; Magnesium oxide; Renal dysfunction

Peer reviewer: Wallace F Berman, MD, Professor, Division of Pediatric GI/Nutrition, Department of Pediatrics, Duke University Medical Center, Duke University School of Medicine, Durham, Box 3009, NC 27710, United States

Tatsuki M, Miyazawa R, Tomomasa T, Ishige T, Nakazawa T, Arakawa H. Serum magnesium concentration in children with functional constipation treated with magnesium oxide. *World J Gastroenterol* 2011; 17(6): 779-783 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/779.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.779>

Abstract

AIM: To determine whether hypermagnesemia recently reported in adult patients possibly develops in children with functional constipation taking daily magnesium oxide.

METHODS: We enrolled 120 patients (57 male and 63 female) aged 1-14 years old (median: 4.7 years) with functional constipation from 13 hospitals and two private clinics. All patients fulfilled the Rome III criteria for functional constipation and were treated with daily oral magnesium oxide for at least 1 mo. The median treatment dose was 600 (500-800) mg/d. Patients were assessed by an interview and laboratory examination to determine possible hypermagnesemia. Serum magnesium concentration was also measured in sex- and age-matched control subjects ($n = 38$).

RESULTS: In the constipation group, serum magnesium concentration [2.4 (2.3-2.5) mg/dL, median and interquartile range] was significantly greater than that

INTRODUCTION

Magnesium-containing cathartics are used worldwide to treat chronic constipation^[1-3]. Approximately 45 million Japanese patients are estimated to undergo treatment with magnesium oxide as an antacid or cathartic annually^[4]. Many children with functional constipation are taking these drugs for long periods of time; sometimes over several years.

Hypermagnesemia is a rare clinical condition^[5,6]. Most cases are iatrogenic and due to increased intake of magnesium, which occurs after intravenous administration of magnesium^[7,8] or oral ingestion of high doses of magnesium-containing antacids or cathartics^[9-12]. Magnesium homeostasis is dependent mainly on gastrointestinal absorption and renal excretion. The kidney is the principal organ involved in magnesium regulation. Renal magnesium excretion is very efficient, because the thick ascending limb of Henle has the capacity to reject completely magnesium reabsorption under conditions of hypermagnesemia^[5,6], and therefore, hypermagnesemia commonly arises in patients with renal dysfunction.

In 2008, the Ministry of Health, Labour and Welfare (MHLW) of Japan reported 15 adult patients with hypermagnesemia, including two cases of death due to oral ingestion of magnesium oxide from April 2005 to August 2008^[4]. Although most of these elderly patients with constipation had dementia, schizophrenia, or renal dysfunction, the MHLW has recommended measuring the serum level of magnesium in patients who regularly use magnesium oxide.

It is now important for pediatricians to know whether hypermagnesemia can develop in children without abnormal renal function after administration of a common or high dose of magnesium oxide. The purpose of this study was to determine serum magnesium concentration in children with functional constipation treated with daily magnesium oxide.

MATERIALS AND METHODS

Patients

We enrolled 120 patients (57 male and 63 female) aged 1-14 years with functional constipation from 13 hospitals and two private clinics in Japan. At entry, all patients fulfilled the Rome III criteria for functional constipation, which meant that they had at least two of the following characteristics: fewer than three bowel movements weekly; more than one episode of fecal incontinence weekly; large stools in the rectum shown by digital rectal examination or palpable on abdominal examination; occasional passage of large stools; retentive posturing and withholding behavior; and painful defecation. All patients had been treated for at least 1 mo with daily magnesium oxide as an oral laxative. The medication was given once daily or in split doses. The dose was dependent on the patient's condition.

Children with known organic causes of constipation, including Hirschsprung disease, spinal and anal congenital abnormalities, previous colon surgery, inflammatory bowel disease, allergy, metabolic or endocrine diseases, renal dysfunction, and severe neurological disability were excluded from the study. Patients with poor drug compliance were also excluded.

In each patient, we recorded the date of initiation of constipation, daily dose of magnesium oxide, and duration of treatment. We also determined whether the patient had symptoms that could be side effects of hypermagnesemia, such as vomiting, nausea, thirst, blushing, feeling of

Table 1 Subject characteristics and serum magnesium concentrations

	Control group (<i>n</i> = 38)	Constipation group (<i>n</i> = 120)	<i>P</i> value
Age (yr)	5.5 (2.0-10.8)	4.7 (3.0-6.8)	NS ^a
Gender, male (%)	63.2	47.5	NS ^b
Serum magnesium concentration (mg/dL)	2.2 (2.0-2.3)	2.4 (2.3-2.5)	< 0.001 ^a

^aMann-Whitney *U* test; ^b χ^2 test. NS: Not significant.

exhaustion, or somnolence. The laboratory examinations carried out were as follows: serum level of magnesium, calcium, phosphorus, blood urea nitrogen (BUN), and creatinine concentration. Urinary concentrations of magnesium and creatinine were also measured. Magnesium clearance and fractional excretion of magnesium (FEMg) were calculated as follows: Magnesium clearance = urine magnesium (mg/dL)/urine creatinine (mg/dL); FEMg = urine magnesium (mg/dL)/serum magnesium (mg/dL) \times serum creatinine (mg/dL)/urine creatinine (mg/dL).

Control group

Serum magnesium concentrations were also measured in the control group which comprised 38 children (24 male and 14 female) aged 1-15 years who visited the Department of Pediatrics at Gunma University Hospital, and were without any history of hematological disease, tumor, heart failure, metabolic or endocrine diseases, renal dysfunction, or severe neurological disability. None of these children were treated with magnesium oxide.

Statistical analysis

Laboratory values, duration of treatment, and daily dose of magnesium oxide are shown as the median and interquartile ranges. Statistical significance of differences was tested by χ^2 test or Mann-Whitney *U* test, as appropriate. Spearman's rank correlation coefficients were calculated for the correlation between the serum level of magnesium and age, duration of treatment, and drug dose. *P* < 0.05 was regarded as significant. All analyses were carried out using SPSS for Windows (SPSS statistics 17.0).

RESULTS

Subject characteristics and serum magnesium concentrations are shown in Table 1. In the constipation group, the median treatment duration with magnesium oxide was 1.3 (0.4-2.6) years and median daily dose was 600 (500-800) mg/d; 33 (25-45) mg/kg per day. After administration of magnesium oxide, the outcome of constipation was investigated in 83 patients. Bowel habits in all patients were improved, and 75% of patients were stable. However, 11% of children had fewer than three bowel movements weekly, 28% of them had withholding behavior, and 34% had painful defecation during the follow-up period. None of the patients still had overflow-incontinence.

The median serum magnesium concentration in the

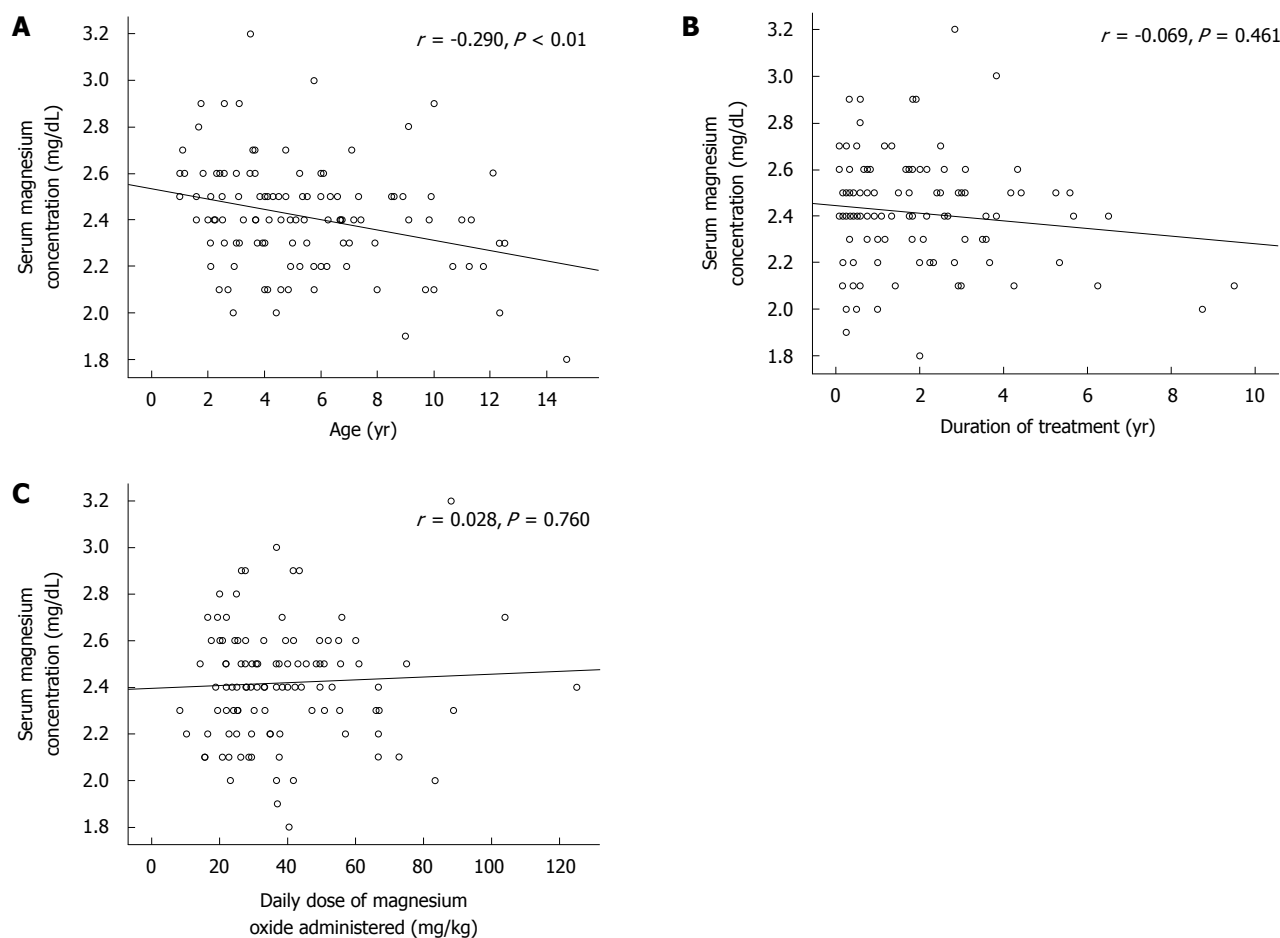


Figure 1 Correlation between serum level of magnesium and age (A), and duration of treatment with magnesium oxide (B), and dose of magnesium oxide (C).

constipation group was significantly greater than that in the control group (Table 1). The highest magnesium concentration was 3.2 mg/dL in a 3.5-year-old patient treated with 1320 mg/d; 88 mg/kg per day magnesium oxide for 2.8 years.

The median urinary magnesium to creatinine ratio in the constipation group was significantly elevated compared with that reported previously [0.23 (0.15-0.37) ($n = 76$) vs 0.15 (0.12-0.20) ($n = 16$), $P < 0.05$]^[13]. The median FEMg in the constipation group was 0.03 (0.02-0.05).

Serum magnesium concentration in the constipation group decreased significantly with age ($P < 0.01$) (Figure 1A). There was no significant correlation between the serum level of magnesium and duration of treatment (Figure 1B). The treatment dose had no effect on serum magnesium level (Figure 1C).

Serum level of calcium [9.9 (9.5-10.2) mg/dL], phosphorus [5.2 (4.8-5.6) mg/dL], creatinine [0.3 (0.3-0.4) mg/dL], and BUN [13.0 (10.8-15.5) mg/dL] were not abnormal in any of the patients. None of the patients had side effects associated with hypermagnesemia.

DISCUSSION

In 2008, the MHLW of Japan reported that 15 patients aged 32-98 years (median, 71 years) who had been treated with magnesium oxide developed severe side effects of

magnesium toxicity, such as hypotension, bradycardia, electrocardiographic changes (atrial fibrillation), loss of consciousness, coma, respiratory depression, and cardiac arrest. The serum magnesium concentration in two fatal cases was 20.0 mg/dL and 17.0 mg/dL. As a result of these reported cases, the MHLW has recommended that the serum concentration of magnesium in subjects on continuous magnesium therapy should be determined^[4].

Most cases of hypermagnesemia in adults result from large intravenous doses of magnesium^[7,8] or from excessive enteral intake of magnesium-containing cathartics^[9-12]. Symptomatic hypermagnesemia is likely to occur in patients with renal dysfunction. In fact, 10 of the 15 Japanese patients reported as cases of hypermagnesemia by the MHLW had renal dysfunction.

Several cases of hypermagnesemia from enteral magnesium intake in patients with normal renal function have been reported previously^[14-17]. It is known that non-renal risk factors for hypermagnesemia are age, gastrointestinal tract disease, and administration of concomitant medications, particularly those with anticholinergic and narcotic effects^[18]. Five elderly Japanese patients without renal dysfunction had intestinal necrosis, severe constipation, and abnormal abdominal distention with intestinal expansion, and these abdominal risk factors could have increased the serum concentration of magnesium.

Hypermagnesemia has also been reported in pediatric

practice^[14,19]. These case reports include a 14-year-old girl without renal dysfunction who was taking magnesium hydroxide because of severe constipation^[14], and a 2-year-old boy with neurological impairments who was taking 2400 mg/d of magnesium oxide administered as part of a regimen of megavitamin and megamineral therapy^[19]. These cases developed increased serum magnesium levels as high as 14.9 mg/dL and 20.3 mg/dL, respectively.

Magnesium oxide is commonly used in patients with chronic constipation; however, not only has the optimum dose for children not been established, but there has been no study to evaluate the concentration of serum magnesium after oral administration of magnesium oxide. The aim of the present study was to determine the serum magnesium concentration in pediatric cases receiving magnesium cathartics for chronic constipation.

In our study, the median serum magnesium concentration was 2.4 mg/dL in the constipation group, which was significantly greater than that in the control group (2.2 mg/dL). Thirty patients (25%) in the constipation group and none in the control group had a serum magnesium concentration greater than the maximum value of the normal range in healthy Japanese children (2.6 mg/dL). The high critical limit of serum magnesium concentration has been reported as 4.9 ± 2.0 mg/dL in adults and 4.3 ± 1.1 mg/dL in children^[20]. The highest value in our study was 3.2 mg/dL, and none of our patients reached the critical limit of serum magnesium concentration or developed symptoms due to hypermagnesemia. The median urinary magnesium to creatinine ratio in the constipation group was significantly elevated compared with that in healthy subjects, which suggests that serum magnesium level is regulated by an increase in renal excretion in those children with normal renal function, and is maintained within its appropriate range.

In our study, serum magnesium level in constipated children treated with magnesium oxide, but not in the control children, decreased significantly with age. No correlation was found between duration of treatment or daily dose of magnesium oxide and serum magnesium concentration. These data are consistent with those reported by Woodard *et al.*^[21] They reported that the increase in serum magnesium concentration in 102 adults who received multiple doses of magnesium citrate did not correlate with the quantity of magnesium administered^[21]. Elderly patients are at risk of magnesium toxicity as kidney function declines with age, but it is not clear whether young children have a higher risk of hypermagnesemia. Alison *et al.*^[22] reported on a 6-week-old infant who had increased serum magnesium level (14.2 mg/dL) and life-threatening apnea due to 733 mg/d magnesium hydroxide that was used to treat constipation. Brand *et al.*^[23] and Humphrey *et al.*^[24] reported on premature infants with hypermagnesemia following antacid administration in order to decrease the risk of gastrointestinal hemorrhage. One infant had an increased serum magnesium level of 13.3 mg/dL and developed intestinal perforation. These reports indicate that infancy, prematurity or young age might be a possible risk factor for hypermagnesemia.

According to our results, we conclude that serum magnesium concentrations increase significantly after daily magnesium oxide intake, but the magnitude of the increase appears modest. Younger age, but not prolonged use of daily magnesium oxide might be a relative risk factor, and it should be determined by further studies whether serum magnesium concentration should be assessed in these subjects.

ACKNOWLEDGMENTS

The following doctors are members of the Japanese Pediatric Constipation Study Group and have provided data and samples for this study: Dr. Mitsuru Kubota (Department of Pediatrics, Teine Keijinkai Hospital, Department of Pediatrics, Hokkaido University Graduate School of Medicine), Dr. Mika Sasaki, Dr. Hisayuki Tsukahara, and Dr. Mutsumi Chiba (Iwate Medical University), Dr. Kazuko Okada (Okada Pediatric Clinic), Dr. Yasuko Wakabayashi (Department of Pediatrics, Tokyo Women's Medical University Medical Center East), Professor Toshiaki Shimizu (Department of Pediatrics, Juntendo University School of Medicine), Dr. Yoshiko Nakayama (Department of Pediatrics, Showa Inan General Hospital, Department of Pediatrics Shinshu University School of Medicine) and Dr. Hitoshi Tajiri (Department of Pediatrics, Osaka General Medical Center).

COMMENTS

Background

Magnesium-containing cathartics are commonly used to treat chronic constipation. Although hypermagnesemia is a rare clinical condition, it can occur as a side effect of increased intake of magnesium salts.

Research frontiers

The Japanese government has recently reported fatal cases of hypermagnesemia in adults treated with magnesium oxide. In our study, serum magnesium concentrations increased significantly after daily magnesium oxide intake, but the magnitude of the increase appeared modest. Serum magnesium levels in constipated children treated with magnesium oxide, but not in the control children, decreased significantly with age. No correlation was found between duration of treatment or daily dose of magnesium oxide and serum magnesium concentration.

Innovations and breakthroughs

Recent reports have highlighted that serum magnesium concentration increases significantly, but not critically, after daily treatment with magnesium oxide in children with normal renal function.

Applications

The present study indicated the safety of daily magnesium oxide treatment for children with chronic constipation.

Peer review

The authors are to be congratulated for providing evidence of the apparent safety of a commonly used and effective therapy to treat an important health issue seen commonly in children.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP

Higher parity associated with higher risk of death from gastric cancer

Chih-Ching Chang, Chih-Cheng Chen, Hui-Fen Chiu, Chun-Yuh Yang

Chih-Ching Chang, Department of Environmental and Occupational Health, National Cheng Kung University, Tainan 70154, Taiwan, China

Chih-Cheng Chen, Department of Pediatrics, Chang-Gung Memorial Hospital, Kaohsiung Medical Center, Chang-Gung University, College of Medicine, Kaohsiung 80708, Taiwan, China

Hui-Fen Chiu, Department of Pharmacology, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China

Chun-Yuh Yang, Faculty of Public Health, College of Health Sciences, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China

Chun-Yuh Yang, Division of Environmental Health and Occupational Medicine, National Health Research Institute, Miaoli 36051, Taiwan, China

Author contributions: Chang CC did the statistical analysis and wrote the manuscript; Chen CC and Chiu HF provided essential insight into the interpretation of the results; Yang CY contributed to the study design and interpretation of the data. He had full access to all of the data in the study, and took responsibility for the integrity of the data and the accuracy of the data analysis.

Supported by A grant from the National Science Council, Executive Yuan, Taiwan, No. NSC-94-2314-B-037-056

Correspondence to: Chun-Yuh Yang, PhD, MPH, Faculty of Public Health, Kaohsiung Medical University, 100 Shih-Chuan 1st RD, Kaohsiung 80708, Taiwan, China. chunyh@kmu.edu.tw
 Telephone: +886-7-3121101 Fax: +886-7-3110811

Received: October 14, 2010 Revised: December 27, 2010

Accepted: January 4, 2011

Published online: February 14, 2011

Abstract

AIM: To examine the association between parity and gastric cancer (the cases are almost all premenopausal women) risk in a cohort of young parous women.

METHODS: The study cohort consisted of all women with a record of a first and singleton childbirth in the Birth Register between 1978 and 1987. We tracked each woman from the time of her first childbirth to December 31, 2008. Their vital status was ascertained by linking records to the computerized mortality database.

Cox proportional hazard regression models were used to estimate hazard ratios of death from gastric cancer associated with parity.

RESULTS: There were 1090 gastric cancer deaths (85.87% of them were premenopausal) during 33686828 person-years of follow-up. The mortality rate of gastric cancer was 3.24 cases per 100000 person-years. A trend of increasing risk of gastric cancer was seen with increasing parity. The adjusted hazard ratio was 1.24 [confidence interval (95% CI): 1.02-1.50] for women who had borne two to three children, and 1.32 (95% CI: 1.01-1.72) for women with four or more births, when compared with women who had given birth to only one child.

CONCLUSION: These results suggest that higher parity may increase the risk of death from gastric cancer among premenopausal women.

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Key words: Gastric cancer; Parity; Mortality; Cohort study

Peer reviewer: Mitsuyoshi Urashima, MD, PhD, MPH, Division of Molecular Epidemiology, Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan

Chang CC, Chen CC, Chiu HF, Yang CY. Higher parity associated with higher risk of death from gastric cancer. *World J Gastroenterol* 2011; 17(6): 784-788 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/784.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.784>

INTRODUCTION

In Taiwan, gastric cancer (GC) is the fifth leading cause of cancer mortality for males and females^[1]. The age-adjusted mortality rate for gastric cancer was 14.1 per 100000 among males and 7.4 among females in 2007. There is

substantial geographic variation in gastric cancer mortality within the country. In most areas, however, its mortality rate is about two-fold higher among men than women^[1]. Known risk factors, such as *Helicobacter pylori* (*H. pylori*) infection, tobacco smoking, and low fruit and vegetable intake cannot entirely explain the gender difference^[2].

The difference between male-to-female incidence rates is greatest during the reproductive ages, and the rates become more similar after menopause; it has been hypothesized that sex hormones play a role in the development or progression of gastric cancer^[3]. The influence of sex hormones on gastric cancer risk is supported by the presence of steroid-hormones receptors in the gastric mucosa and gastric cancer tissues^[4,5]. In rat experimental studies, there is also a greater preponderance of GC in males compared with females^[6,7].

Few epidemiological studies have investigated the association between parity and gastric cancer, and results have been inconsistent. Parity was associated with increased risk of gastric cancer in four studies^[8-11]. Three studies found a suggestive inverse relationship, but no significant dose-risk trend^[12-14], whereas five others reported no association^[15-19].

A previous study on gastric cancer in young individuals indicated that gastric cancer diagnosed in women within two years after delivery was more progressive, and proposed that pregnancy or delivery might accelerate the growth of gastric cancer^[20]. Maeta *et al.*^[21] found that the pathological features of gastric cancer that occurred more frequently in young women were more common among pregnancy-related cases. These results suggested the need for separate analysis of pre- and postmenopausal women when examining the relationship between parity and gastric cancer risk.

Four of the above-mentioned studies, which studied the relationship between parity and gastric cancer risk, were restricted to postmenopausal women^[11,12,17,18]. Only one recent Swedish study has examined the relationship between parity and gastric cancer separately for pre- and postmenopausal women^[14]. Other studies did not categorize the gastric cancer cases into pre- or postmenopausal because of the lack of sufficient premenopausal gastric cancer cases to support a complete analysis.

The objective of this study was to examine the effect of parity on the risk of gastric cancer in a cohort of 1 292 462 young parous women in Taiwan, followed over a period of 31 years.

MATERIALS AND METHODS

Data source

Registration of births is required by law in Taiwan. It is the responsibility of the parents or the family to register infant births at a local household registration office within 15 d. The Birth Registration System, which is managed by the Department of the Interior, released computerized data on live births since 1978. The registration form, which requests information on maternal age, education, parity, gestational age, date of delivery, infant gender, and

birth weight, is completed by the physician attending the delivery. Most deliveries in Taiwan take place in either a hospital or a clinic^[22], the birth certificates are completed by physicians attending the delivery, and it is mandatory to register all live births at local household registration offices; therefore the birth registration data are considered complete, reliable, and accurate^[22].

Study population

The study cohort consisted of 1 292 462 women with a record of a first and singleton childbirth in the Birth Register between January 1, 1978 and December 31, 1987. Information on any subsequent births was also retrieved from the Birth Register.

Follow-up

Each woman has her own unique personal identification number, which was used to track the women from the time of their first childbirth to December 31, 2008. Their vital status was ascertained by linking records with the computerized mortality database, identifying the date of any deaths.

Statistical analysis

We categorized parity (the number of children recorded in the last childbirth record of each woman registered during follow-up) into three categories: one, two to three, and four or more. We compared selected baseline characteristics of the cohort by parity using χ^2 tests or analysis of variance, as appropriate. Death rates were calculated by dividing the number of deaths from gastric cancer (ICD-9 code 151) by the number of person-years of follow-up. Cox proportional hazard regression models were used to estimate the hazard ratio of death from gastric cancer associated with parity. The 95% confidence intervals (CIs) for the hazard ratios were also calculated. We used two Cox proportional hazard models: an age-adjusted model and a multivariate-adjusted model, which was additionally adjusted for marital status (married, unmarried), years of schooling (≤ 9 , > 9 years), and birthplace (hospital/clinic, home/other). The proportion hazards assumption was assessed for all above-mentioned variables and no violations were observed. Analyses were performed using the SAS statistical package (version 8.02, SAS Institute Inc). All statistical tests were two-sided. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The study cohort was comprised of 1 292 462 primiparous women with complete information. A total of 33 686 828 person-years were observed in this study. The mean follow-up period was 26.09 (standard deviation = 3.28) years. During the follow-up period, 1090 gastric cancer deaths were recorded, yielding a mortality rate of 3.24 cases per 100 000 person-years.

Table 1 presents the baseline characteristics of the study population by parity. Compared with women who had given birth to only one child, women with four or

Table 1 Demographic characteristics of the study cohort (mean \pm SD) *n* (%)

	Parity			P-value
	1 (<i>n</i> = 157 207)	2-3 (<i>n</i> = 1 000 977)	4+ (<i>n</i> = 134 278)	
Age at recruitment (1st birth)	26.38 \pm 4.43	24.26 \pm 3.22	22.44 \pm 2.95	< 0.001
Marital status				
Married	146 022 (92.89)	984 049 (98.31)	130 544 (97.22)	< 0.001
Not married	11 185 (7.11)	16 928 (1.69)	3 734 (2.78)	
Years of schooling				
\leq 9	72 090 (45.86)	544 098 (54.36)	106 330 (79.19)	< 0.001
> 9	85 117 (54.14)	456 879 (45.64)	27 948 (20.81)	
Birth place				
Hospital/clinic	153 167 (97.43)	970 422 (96.95)	122 336 (91.11)	< 0.001
Home/other	4 040 (2.57)	30 555 (3.05)	11 942 (8.89)	

Table 2 Association between parity and hazard ratio of death from gastric cancer over a 31-year follow-up period

Parity	No. of subjects	Follow-up person-years	No. of gastric cancer (per 100 000)	Age-adjusted HR (95% CI)	Multivariate-adjusted HR ¹ (95% CI)
1	157 207	4 020 271.75	128 (3.18)	1.00	1.00
2-3	1 000 977	26 036 992.42	848 (3.25)	1.23 (1.01-1.49)	1.24 (1.02-1.50)
4+	134 278	3 629 563.83	114 (3.14)	1.33 (1.02-1.73)	1.32 (1.01-1.72)
				<i>P</i> = 0.030 for linear trend	<i>P</i> = 0.035 for linear trend

¹Adjusted for age, marital status, years of schooling, and birth place. HR: Hazard ratio; CI: Confidence interval.

more children were more likely to have lower educational level, younger age at first birth, and a lower chance of being born in a hospital or clinic.

Table 2 presents the hazard ratios of gastric cancer mortality by parity. After adjustment for age at first birth, the hazard ratio for gastric cancer death was 1.23 (95% CI: 1.01-1.49) for women who had two to three children, and 1.33 (95% CI: 1.02-1.73) for women with four or more births, when compared with women who had given birth to only one child. In the multivariate-adjusted model, the hazard ratios were only slightly altered. The adjusted hazard ratio was 1.24 (95% CI: 1.02-1.50) for women who had borne two to three children, and 1.32 (95% CI: 1.01-1.72) for women with four or more births, when compared with women who had given birth to only one child. There was a significant increasing trend in the adjusted hazard ratios of gastric cancer with increasing parity (*P* for trend = 0.035).

DISCUSSION

To our knowledge, this is the largest cohort (*n* = 1 292 462 women) published to date to examine the relationship between parity and gastric cancer risk. In this prospective cohort study, we found a positive association between parity and gastric cancer risk. Our finding of an increased risk of gastric cancer associated with higher parity agrees with some previous studies^[8-11], but not with other studies that reported the reverse effect^[12-14] or no association^[15-19] with parity. Pregnancy elevates serum estrogen levels by about 100 fold^[23]. Increasing parity is associated with an overall increase in lifetime exposure to sex hormones. There is experimental evidence that gastric cancer carcinogenesis might be inhibited by estrogens^[6,7]. Thus, if estrogens are associated with a

reduced risk of gastric cancer, we would expect pregnancy to offer some protection from gastric cancer. Our data did not provide support for this hypothesis.

On the other hand, it has been reported that estrogen stimulates the growth of gastric cancer cell lines^[24], and there is evidence that pregnancy or delivery might accelerate the growth of gastric cancer^[20]. The mean age at death for gastric cancer was 42.90 \pm 7.08 years in this study. The majority of gastric cancer deaths (85.87%) were premenopausal (using age 50 as the cut-off value^[14]). Women included in this study tended to be younger (with the large majority of the gastric cancer deaths occurring before menopausal age) than in previous studies. The mean time of gastric cancer was about 14 years after last delivery (age at the birth of the last child = 28.97 \pm 4.01; age at the death for gastric cancer = 42.90 \pm 7.08). It is possible that these premenopausal gastric cancers were influenced by hormonal conditions caused by the actual events of pregnancy or delivery^[17]. Our finding of an increased risk of gastric cancer associated with higher parity may therefore plausibly be related to a short-term increase in risk after a delivery^[16]. The incidence of gastric cancer in premenopausal women is low; therefore, such effects are easily lost in overall analyses^[17]. Some studies have been restricted to postmenopausal women^[11,12,17,18]. To our knowledge, this is the first cohort study to indicate that a positive association between parity and gastric cancer may be only restricted to premenopausal women. However, because there is no consistent evidence to date for an association between parity and risk of death from gastric cancer, the possibility that this is a chance finding must also be considered. Clearly, more work will be needed before the influence of parity on the risk of gastric cancer is understood.

In the event of a death in Taiwan, the decedent's family is required to obtain a death certificate from the hospital or local community clinic, which then must be submitted to the household registration office to cancel the decedent's household registration. The death certificate is required to have the decedent's body buried or cremated. Death certificates must be completed by physicians in Taiwan. It is also mandatory to register all deaths at local household registration offices; thus, the death registration is reliable and complete^[22]. The complete population coverage and follow-up made possible by the national identification number has left the study without selection bias. Information bias is also unlikely to be important for parity.

Taiwan is a small island with a convenient communication network. It is believed that all gastric cancer cases had access to medical care. Mortality data rather than data on inpatient cases was used to assess the association between parity and gastric cancer in this study. The mortality of a disease is a function of its incidence and fatality. Gastric cancer has been reported to have the fifth poorest five-year relative survival rate among all cancer sites^[25]. Deaths from gastric cancer may therefore be regarded as a reasonable indicator of the incidence of gastric cancer.

Hormone replacement therapy (HRT) has been reported to reduce the risk of gastric cancer in population with higher HRT use^[26]. We were unable to adjust for this factor in the current study because of the lack of available data. HRT use is low in Taiwan compared with Western countries^[27]; therefore, the confounding effect resulting from this factor should be small, if it exists at all. Furthermore, if the association between this potential confounding variable and the risk of gastric cancer is not as strong as the one that has been observed for parity, adjustment of this variable will not qualitatively change the conclusion.

Cigarette smoking^[2] and a family history of gastric cancer^[28] have been documented as risk factors for gastric cancer in Taiwan. Unfortunately, there is no information available on these variables for the individual study subjects and, thus, they could not be adjusted in the analysis. However, there is no reason to believe that there would be any correlation between these two variables and parity.

An increased susceptibility to infection by *H. pylori* during pregnancy might affect the increased risk of gastric cancer^[29]. We could not adjust for this variable because of the lack of information on *H. pylori* infection. However, it has been reported that most women acquired the infection in childhood rather than during pregnancy^[30]. Furthermore, there is no reason to believe that *H. pylori* status would be associated with parity, and, therefore, the estimated effect of parity is likely to be free of a confounding effect of *H. pylori* status.

The birth registration system in Taiwan covers only live births and did not include stillbirths and abortions. Therefore we were unable to examine the possible role of gravidity on the risk of gastric cancer. Our study design only allowed for the study of mortality among parous women. Again, we were unable to examine the possible role of nulliparity on the risk of gastric cancer because the birth registry ascertained births rather than pregnan-

cies. The generalizability of this findings is thus limited. Misclassification of menopausal status may have occurred by using age 50 as the cut-off point. Any effect of this is, however, is probably nondifferential (the misclassification is unlikely to be related to parity) and would probably lead to underestimation of the results.

In summary, we found that there was a trend for increasing parity to be associated with increasing risk for gastric cancer among a cohort of young parous women. This study suggests that the relation between parity and risk of gastric cancer should be considered separately for pre- and postmenopausal women.

COMMENTS

Background

Previous studies that examined this association rarely categorized the gastric cancer cases into pre- or postmenopausal because of the lack of sufficient premenopausal gastric cancer cases to support a complete analysis.

Research frontiers

This study was undertaken to examine the association of parity and gastric cancer (the cases are almost all premenopausal women) risk in a cohort of young parous women.

Innovations and breakthroughs

The results of this study suggest that higher parity may increase the risk of death from gastric cancer among premenopausal women.

Applications

This study suggests that the relation between parity and risk of gastric cancer should be considered separately for pre- and postmenopausal women.

Peer review

The study is worthy of being accepted. The study population is large enough to detect minor differences.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Lin YP

An end-to-end anastomosis model of guinea pig bile duct: A 6-mo observation

Xiao-Qing Zhang, Yuan-Hu Tian, Zhi Xu, Li-Xin Wang, Chun-Sheng Hou, Xiao-Feng Ling, Xiao-Si Zhou

Xiao-Qing Zhang, Yuan-Hu Tian, Zhi Xu, Li-Xin Wang, Chun-Sheng Hou, Xiao-Feng Ling, Xiao-Si Zhou, Department of General Surgery, Peking University Third Hospital, Beijing 100191, China

Author contributions: Zhang XQ and Tian YH contributed equally to this work, and performed the majority of experiments; Xu Z, Wang LX and Hou CS designed the experiment and provided the professional consults to this research; Zhang XQ wrote the manuscript; Xu Z, Ling XF and Zhou XS revised the manuscript.

Supported by A grant from National 863 Program of China, No. 2007AA04Z313

Correspondence to: Zhi Xu, Professor, Department of General Surgery, Peking University Third Hospital, 49 North Garden Rd, Haidian District, Beijing 100191, China. xuzhi123456@sohu.com

Telephone: +86-10-82267331 Fax: +86-10-62010334

Received: October 17, 2010 Revised: November 14, 2010

Accepted: November 21, 2010

Published online: February 14, 2011

CONCLUSION: A simple and reliable EEA model of guinea pig bile duct can be established with a good reproducibility and a satisfactory survival rate.

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Key words: Animal model; Guinea pig; Anastomosis; Common bile duct; Wound healing

Peer reviewer: Emiko Mizoguchi, MD, PhD, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

Zhang XQ, Tian YH, Xu Z, Wang LX, Hou CS, Ling XF, Zhou XS. An end-to-end anastomosis model of guinea pig bile duct: A 6-mo observation. *World J Gastroenterol* 2011; 17(6): 789-795 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/789.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.789>

Abstract

AIM: To establish the end-to-end anastomosis (EEA) model of guinea pig bile duct and evaluate the healing process of bile duct.

METHODS: Thirty-two male guinea pigs were randomly divided into control group, 2-, 3-, and 6-mo groups after establishment of EEA model. Histological, immunohistochemical and serologic tests as well as measurement of bile contents were performed. The bile duct diameter and the diameter ratio (DR) were measured to assess the formation of relative stricture.

RESULTS: Acute and chronic inflammatory reactions occurred throughout the healing process of bile duct. Serology test and bile content measurement showed no formation of persistent stricture in 6-mo group. The DR revealed a transient formation of relative stricture in 2-mo group in comparison to control group (2.94 ± 0.17 vs 1.89 ± 0.27 , $P = 0.004$). However, this relative stricture was released in 6-mo group (2.14 ± 0.18 , $P = 0.440$).

INTRODUCTION

Bile duct injury (BDI) is a severe consequence of gastrointestinal surgery. Unrecognized or improperly treated biliary injuries can lead to severe complications such as biliary cirrhosis, hepatic failure, and death^[1,2]. Treatment of BDI remains a challenge for gastrointestinal surgeons. Reconstruction of bile ducts following iatrogenic injuries is associated with a high risk of stricture and stricture recurrence in the anastomosis^[3,4]. Therefore, effective and safe bile duct reconstruction is very important.

Although the method of biliary tract reconstruction has been extensively studied, no consensus is reached concerning the ideal model of biliary tract reconstruction. The most frequently recommended procedure is Roux-Y hepaticojejunostomy (HJ) for its reconstruction^[3-5].

However, Roux-Y HJ has its obvious drawbacks, including a large number of postoperative complications, such as a high occurrence of biliary tract stenosis leading to secondary biliary cirrhosis. The diagnostic and thera-

peptic endoscopic access to the biliary tract becomes impaired or hindered since the reconstruction of biliary tract with Roux-en-Y HJ is not anatomical^[6]. The changed bile flow pathway is also a cause of disturbance in fat metabolism^[7]. In case of poor drainage of the excluded loop, especially when applied in thin biliary tract with an intense inflammatory process, ascending cholangitis may also ensue. As the reconstruction of biliary tract with Roux-en-Y HJ is not physiological, the bile bypass induces gastric hypersecretion leading to a pH change secondary to altered bile synthesis and release of gastrin, therefore peptic ulcer occurs frequently in the long term^[8,9].

End-to-end anastomosis (EEA) of bile duct is seldom used in surgical treatment of BDI. However, this procedure is routinely performed during hepatic transplantation with good results^[10,11] and can achieve a better long-term outcome than Roux-en-Y HJ. Establishing a physiological bile pathway allows proper digestion and absorption. Also, control endoscopic examination in these patients is possible. Therefore, some authors recommend EEA as the first choice of bile duct reconstruction^[6,12].

However, no large-scale clinical trial and a suitable animal model of EEA are available to evaluate the bile duct healing process. Therefore, to gain a better understanding of the healing process after EEA, and provide some valuable information for the etiology, development and prophylaxis of BDI, an animal model of bile duct reconstruction with EEA was established after total resection of common bile duct (CBD) in guinea pigs in this study. Guinea pigs were raised for 2, 3, and 6 mo after operation to observe the short- and long-term healing and possible complications. General conditions, survival rate and histological characteristics of the animals were detected and serology test was performed, as well as content and size of bile duct were measured before and after operation.

MATERIALS AND METHODS

Animal model and experimental design

Thirty-two male guinea pigs weighing 350-400g, purchased from Laboratory of Experimental Animals, Peking University Health Science Center, Beijing, were housed under controlled conditions at a temperature of $21 \pm 2^\circ\text{C}$ and a relative humidity of 30%-70% in a 12-h dark and light cycle. The animals were fasted with free access to water 8 h before and after operation. This study was performed in accordance with the rules for the protections of animals and approved by the Animal Ethical and Welfare Committee of Peking University Health Science Center (LA2008-021).

The animals were randomly divided into control group (group 1), and 2-, 3-, 6-mo groups (groups 2-4) after EEA model was established, 8 in each group.

Microsurgical reconstruction of bile duct

Surgical microscope (XTS-4A Jiangsu Surgical Instruments Company, China), microsurgical instruments (SSW-4, Shanghai Surgical Instruments Company, China), and 10-0 monofilament sutures (Double Arrow, China) were used.

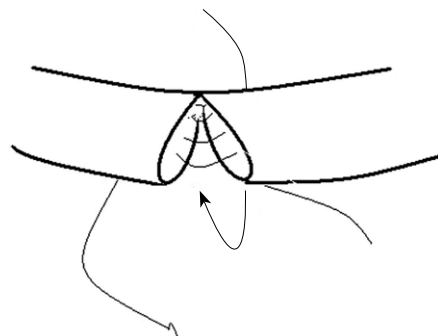


Figure 1 Microsurgical reconstruction of common bile duct with end-to-end anastomosis.

The animals were anesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection under sterile conditions. Peritoneal cavity was accessed *via* a midline incision (approximately 3 cm). After the liver lobes were pressed to the upper region and the duodenum was tracked toward left, the CBD was identified. Gallbladder was drained through a cystic duct joined with hepatic duct into the CBD. The CBD in control group was exposed and freed with forceps. A complete transection between the portal hilus and duodenum was performed with sharp dissection in the other 3 groups. The bile duct was reconstructed with the microsurgical instruments.

EEA was performed with interrupted sutures passing through the layers of the duct wall. The first stitch was placed at the side wall to join the two cut ends. Then, posterior walls of the proximal and distal cut ends were clockwise sutured. After the posterior wall was sutured, anterior wall was sutured in the same way (Figure 1). The proximal and distal cut ends were connected precisely and all stitches were distributed evenly with no tension on the approximate distal and proximal ends. Leaked bile was constantly wiped out to keep the operation area clean. The peritoneal cavity was flushed with normal saline to wash away the remaining bile after suturing was completed. The whole process took about 40 min with 8-10 sutures. No clasper was used to disturb the blood supply and bile flow during operation. No T-tube or drainage was placed.

General conditions and survival rate of animals

The general conditions and body weights of guinea pigs were carefully observed. The causes of death of guinea pigs were examined by autopsy. Overall survival rates of the animals were calculated and recorded.

Measurement of bile duct diameter

By the end of two, three and six months after the EEA model was established, guinea pigs in the 4 groups were sacrificed by exsanguination. The maximum diameter of the proximal end (MDP) of CBD and the maximum diameter of anastomosis (MDA) were measured using a sliding caliper. The diameter ratio (DR, $\text{DR} = \text{MDP}/\text{MDA}$) in every group was calculated to assess the formation of relative stricture. Tissue samples were harvested from the anastomosed bile duct for histological examination.

Histological and immunohistochemical examination

Tissue samples, taken from the anastomosed bile duct, were fixed in 10% formalin, embedded in paraffin, and cut into 5- μ m thick cross-sections which were stained with hematoxylin and eosin (HE). Immunohistochemical staining was performed using proliferating cell nuclear antigen (PCNA monoclonal antibody, Medical and Biological Laboratories, Japan). Histological characteristics were reported by the professional pathologist.

Cells were considered positive when their nuclei were stained distinctly brown. Negative control sections were fixed with a safe buffer and positive control sections were used.

The sections of PCNA stained with immunohistochemistry were examined under a light microscope and graded using a modified 0-4 numerical scale provided by Hunt *et al.*^[13] (Table 1).

Serology examination

Serum alkaline phosphatase (ALP) and γ -glutamyltransferase (GGT) levels were measured and analyzed with an automatic biochemical analyzer (Type 5421-04, MISHIMA OLYMPUS CO., Shizuoka-ken, Japan).

Measurement of bile content

The changes of bile contents in 3- and 6-mo groups were analyzed, and the presence of biliary sludge and gallstones was detected. The bile was drained in sterile conditions to examine its pH value. The levels of total bilirubin (TBIL), total bile acid (TBA), and calcium ions were measured with an automatic biochemical analyzer (Type 5421-04, Mishima Olympus Co., Shizuoka-ken, Japan).

Statistical analysis

Levene's test for equality of variances was performed to assess the equality between groups. Independent sample *t* test and Mann-Whiney *U* test were used to compare the differences in the 4 groups. Data were expressed as mean \pm SE. Statistical analysis was conducted using the SPSS for Windows (Chicago, Illinois, USA). $P < 0.01$ was considered statistically significant.

RESULTS

General conditions and survival rate of animals

The animals in 4 groups survived throughout the experiment with a survival rate of 97%. One guinea pig in the control group died of anaesthetic intolerance before surgery. The total survival rate was 91% and two guinea pigs (one in 3-mo group and 1 in 6-mo group) died of bile leak age within seven days after operation. No fever, bile leakage, jaundice, infection, cholangitis, peritonitis, or other postoperative complications were noted in the surviving animals during the follow-up. All animals fed with usual diet gained their weight gradually (Table 2) and remained in a good condition till euthanized.

Measurement of bile duct

The mean value of MDA in groups 2-4 was 3.51 ± 0.12 mm,

Table 1 Immunohistochemical grading scale

0	No evidence
1	Occasional evidence
2	Light scattering
3	Abundant evidence
4	Prominent distribution

Modified from^[13] for the immunohistochemical grading of proliferating cell nuclear antigen.

Table 2 Body weight of guinea pigs

Groups	<i>n</i>	mean \pm SE (g)	<i>P</i>
Group 1	7	380.86 \pm 3.04	
Group 2	8	871.38 \pm 15.23	0.002 ^{1,b}
Group 3	7	963.00 \pm 39.90	0.002 ^{1,b}
Group 4	7	1101.71 \pm 31.96	0.002 ^{1,b}

¹Mann-Whitney *U* test; ^b $P < 0.01$ vs group 1.

3.15 ± 0.23 mm, and 3.47 ± 0.16 mm, respectively, which was significantly higher than that (1.71 ± 0.17 mm) in control group ($P < 0.01$). The mean value of MDP in groups 2-4 was 10.24 ± 0.48 mm, 8.66 ± 0.47 mm, and 8.05 ± 0.52 mm, respectively, which was also significantly higher than that (2.95 ± 0.15 mm) of control group ($P < 0.01$). The MDP value in group 2 was the highest. The MDP values were comparable between groups 3 and 4.

The DR indicates the formation of relative stricture and the postoperative DR suggests the remodeling status and restoration of physiological function. A significant difference was observed in DR between the control and 2-mo groups (1.89 ± 0.27 vs 2.94 ± 0.17 , $P = 0.004$). The DR in groups 3 and 4 was 3.24 ± 0.65 and 2.14 ± 0.18 , respectively (Table 3).

Histological examination

Gross inspection of the bile duct after operation revealed that the anastomosed bile duct was narrowed due to local inflammation and edema. The thickened bile duct wall looked like a "stricture ring" (Figure 2).

In this study, normal bile duct contained abundant elastic fibers with a good contract property. Its intima was lined with a single glandular epithelium. Lamina propria contained a small amount of gland elements. Scattered smooth muscle cells were distributed unevenly in the media wall. Adventitia contained fibroblasts and other connective tissues (Figure 3A).

Two months after operation, significant epithelial proliferation, intra- and extramural glandular hyperplasia, fibrous thickening of the duct and dense infiltration of inflammatory cells were noted in the anastomosis (Figure 3B). Inflammatory reactions were gradually subsidized in 3- and 6-mo groups. Hyperplasia of gland elements was observable in 6-mo group (Figure 3C and D).

Immunohistochemical examination

PCNA, a 36-kDa nuclear protein, is an auxiliary protein for DNA polymerase -delta. PCNA expression and distribution

Table 3 Measurement of common bile duct in guinea pigs

Groups	<i>n</i>	MDA (mm)		MDP (mm)		DR	
		mean \pm SE	<i>P</i>	mean \pm SE	<i>P</i>	mean \pm SE	<i>P</i>
Group 1	7	1.71 \pm 0.17		2.95 \pm 0.15		1.89 \pm 0.27	
Group 2	8	3.51 \pm 0.12	0.000 ^{1,b}	10.24 \pm 0.48	0.001 ²	2.94 \pm 0.17	0.004 ^{1,b}
Group 3	7	3.15 \pm 0.23	0.000 ^{1,b}	8.66 \pm 0.47	0.002 ²	3.24 \pm 0.65	0.079 ¹
Group 4	7	3.47 \pm 0.16	0.000 ^{1,b}	8.50 \pm 0.52	0.002 ²	2.14 \pm 0.18	0.440 ²

¹Student's *t* test; ²Mann-Whitney *U* test; ^b*P* < 0.01 vs group 1. MDA: Maximum diameter of anastomosis; MDP: Maximum diameter of the proximal end; DR: Diameter ratio.

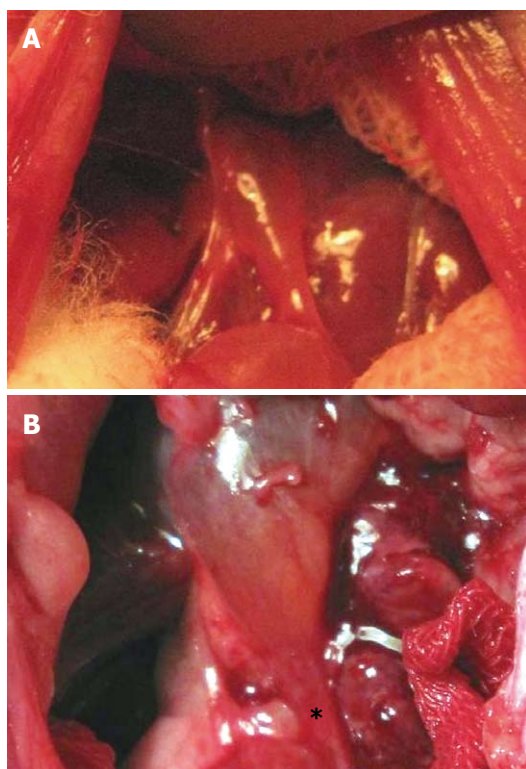


Figure 2 Gross observation of normal common bile duct (A) and its diameter (B) 2 mo after end-to-end anastomosis. Asterisk indicates anastomosed region in 2-mo group.

are correlated with cell proliferation, DNA synthesis, and cell proliferative activity^[14,15]. In this study, positive PCNA cells were concentrated on the glandular-epithelia and peribiliary glands of control group. After bile duct reconstruction in 2- and 3-mo groups, an increased number of positive cells were distributed in all layers of the duct wall. By the end of six months, positive PCNA cells were located mainly in glandular elements and epithelial cells (Figure 4). The number of proliferated PCNA cells was significantly greater in groups 2-3 than in control group (*P* < 0.01) (Table 4).

Serum ALP and GGT levels

One serum sample taken from control group was abandoned due to contamination. The mean serum ALP and GGT level was 55.33 \pm 8.44 U/L and 14.67 \pm 2.76 U/L, respectively, in control group and groups 2-4 (*P* < 0.01, Table 5).

Table 4 Semi-quantitative analysis of proliferating cell nuclear antigen expression in bile duct of guinea pigs

Groups	<i>n</i>	mean \pm SE	<i>t</i>	<i>P</i>
Group 1	7	1.57 \pm 0.535		
Group 2	8	3.63 \pm 0.518	-7.511	0.000 ^b
Group 3	7	3.14 \pm 0.378	-6.351	0.000 ^b
Group 4	7	1.71 \pm 0.488	-0.522	0.611 ^b

^b*P* < 0.01 vs group 1.

Table 5 Serum alkaline phosphatase and γ -glutamyltransferase levels in guinea pig (U/L)

Groups	<i>n</i>	ALP (U/L)		GGT (U/L)	
		mean \pm SE	<i>P</i>	mean \pm SE	<i>P</i>
Group 1	6	53.33 \pm 8.44		14.67 \pm 2.76	
Group 2	8	76.63 \pm 9.95	0.145 ¹	13.00 \pm 1.04	0.560 ²
Group 3	7	63.29 \pm 5.18	0.424 ¹	14.00 \pm 1.50	0.829 ¹
Group 4	7	62.00 \pm 13.22	0.691 ¹	16.43 \pm 1.04	0.774 ²

¹Student's *t* test; ²Mann-Whitney *U* test. ALP: Alkaline phosphatase; GGT: γ -glutamyltransferase.

Measurement of bile contents

The bile contents were measured to show whether there is a tendency to form gallstones. No biliary sludge or gallstones were found in the biliary system of all groups, and no significant difference was observed in levels of TBIL, TBA, calcium ions and pH value among the 4 groups (Table 6).

DISCUSSION

In this study, EEA was performed instantly after total transection of CBD in guinea pigs. A few weeks after EEA, the anastomosed bile duct was narrowed due to local inflammation, edema and proliferation of glandular elements. The thickened bile duct wall looked and functioned as a "stricture ring", leading to a significantly higher hydrodynamic pressure on the proximal bile duct end and noticeable dilation of the proximal bile duct end above the stenosis zone. The increased pressure of the proximal bile duct end would pass on the pressure to the anastomosed area of bile duct and influence its remodeling. The "stricture ring" took on adaptive changes with inflammation gradually subsidized after a

Table 6 Bile contents in guinea pigs

Groups	n	TBIL ($\mu\text{mol/L}$)		TBA ($\mu\text{mol/L}$)		Ca ²⁺ (mmol/L)		pH	
		mean \pm SE	P	mean \pm SE	P	mean \pm SE	P	mean \pm SE	P
Group 1	7	32.44 \pm 3.43		11 668.38 \pm 1549.42		0.78 \pm 0.06		8.87 \pm 0.07	
Group 3	7	28.5 \pm 7.92	0.622 ¹	15 981.67 \pm 2295.02	0.136 ¹	1.17 \pm 0.13	0.014 ¹	8.86 \pm 0.08	0.908 ¹
Group 4	7	39.19 \pm 9.97	0.565 ²	18 260.25 \pm 4164.40	0.164 ¹	1.24 \pm 0.17	0.064 ²	8.82 \pm 0.07	0.642 ¹

¹Student's *t* test; ²Mann-Whitney *U* test. TBIL: Total bilirubin; TBA: Total bile acid.

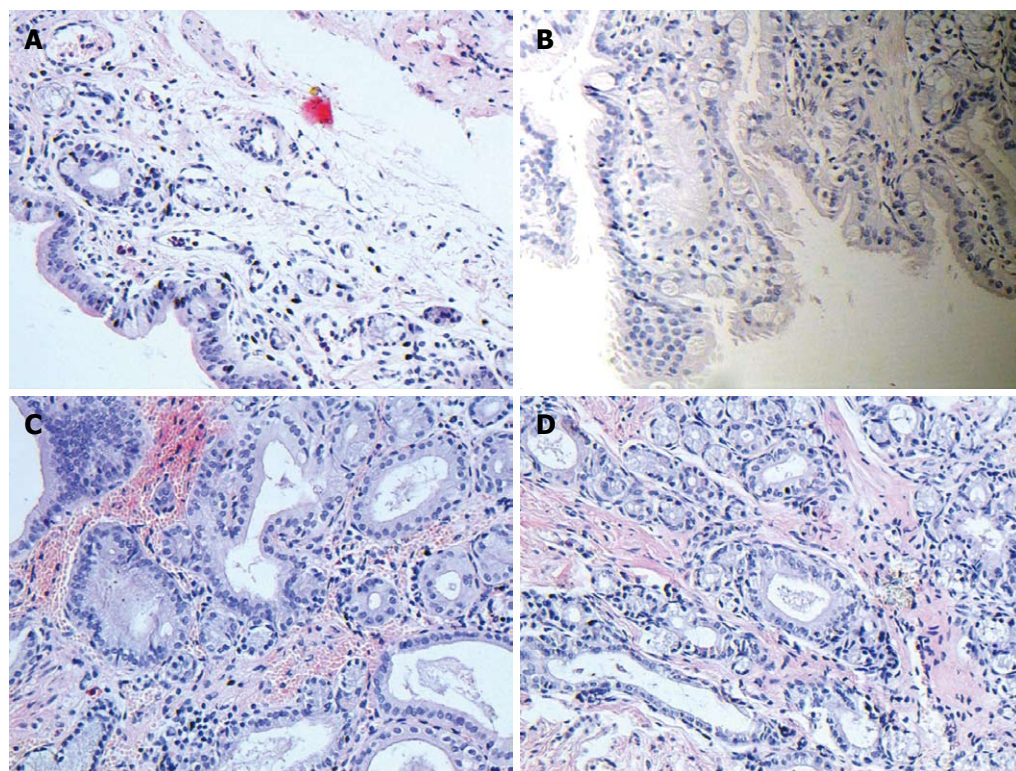


Figure 3 Histological examination of bile duct in all groups (original magnification, $\times 200$). A: Normal bile duct intima; B: Gland proliferation and infiltration of inflammatory cells in 2-mo group; C: Fibrous thickening of bile duct and dense infiltration of inflammatory cells in 3-mo group; D: Epithelial proliferation as well as intra- and extramural glandular hyperplasia in bile duct.

few months. The MDA and MDP values were significantly higher in groups 2-4 than in control group. Therefore, a single MDA parameter could not reliably reflect the remodeling process of the anastomosed bile duct. Moreover, the body weight of guinea pigs increased throughout the experiment in 6-mo group. The DR parameter of ($\text{DR} = \text{MDP}/\text{MDA}$) was used to assess the remodeling status and evaluate whether there is a relative stricture formation. If the DR was significantly higher in groups 2-4 than in control group, a relative stricture would form.

The DR was significantly higher in 2-mo group than in control group, which might be the indication for relative stricture formation. The animals remained in a good condition with their weight gradually increased. The MDP, MDA and DR values were lower in 6-mo group than in 2-mo group, while comparable to those in 3-mo group. No significant difference was noted in levels of ALP, GGT, and bile contents among 4 groups, indicating that normal bile duct anatomy and physiological function can

be gradually restored after a transient formation of “relative stricture”.

Although the body weight of guinea pigs was notably increased in six months, the bile duct parameters did not increase in proportion, suggesting that the growth of animals is not the only cause of bile duct enlargement. We hypothesize that besides hydrodynamic pressure of bile flow to the side walls and normal growth of the animals, neurological factors may also account for the changes during bile duct remodeling.

Consequently, the postoperative remodeling process in 6-mo group was a synergetic and balanced result of tissue injury and repair, as well as hydrodynamic and neurological changes in injured CBD. Further study is needed to explain the neurological mechanism underlying the postoperative remodeling process of bile duct.

It has been shown that biliary stasis can induce pigment stone formation in animals^[16,17] and in patients with biliary stricture^[18]. It was reported that the increased bile

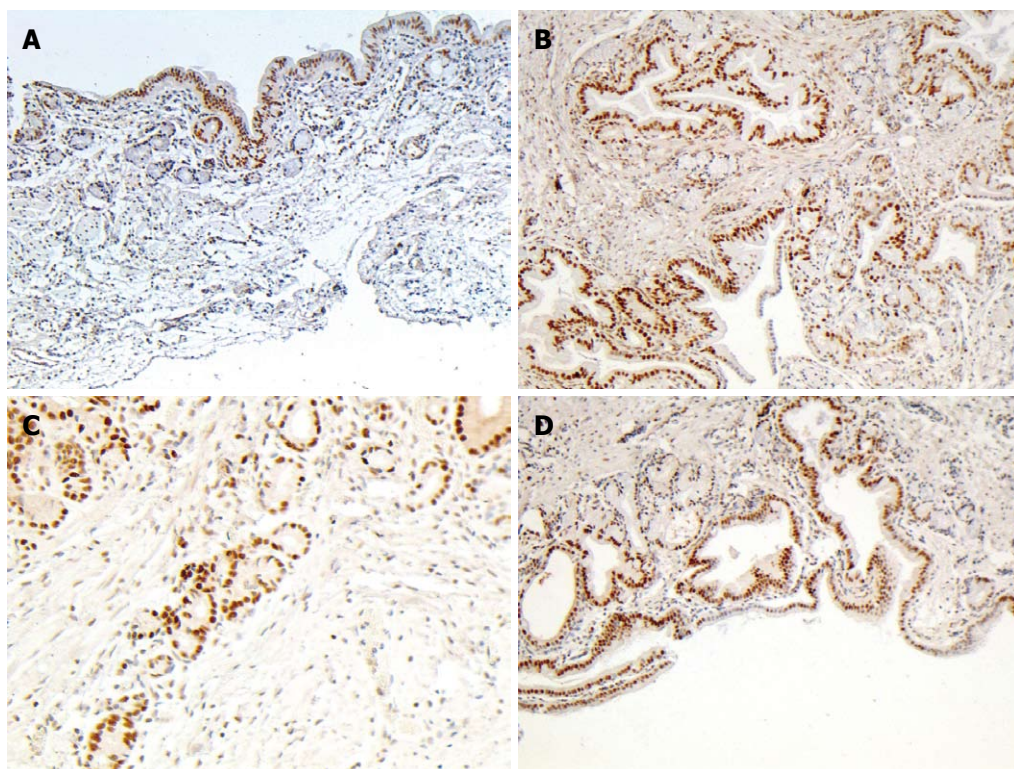


Figure 4 Proliferating cell nuclear antigen immunohistochemical staining. A: Distribution of proliferating cell nuclear antigen (PCNA) positive cells in normal bile duct (original magnification, $\times 100$); B, C: Positive PCNA cells distributed in glandular-epithelia and peribiliary glands in 2- and 3-mo groups, respectively (B: original magnification, $\times 100$; C: original magnification, $\times 200$); D: Positive PCNA cells located mainly in glandular elements and epithelium cells (original magnification, $\times 100$).

pH and changes in bile contents when ligation results in bile duct stricture are the early events, leading to the formation of gallstone^[17,19]. In our experiment, the levels of TBIL, TBA, calcium ions and the pH value were measured. No biliary sludge or gallstones were found in the biliary system of guinea pigs.

Guinea pig is an ideal animal for the reconstruction of bile duct with EEA. The EEA model is frequently used in studies of biliary system, especially in investigation of gallstone formation^[17,19]. Rats have no gallbladder and the diameter of their CBD is only 1 mm^[20]. The anatomy of bile duct in guinea pigs is very similar to that of human beings. Since the diameter of bile duct in guinea pigs is approximately 1.7 mm, it is easy to reconstruct the bile duct with a good reproducibility. The bile contents differ in species. The bile in guinea pigs contains the same bilirubinic acid as in human beings, while the bile in rabbits contains biliverdinic acid which renders it unusable in this respect^[21]. Moreover, although dogs might be the better candidates for the reconstruction of bile duct, guinea pigs were chosen in this preliminary experiment from the animal welfare and ethical point of view.

In the present study, the histological characteristic of normal CBD tissue taken from guinea pigs were similar to those taken from human beings, which are consistent with the reported findings^[22-24], indicating that guinea pigs are ideal for the establishment of EEA models.

The tissue repair of injured biliary tract is a scar healing process. Two important changes in tissue repair can restore the morphological consistency and physiological

function, namely the formation of granulation tissue with contractile properties and the epithelial cell proliferation, migration and the closure of the wound^[25]. MFB is the major constituent of inflammatory and reparative granulation tissues. By forming a net work of contracting system, MFB may last scar contraction and result in stricture formation. MFB disappear due to apoptosis when the epithelialization is completed and the remodeling process becomes stable^[26,27]. The presence of MFB can lead to excessive scarring and fibrotic conditions. Geng and his colleagues established the bile duct anastomosis model of dogs by making an incision on the anterior wall of CBD with one third of its circumference, and found that the number of myofibroblasts can reach its peak 3 mo after operation, and decrease due to apoptosis 6 mo after operation^[28], suggesting that six months is enough for the examination of the healing process of bile duct anastomosis. However, the long-term outcome is critical in surgical treatment of BDI in clinical practice. No biliary anastomosis stricture formation is a proof of successful surgical management. Therefore, further study is needed to observe the longer postoperative outcome.

In this preliminary study, we presented a simple and reliable animal EEA model for bile duct reconstruction with a good reproducibility and satisfactory survival rate. No permanent biliary anastomosis stricture was noticed in 6-mo group and no serology or bile content revealed stricture formation. The overall animal survival rate was 91%. The animals gained their weight with no postoperative biliary obstruction found in all groups.

In conclusion, the EEA animal model of bile duct established in this study can be used in studies of BDI etiology, development, and possible prophylaxis, and provide some valuable information for the postoperative healing process of bile duct.

COMMENTS

Background

Treatment of bile duct injury (BDI) remains a great challenge for gastrointestinal surgeons. No consensus has been reached concerning the ideal method for bile duct reconstruction. No large scale clinical study is available on bile duct reconstruction.

Research frontiers

Some surgeons prefer end-to-end anastomosis (EEA) as a more physiological method in bile duct reconstruction. However, no large-scale clinical study or suitable animal model is available or analyzed. In this study, by establishing a reliable animal EEA model of common bile duct (CBD) and observing the post-operative results of histological, immunohistochemical examination, serologic and bile content analysis, as well as bile duct parameters, the authors demonstrated that EEA can be utilized in treatment of BDI.

Innovations and breakthroughs

The authors provided a simple and reliable animal EEA model of CBD with a good outcome in this study, which may shed light on studies of BDI etiology, development, and possible prophylaxis.

Applications

The animal EEA model of CBD we established in the present study can be utilized in studies on BDI etiology, development, and possible prophylaxis as well as provide some valuable information for the post-operative healing process of EEA.

Terminology

EEA, an end-to-end anastomosis procedure, is a more preferable choice of treatment than Roux-en-Y maneuver. MFB are the myofibroblasts with α -SMA expression in stress fibers. In wound healing, inflammation mediators and mechanical tension lead to generation of actin-containing microfilaments or stress fibers which confer contractile property to fibroblasts, and convert them into terminally differentiated MFB. MFB are the major constituent of inflammatory and reparative granulation tissue and can last scar contraction and stricture formation.

Peer review

In this study, the animals gained their weight and no postoperative biliary obstruction was observed in any group after bile duct reconstruction with EEA in this study, showing that EEA is a simple and reliable procedure for bile duct reconstruction with a satisfactory survival rate, which provides some valuable information for the postoperative healing process of bile duct.

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S- Editor Tian L L- Editor Wang XL E- Editor Lin YP

RRAS: A key regulator and an important prognostic biomarker in biliary atresia

Rui Zhao, Hao Li, Chun Shen, Shan Zheng

Rui Zhao, Hao Li, Chun Shen, Shan Zheng, Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai 201102, China

Author contributions: Zhao R wrote the manuscript and performed the majority of experiments; Li H and Shen C coordinated and provided the collection of all the human materials; Zheng S designed the study and provided the financial support for this work.

Supported by National Natural Science Foundation of China, No. 30973139

Correspondence to: Shan Zheng, MD, Department of Pediatric Surgery, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China. szheng@shmu.edu.cn
 Telephone: +86-21-64931007 Fax: +86-21-64931901

Received: September 14, 2010 Revised: November 3, 2010

Accepted: November 10, 2010

Published online: February 14, 2011

Abstract

AIM: To characterize the differentially expressed gene profiles in livers from biliary atresia (BA) patients including, ascertain genes, functional categories and pathways that play a central role in the pathogenesis of BA, and identify the novel prognostic markers for BA.

METHODS: Liver tissue samples from control patients, neonatal cholestasis patients, and BA patients at the age of < 60 d, 60-90 d, and > 90 d were pooled for DNA microarray analysis. Bioinformatics analysis was performed using, series test cluster of gene ontology, and Pathway-Finder software. Reverse-transcription polymerase chain reaction was performed to confirm changes in selected genes. Relation between RRAS gene expression and prognosis of 40 BA patients was analyzed in a 2-year follow-up study.

RESULTS: The 4 identified significant gene expression profiles could confidently separate BA liver tissue from normal and other diseased liver tissues. The included

genes were mainly involved in inflammation response and reconstruction of cellular matrix. The significant pathways associated with BA were primarily involved in autoimmune response, activation of T lymphocytes and its related cytokines. The *RRAS*, *POMC*, *SLC26A6* and *STX3* genes were important regulatory modules in pathogenesis of BA. The expression of RRAS was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis.

CONCLUSION: Autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The *RRAS* gene is an important regulatory module in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

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Key words: Biliary atresia; DNA microarray; Bioinformatics; RRAS; Prognostic biomarker

Peer reviewer: Beata Jolanta Jabłońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

Zhao R, Li H, Shen C, Zheng S. RRAS: A key regulator and an important prognostic biomarker in biliary atresia. *World J Gastroenterol* 2011; 17(6): 796-803 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/796.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.796>

INTRODUCTION

Biliary atresia (BA) is a devastating disease of infants, invariably leading to cirrhosis, end-stage liver disease, and death if untreated^[1]. A recent review reported that BA may involve a primary perinatal hepatobiliary viral infection and a secondary autoimmune-mediated bile duct inju-

ry^[2]. However, the cause and pathogenesis of BA remain largely unknown.

Microarray technology, emerged as an indispensable research tool for gene expression profiling, has been used to study the mechanism underlying BA, and allows the simultaneous analysis of thousands of transcripts within a single experiment^[3]. Some studies have been performed to investigate the gene expression profiling of livers from BA patients^[4-6]. However, to our knowledge, none of them was designed to identify genes that play a key role in the pathogenesis and prognosis of BA. In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns of normal livers and livers from BA patients at different ages, as well as to ascertain the genes and pathways that play a central role in the pathogenesis of BA. Furthermore, reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm the changes in selected genes. The relation between selected gene expression and prognosis of BA patients was also analyzed.

MATERIALS AND METHODS

Patients and specimens

Biopsy specimens were obtained from livers of 9 patients with BA, 3 patients with neonatal cholestasis and 3 control patients suffering from liver trauma (as normal control) at Children's Hospital of Fudan University from November 2007 to December 2008. Nine patients with BA were further divided into < 60 d group ($n = 3$), 60-90 d group ($n = 3$) and > 90 d group ($n = 3$). Liver samples from 3 groups of BA patients, neonatal cholestasis and control groups were immediately dissolved in a RNeasy lysis reagent (Qiagen, Germany) and then stored at -80°C. Liver samples from each group were pooled and total RNA was isolated from them for DNA microarray experiments. Clinical data about these patients are summarized in Table 1. Liver samples were collected from the other 14 patients with neonatal cholestasis and 40 patients with BA for RT-PCR experiments. All subjects gave their informed consent to participate in the study which was approved by the Research Ethics Committee of Fudan University.

RNA extraction, processing and microarray analysis

Total RNA was extracted from liver tissue samples using the Trizol reagent (Invitrogen) according to its manufacturer's protocol, and then further purified using a NucleoSpin RNA clean-up kit (Macherey-Nagel, Germany). Quantification analysis of RNA was performed on a spectrophotometer and quality of RNA was analyzed by denaturing formaldehyde gel electrophoresis. Five micrograms of total RNA from each group was amplified and labeled with biotin using an Illumina total Prep RNA amp kit (Ambion, Austin, TX, USA) and hybridized to Illumina's Sentrix Human-6 (Version 3) Expression Bead-Chips containing 48000 transcripts (Illumina, San Diego, CA, USA). Three duplicated chips were also used in each

group to test the variations in duplications from the same pooling. The hybridized Illumina chips were scanned on a BeadArray reader (Illumina, San Diego, CA, USA) and microarray analysis was performed using the BeadStudio software (Illumina, San Diego, CA, USA). Raw data were normalized using the cubic spline method and the resulting genes were filtered. Finally, only genes with a differential expression score (Diffscore) greater than 20 or less than -20 were included.

Bioinformatics analysis of differentially expressed genes

Differentially expressed genes were analyzed by a series test of cluster (STC) to search a set of model expression profiles that were distinct in 5 groups as previously described^[7,8]. These profiles were assigned to significant gene ontology categories using series test cluster of gene ontology (STC-GO)^[9], and analyzed with the Pathway-Finder software to obtain the significance of pathway categories^[10,11]. Moreover, dynamic gene networks were constructed to find the key genes that may play a central role in the pathogenesis of BA^[12,13]. The principle and algorithmic details are available in supplementary data.

Validation of microarray data by RT-PCR

RT-PCR was performed on liver tissue samples from 14 patients with neonatal cholestasis and 40 patients with BA to confirm changes in selected genes, including RRAS, POMC, SLC26A6 and STX3. Total RNA was extracted from liver tissue samples and purified as previously described^[14]. Five micrograms of total RNA was reverse transcribed using MMLV reverse transcriptase (Merck, Germany) and random primers in a 20 µL reaction volume at 42°C for 1 h. Oligonucleotide primers for the RRAS, POMC, SLC26A6, STX3 and β-actin are shown in Table 2. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis and UV illuminated on a 2% agarose gel containing ethidium bromide (0.5 µg/mL). The gel image was stored using the UVP gel documentation system 5000 (Ultra-Violet Products Ltd., Cambridge, United Kingdom). Expression levels of the selected genes relative to β-actin were measured with densitometric scanning using the multi-analysis/PC system (Bio-Rad, Hercules, California, USA).

Prognostic biomarker and follow-up research

Fibrosis of liver biopsy specimens from 40 patients with BA was histologically classified into different groups. Elimination rate of jaundice (TB < 20 µmol/L) within 6 mo after operation, 2-year survival rates of cholangitis patients and of 40 BA patients were calculated. The diagnostic criteria for cholangitis included fever, increasing jaundice, acholic stools, with other causes of infection excluded. Follow-up data were obtained from our outpatient and inpatient referrals, as well as from interview by

Table 1 Microarray analysis showing clinical characteristics of biliary atresia patients

Case No.	Gender	Age	TB/DB	ALT/AST	AKP/GGT	Albumin	Disease	Group
1	Female	50 d	171/130	132/254	604/609	35.8	BA	1
2	Male	49 d	291/231	148/155	581/408	39.4	BA	1
3	Male	57 d	148/117	189/166	493/535	35.2	BA	1
4	Female	73 d	145/118	111/153	460/1460	36.3	BA	2
5	Male	84 d	160/127	100/149	713/1278	37.3	BA	2
6	Female	66 d	129/108	121/165	632/1235	39.2	BA	2
7	Female	103 d	151/112	85/62	451/360	39.4	BA	3
8	Female	97 d	118/89	74/78	522/501	34.3	BA	3
9	Male	110 d	155/121	105/97	377/912	35.4	BA	3
10	Female	77 d	102/89	201/137	234/317	39.4	Cholestasis	4
11	Male	64 d	137/108	404/267	584/1044	37.2	Cholestasis	4
12	Female	55 d	144/112	389/266	612/1339	41.0	Cholestasis	4
13	Male	4 yr	16/9	33/35	200/50	39.4	Liver trauma	5
14	Male	6 yr	10/4	20/25	192/44	40.1	Liver trauma	5
15	Male	4 yr	12/4.7	29/37	101/38	37.1	Liver trauma	5

TB: Total bilirubin; DB: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; GGT: γ -glutamyl transferase; BA: Biliary atresia.

Table 2 Sequences of primers in selected genes used in reverse-transcription polymerase chain reaction

Gene name	Primer sequences
RRAS	F: TTGGTCGGGAACAAGGCAGAT R: CTCGTCCACGTTGAGACGCAGT
POMC	F: GAGAGCAGCCAGTGTCAGG R: GAAGTGGCCCATGACGTACT
SLC26A6	F: CGGTATCCIGTGCGTGACT R: GGAAGTGCCAAACAGGAAGT
STX3	F: GGCAAAAAGACAACCGATGA R: TGTCGTGAAGCTCCTTGATG
β -actin	F: GGGAAATCGTGCCTGCATT R: CAGGCAGCTCGTAGCTCTT

telephone or questionnaires. These patients were further classified based on the follow-up data, including presence of jaundice 6 mo after operation, occurrence of cholangitis within 2 years after operation, and 2-year survival rate.

Statistical analysis

Data were expressed as mean \pm SD. Variations in duplications were detected by Fisher's exact test, χ^2 test, *t*-test and Cochran-Mantel-Haenszel test using the STATA 8.0 software (Stata Co., College Station, TX, USA). Pair-wise test was used to confirm the limited variations in duplications from the same pooling. $P < 0.05$ was considered statistically significant.

RESULTS

Differentially expressed gene profiles in liver tissue samples from BA patients

Total RNA was extracted from liver tissues of the normal group, neonatal cholestasis group, and 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). Denaturing formaldehyde gel electrophoresis showed no degradation (data not shown). Illumina's Sentrix Hu-

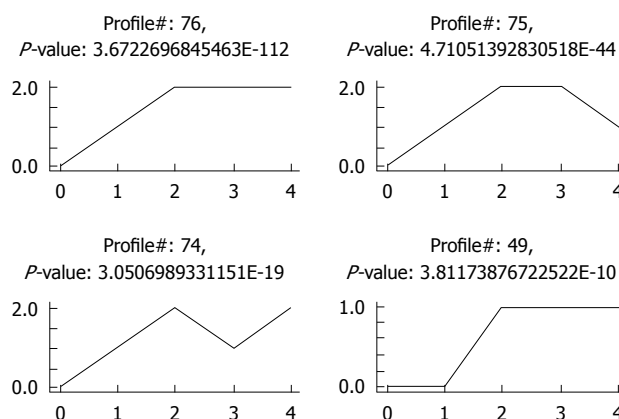


Figure 1 Four most significant expression profiles in liver samples from biliary atresia patients. 0: Normal control group; 1: Neonatal cholestasis group; 2: Group of biliary atresia (BA) patients > 90 d; 3: Group of BA patients at the age of 60-90 d; 4: Group of BA patients < 60 d. Y axis represents the expression change expressed as $\log_2 [v(i)/v(0)]$.

man-6 (Version 3) Expression BeadChip was used for each of pooled liver tissue samples from the 5 groups. Pair-wise test confirmed a limited variation in duplications from the same pooling (data not shown). A total of 795 differentially expressed genes were identified from different groups with a Diffscore greater than 20 or less than -20. Further STC analysis yielded 80 expression profiles. Of these expression profiles, 20 were statistically significant ($P < 0.05$) and 4 had the lowest P value (Figure 1). These 4 expression profiles could confidently separate livers in groups of BA patients from those in normal and neonatal cholestasis groups. Specifically, all the differentially expressed genes in 5 groups were included in profile 76, which showed no significant difference in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). The 611 genes represented in profile 75 were mainly involved in inflammation mediated by activation of T lymphocytes, and reconstruction of extracellular matrix. As shown in Figure 1, the global expression level of

Table 3 Significant pathways involved in pathogenesis of biliary atresia

Pathway name	P value	Profile No.
Cell adhesion molecules	0.000118	Profile49
Regulation of actin cytoskeleton	0.000739	Profile49
T Leukocyte transendothelial migration	0.001738	Profile49
Asthma	0.002023	Profile49
Allograft rejection	0.003243	Profile49
Systemic lupus erythematosus	2.44E-05	Profile74
Lysosome	0.0002109	Profile74
NF-kappa B signaling pathway	0.0036605	Profile74
MAPK signaling pathway	0.0052322	Profile74
Allograft rejection	0.0058515	Profile74
Graft-versus-host disease	0.0071277	Profile74
Type 1 diabetes mellitus	0.00781	Profile74
Chemokine signaling pathway	1.81E-08	Profile75
Matrix_Metalloproteinases	6.52E-07	Profile75
Cytokine-cytokine receptor interaction	3.59E-05	Profile75
T cell receptor signaling pathway	4.34E-05	Profile75
Antigen processing and presentation	0.000337	Profile75
Leukocyte transendothelial migration	0.0010211	Profile75
Lysosome	2.09E-07	Profile76
Toll-like receptor signaling pathway	0.000284	Profile76
T cell receptor signaling pathway	0.000388	Profile76
Chemokine signaling pathway	0.000755	Profile76
Asthma	0.000841	Profile76
Matrix_Metalloproteinases	0.001402	Profile76
Allograft rejection	0.001697	Profile76

the genes in profile 75 was much lower in livers from the group of BA patients at the age of < 60 d than from the groups of BA patients at the age of 60-90 d and > 90 d. The 372 genes represented in profile 74 were associated with an apoptotic pathway and inflammatory response mediated by nuclear factor- κ B (NF- κ B). The global expression level of profile 74 genes was much lower in the group of BA patients at the age of 60-90 d than in the groups of BA patients at the age of < 60 d and > 90 d. Moreover, the 285 genes represented in profile 49 were mainly involved in inflammatory response mediated by the major histocompatibility complex (MHC) class II antigen. The global expression level of profile 49 genes was much higher in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) than in the normal and neonatal cholestasis groups. Little variance in gene expression was observed neither in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) nor in the normal and neonatal cholestasis groups (See supplementary data for a complete list of these 4 expression profiles).

Involvement of significant pathways in BA focused on autoimmune response associated with inflammatory response of T lymphocytes

Based on the Kyoto Encyclopedia of Genes and Genomes Database and the most significant 4 gene expression profiles, Fisher's exact test and χ^2 test were performed to identify the significant pathways involved in BA as previously described^[15]. The significant pathways ($P < 0.01$) highly associated with BA were mainly focused on (1) autoimmune response associated with asthma, systemic

Table 4 Genes with the highest degree and k-core in dynamic gene networks

Gene symbol	Definition	Degree	k-core
RRAS	Homo sapiens related RAS viral (r-ras) oncogene homolog (RRAS), mRNA	12	6
POMC	Homo sapiens POMC, transcript variant 1, mRNA	12	6
SLC26A6	Homo sapiens SLC26A6, transcript variant 3, mRNA	12	6
STX3	Homo sapiens STX3, mRNA	10	6

Table 5 Reverse-transcription polymerase chain reaction showing relative expression levels of RRAS, POMC, SLC26A6 and STX3 (mean \pm SD)

Group	POMC	SLC26A6	RRAS	STX3
Biliary atresia (n = 40)	0.58 \pm 0.090	0.43 \pm 0.054	0.89 \pm 0.103	0.61 \pm 0.074
Neonatal cholestasis (n = 14)	0.41 \pm 0.081	0.30 \pm 0.029	0.47 \pm 0.074	0.51 \pm 0.045
P-value	0.031	0.023	0.004	0.017

lupus erythematosus, allograft rejection graft-versus-host disease, type I diabetes mellitus, antigen processing and presentation; (2) activation of T lymphocytes and inflammatory response including transendothelial migration of T leukocytes, cell adhesion molecules, NF- κ B and MAP kinase (MAPK) signaling pathways, chemokine signaling pathway, cytokine-cytokine receptor interaction, transendothelial migration of leukocytes, Toll-like receptor signaling pathway; and (3) reconstruction of extracellular matrix, including matrix-metalloproteinases. The significant pathways are shown in Table 3.

Construction of dynamic gene networks

Dynamic gene networks^[16] were constructed to find the key regulators that may play a central role in the pathogenesis of BA (Figure 2). Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, size of circles indicates their interactions with other molecules, coloring is classified according to the k core and red indicates high k core. The genes with the highest degree and k-core from Figure 2, are listed in Table 4, including the related RAS viral (r-ras) oncogene homolog (RRAS), POMC, SLC26A6 and STX3 genes, indicating that they play a crucial role in the pathogenesis of BA.

Expression of RRAS, POMC, SLC26A6 and STX3 genes in liver tissue samples from patients with BA confirmed by RT-PCR

The mRNA expression levels of RRAS, POMC, SLC26A6 and STX3 in liver tissue samples from 40 patients with BA and 14 patients with neonatal cholestasis, measured in order to validate the results derived from microarray data, were significantly higher in liver tissue samples from patients with BA than from those of neonatal patients

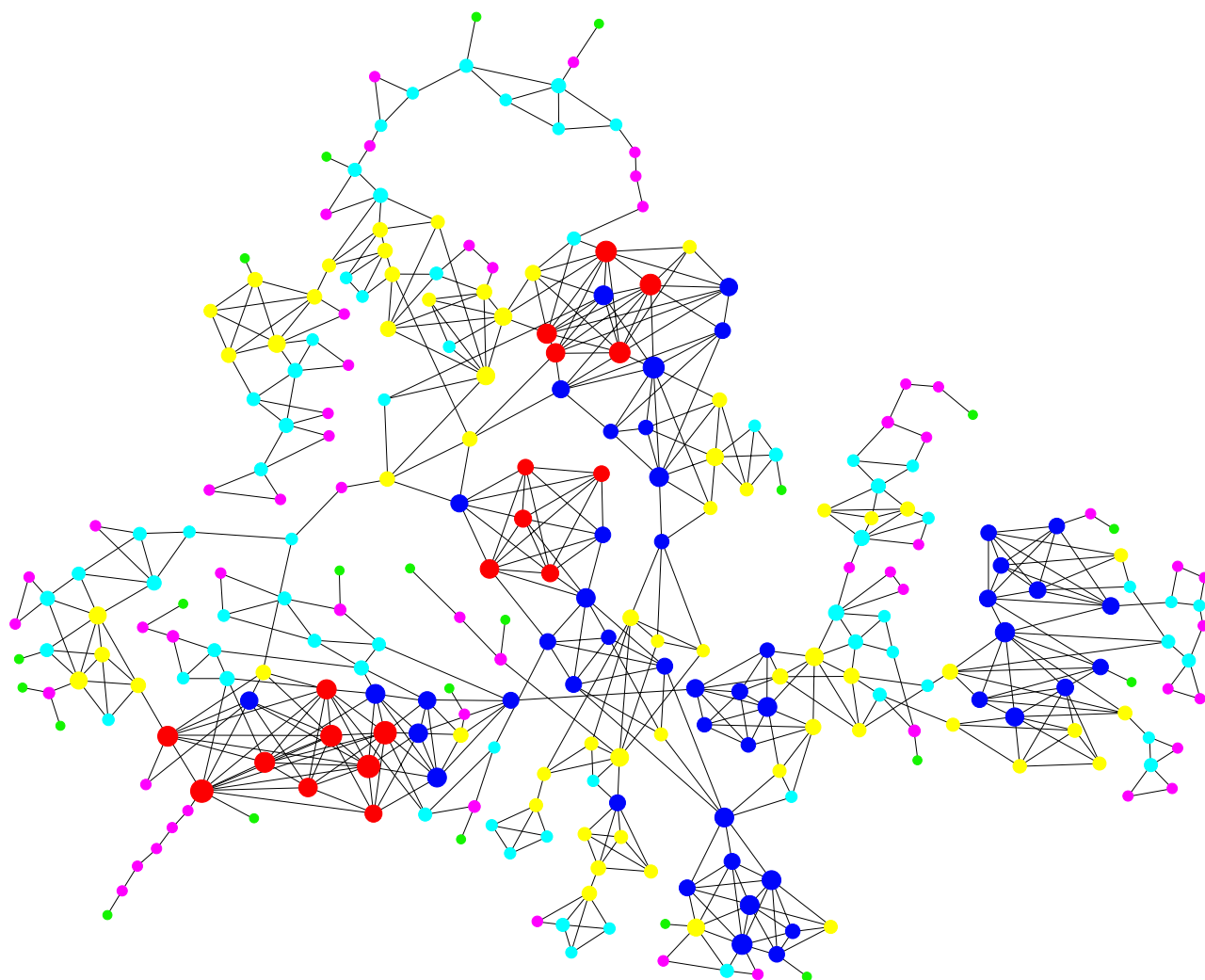


Figure 2 Dynamic gene networks constructed showing the key regulators that may play a central role in the pathogenesis of biliary atresia. Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, and size of circles indicates their interactions with other molecules. Coloring is classified according to the k core, and red indicates high k core.

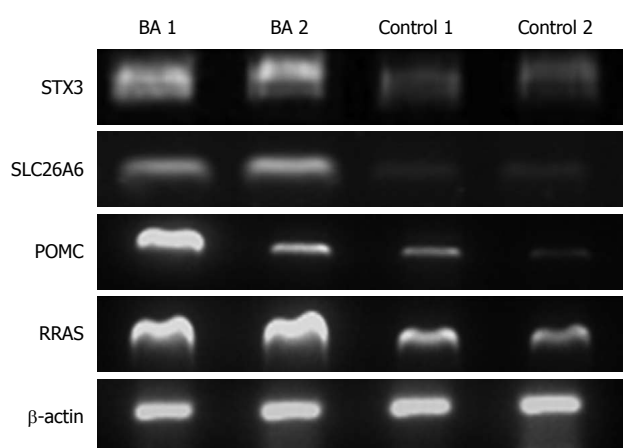


Figure 3 Reverse-transcription polymerase chain reaction showing expression levels of RRAS, POMC, SLC26A6, and STX3 genes. BA: Biliary atresia; Control: Neonatal cholestasis.

with cholestasis ($P < 0.05$, Table 5, Figures 3 and 4). The mRNA expression level of the RRAS gene increased 1.9-fold in BA patients ($P < 0.05$).

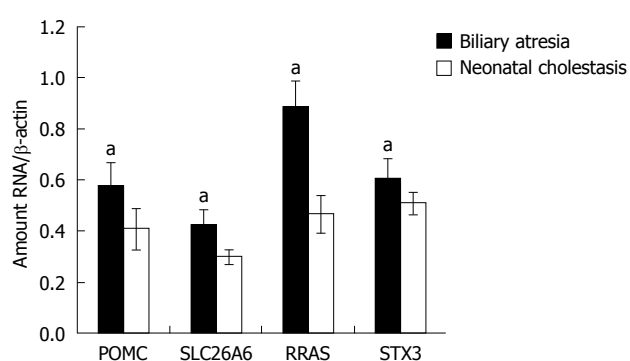


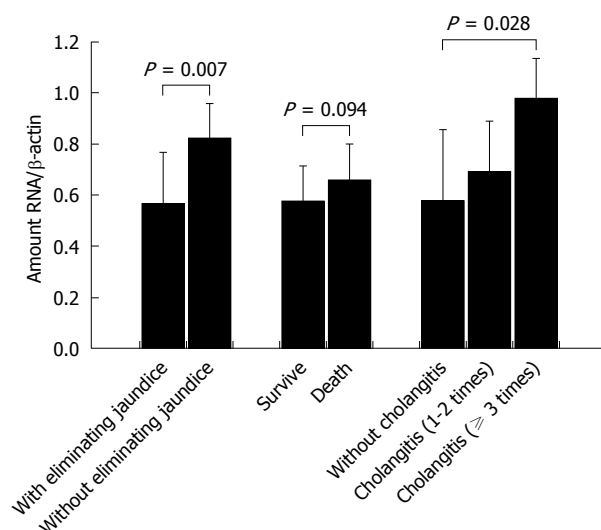
Figure 4 Reverse-transcription polymerase chain reaction showing significantly higher relative expression levels of RRAS, POMC, SLC26A6 and STX3 genes in biliary atresia patients ($n = 40$) than in neonatal cholestasis patients ($n = 14$). ^a $P < 0.05$ vs liver tissue samples from neonatal cholestasis patients.

Correlation between expression of RRAS in liver tissue samples and prognosis of BA patients

To address whether the RRAS expression is associated with the prognosis of BA patients, we performed a 2-year

Table 6 Fibrosis scores for different groups of biliary atresia patients at different ages

Group	Fibrosis score (n)					Total
	0	1	2	3	4	
< 60 d	1	3	8	2	0	14
60-90 d	0	2	3	6	4	15
> 90 d	0	0	1	4	6	11
Total	1	5	12	12	10	40

**Figure 5** Correlation between expression of RRAS in liver tissue samples and prognosis of biliary atresia patients within 2 years.

follow-up study in 40 patients with BA. Six months after operation, The RRAS expression level was significantly higher in patients with their jaundice not eliminated than in those with their jaundice eliminated ($P = 0.007$). Furthermore, the expression level of RRAS was significantly higher in patients with cholangitis reoccurred 3 or more times than in those with no cholangitis recurred ($P = 0.028$). However, no significant difference in RRAS expression was found between the surviving and dead patients ($P = 0.094$, Figure 5). In addition, the fibrosis was more serious in group of BA patients at the age of > 60 d than in group of BA patients at the age of < 60 d (Table 6), which was consistent with that observed in profile 75.

DISCUSSION

The pathogenesis of BA has not yet been delineated. It has been shown that factors such as genetic susceptibility, congenital heteroplasia, and infectious and abnormal immune response lead to BA^[17-23], and its clinical course and surgical outcome are correlated with the age of such patients^[24-26]. Although there are some studies involving DNA microarrays in BA^[4-6], very few studies are available on gene expression profiling of BA at its different stages of clinical course. That is why the clinicopathologic characteristics of BA vary with the age of such patients.

The differential gene expression patterns of RRAS in

liver tissue samples from BA patients at different ages, as well as normal liver tissue samples and liver tissue samples from neonatal cholestasis patients were characterized in this study using the expression DNA microarray technology and bioinformatics. The 4 significant expression profiles identified using STC could confidently separate BA liver tissues from normal and diseased liver tissues. STC-GO analysis revealed that the genes represented in the 4 profiles were mainly involved in inflammatory response and reconstruction of extracellular matrix. Notably, as validated by fibrosis classification, profile 75 showed that the expression level of genes involved in fibrosis and inflammation was much lower in BA patients at the age of < 60 d than in those at the age of > 60 d, which may explain why BA patients at the age of < 60 d often have a good prognosis after a Kasai's operation^[27]. Additionally, this phenomenon may also result from fibrosis due to the continuous hepatic inflammatory response-induced activation of stellate cells^[28]. Moreover, a set of genes were involved in apoptosis represented in profile 74, which is in agreement with the reported findings^[29]. In this profile, the BA patients at the age of > 90 d and < 60 d showed obvious inflammatory response and apoptosis mediated by NF-κB, which might be associated with the inflammatory response of local bile ducts in BA patients at the age of < 60 d and severe inflammatory response induced by fibrosis in BA patients at the age of > 90 d.

Mack *et al.*^[30,31] showed that CD4⁺ Th1-mediated bile duct inflammation is responsible for the development of BA. Profile 74 in the present study contains a set of genes associated with MHC class II antigen-mediated Th1 inflammatory response, which is in agreement with the findings of Mack *et al.*^[31] and Osada *et al.*^[32]. It is well known that antigens associated with MHC class II can bind to T cell receptors of CD4⁺ Th1 cells, and thereby produce functional T lymphocytes. Furthermore, the significant pathways highly associated with BA were mainly focused on the autoimmune response, activation of T lymphocytes and its related cytokines, suggesting that autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA, which is consistent with the widely accepted hypothesis of BA^[2,22,23,32].

In this study, the RRAS, POMC, SLC26A6 and STX3 genes were found to be important regulatory modules in BA. The RRAS gene is a component of the MAPK signaling pathway with GTP kinase activity^[33,34]. The MAPK pathway is associated with BA^[35]. Based on the results of this study, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA. The human POMC gene is located on chromosome 2p23.3 encoding a preprohormone. The adrenocorticotropin hormone and α melanocyte-stimulating hormone are cleavage products of POMC, which are associated with immune regulation and participate in the pathogenesis of experimental autoimmune encephalomyelitis^[36,37]. The SLC26A6 is an anion exchanger involved in the secretion of bile acid. The STX3 has been implicated in the development and differentiation of dendritic cells^[38,39]. Nonetheless,

the precise role of these genes in the pathogenesis of BA needs to be further elucidated.

Given the key role of RRAS gene in the pathogenesis of BA, we evaluated the relation between the expression of RRAS and prognosis of BA patients through a 2-year follow-up study. The RRAS expression was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis, indicating that up-regulation of RRAS expression may inhibit the recovery of BA from jaundice and cholangitis *via* activation of the MAPK pathway, continuous inflammatory response, inflammatory cell infiltration, as well as activation of stellate cells^[40]. However, no significant difference was found in the 2-year survival rate of patients with different expression levels of RRAS. Validation may require a long-term follow-up and a larger number of subjects.

In summary, autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The RRAS gene and its related MAPK pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

ACKNOWLEDGMENTS

The authors thank Dr. Duan Ma for his technical assistance and Song Zai for his collection of clinical samples.

COMMENTS

Background

Biliary atresia (BA) is an inflammatory obliterative cholangiopathy with unknown etiology, leading to progressive fibrosis and cirrhosis. Although there are some studies involving DNA microarrays on BA, very few studies are available on gene expression profiling of BA at different stages of its clinical course, which is why the clinicopathologic characteristics of BA vary with the age.

Research frontiers

Microarray technology and bioinformatics, emerged indispensable research tools for gene expression profiling, have been used to study the pathogenesis of BA and allow the simultaneous analysis of thousands of transcripts within a single experiment. In this study, genes that play a key role in the pathogenesis and prognosis of BA were identified.

Innovations and breakthroughs

In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns in normal livers and livers from BA patients at different ages, as well as to ascertain genes and pathways playing a central role in the pathogenesis of BA. The results demonstrate that RRAS gene and its related MAP kinase (MAPK) pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

Applications

By identifying genes and pathways playing a central role in the pathogenesis of BA, this study may represent a future strategy for therapeutic intervention in treatment of BA.

Terminology

RRAS gene is a component of the MAPK signaling pathway with GTP kinase activity. The MAPK pathway is associated with BA. Consequently, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA.

Peer review

This paper is interesting and valuable for other researchers. BA is a pediatric liver disease, which can lead to liver-related death and is the most common indication for liver transplantation in children. Therefore, the early proper treat-

ment of BA with Kasai procedure is important in this group of patients. Early diagnosis of BA and knowledge of its prognostic factors can improve the treatment outcome of BA. Different prognostic factors have been described in the literature, but no report is available on RRAS as a key regulator and an important prognostic biomarker for BA identified by DNA microarray and bioinformatics.

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S- Editor Sun H L- Editor Wang XL E- Editor Zheng XM

HBV infection decreases risk of liver metastasis in patients with colorectal cancer: A cohort study

Hai-Bo Qiu, Li-Yi Zhang, Zhao-Lei Zeng, Zhi-Qiang Wang, Hui-Yan Luo, Rajiv Prasad Keshari, Zhi-Wei Zhou, Rui-Hua Xu

Hai-Bo Qiu, Zhao-Lei Zeng, Zhi-Qiang Wang, Hui-Yan Luo, Rajiv Prasad Keshari, Zhi-Wei Zhou, Rui-Hua Xu, State Key Laboratory of Oncology in South China, Guangzhou 510060, Guangdong Province, China

Hai-Bo Qiu, Zhi-Qiang Wang, Hui-Yan Luo, Rui-Hua Xu, Department of Medical Oncology, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Hai-Bo Qiu, Rajiv Prasad Keshari, Zhi-Wei Zhou, Department of Gastric and Pancreatic Surgery, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Li-Yi Zhang, Department of Clinical Oncology, University of HongKong, Pokfulam, HongKong, China

Zhao-Lei Zeng, Department of Research, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Author contributions: Qiu HB and Zhang LY contributed equally to this work; Qiu HB and Xu RH defined the research theme; Qiu HB, Zhang LY and Zeng ZL designed the methods and wrote the paper; Keshari RP revised the manuscript; Zhang LY, Wang ZQ and Luo HY analyzed the data and interpreted the results; Qiu HB and Zhou ZW collected, interpreted the data, and discussed the analyses; all authors approved the manuscript.

Supported by National Natural Science Foundation of China, No. 30672408

Correspondence to: Rui-Hua Xu, MD, PhD, Professor of Medical Oncology, Vice President, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China. xurh@sysucc.org.cn

Telephone: +86-20-87343468 Fax: +86-20-87343228

Received: October 7, 2010 Revised: November 8, 2010

Accepted: November 15, 2010

Published online: February 14, 2011

Abstract

AIM: To evaluate the effect of hepatitis B virus (HBV) infection on liver metastasis of colorectal cancer.

METHODS: A total of 1298 colorectal cancer patients were recruited from January 2001 to March 2005 in this study. Enzyme-linked immunosorbent assay was used to

test serum HBV markers for colorectal cancer. Patients were divided into study (infection) group and control (non-infection) group. Clinical features of patients in two groups were compared.

RESULTS: Liver metastasis was found in 319 out of the 1298 colorectal cancer patients. The incidence of liver metastasis was significantly lower in study group than in control group (14.2% vs 28.2%, $P < 0.01$). HBV infection significantly decreased the risk of liver metastasis [hazard ratio (HR): 0.50, 95% confidence interval (95% CI): 0.38-0.66], but the incidence of extrahepatic metastasis was significantly higher in study group than in control group (31.9% vs 17.0%, $P < 0.01$). The HR was the lowest in chronic hepatitis B group (HR: 0.29, 95% CI: 0.12-0.72). The number of liver metastatic lesions was significantly less in study group than in control group with a higher surgical resection rate. However, no significant difference was found in survival rate between the two groups ($P = 0.95$).

CONCLUSION: HBV infection decreases the risk of liver metastasis in patients with colorectal cancer and elevates the surgical resection rate of liver metastatic lesions.

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Key words: Hepatitis B virus; Colorectal cancer; Liver metastasis; Risk

Peer reviewers: Dr. Lucia Ricci Vitiani, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, Rome 00161, Italy; Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Qiu HB, Zhang LY, Zeng ZL, Wang ZQ, Luo HY, Keshari RP, Zhou ZW, Xu RH. HBV infection decreases risk of liver metastasis in patients with colorectal cancer: A cohort study. *World*

J Gastroenterol 2011; 17(6): 804-808 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i6/804.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.804>

INTRODUCTION

Colorectal cancer (CRC) accounts for 10%-15% of all cancers and is the second leading cause of cancer-related deaths in Western countries^[1]. Approximately half of CRC patients develop metastatic disease^[2]. Of the CRC patients, 15%-25% present with synchronous liver metastasis and 80%-90% are initially found to have unresectable liver metastatic disease^[3]. Metastatic liver disease more frequently develops metachronous metastasis following treatment of CRC. It is estimated that over half of dead CRC patients have liver metastasis at autopsy^[4].

Hepatitis B virus (HBV) infection is the most common cause of chronic liver diseases worldwide, an estimated 350 million persons are chronically infected with HBV worldwide, and China is a highly endemic area of HBV infection with approximately 170 million HBV carriers^[5]. It has been demonstrated that HBV infection plays an important role in the development of hepatocellular carcinoma (HCC)^[6]. It was reported that HBV infection finally reduces the risk of intrahepatic metastasis in HCC patients with a higher survival rate and therefore can be considered an important prognostic factor for HCC patients^[7].

Rare reports are available on the relation between HBV infection and hepatic metastasis of CRC. Utsunomiya *et al.*^[8] reported that CRC seldom metastasizes to liver of patients infected with HBV or hepatitis C virus (HCV), but most patients in their study were infected with HCV. Song *et al.*^[9] showed that chronic HBV infection with viral replication reduces hepatic metastasis of CRC and prolongs the survival time of CRC patients. However, their study was hard to demonstrate the relation between HBV infection and hepatic metastasis of CRC due to its small sample size. Alternatively, investigation of experimentally induced hepatic metastasis of colon cancer demonstrated that activated immune cells residing in livers can effectively kill metastatic tumor cells, indicating that alterations in liver-associated immunity play an important role in hindering hepatic metastasis^[10]. Thus, we designed this cohort study to observe the relation between HBV infection and liver metastasis of CRC.

MATERIALS AND METHODS

Patients

A total of 1298 CRC patients at the age of > 16 years, admitted to Sun Yat-Sen University Cancer Center (Guangzhou, China) from January 2001 to March 2005, were recruited in this study and divided into study (infection) group and control (non-infection) group. All patients gave their written informed consent to receive a test for HBV infection at their first visit. The study was approved by The Ethics Committee of Sun Yat-Sen University Cancer Center.

Serologic assay for viral infection

HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc were detected by enzyme-linked immunosorbent assay and HBV deoxyribonucleic acid (HBV-DNA) was detected by polymerase chain reaction.

Treatment

Primary colorectal adenocarcinoma was completely removed from all eligible patients with no prior chemotherapy or radiotherapy, and staged according to AJCC Cancer Staging Manual, 6th edition^[11]. All patients received 5-fluorouracil-based FOLFOX6 or XELOX regimen. Patients with liver metastasis underwent palliative treatment (including chemotherapy, radiotherapy, surgical resection and radio-frequency ablation) according to the update NCCN Guidelines for CRC^[12].

Assessment of study and follow-up of patients

Patients were assessed by abdominal and pelvic computed tomography (CT) scan or magnetic resonance imaging (MRI), thoracic radiography or thoracic CT or MRI before surgery. Patients who underwent surgery were assessed again during operation. All patients, after discharged from hospital, were followed up according to a standard protocol^[13]. The patients were followed up every 3 mo in the first 2 years after surgery, during which clinical examination, routine blood test, assessment of tumor markers, and abdominal ultrasonography or CT scan and endoscopy were performed. In the next 3 years, the patients were followed up every 6 mo and underwent endoscopy every 12 mo. The relapse of CRC (defined as local recurrence or metastasis at distant sites) at other sites was detected and staged. The follow-up was terminated in April 2010.

Statistical analysis

Differences in baseline clinical parameters and treatment outcomes between the two groups were evaluated by chi-square test or Student *t* test. Hazard ratio (HR) and 95% confidence interval (95% CI) were calculated with the Cox proportional-hazards model. Overall survival (OS) and disease-free survival (DFS) curves were plotted with the Kaplan-Meier method, and compared by log-rank test. OS rate was calculated from the date of discharge to death. DFS time was defined as the time between discharge and first relapse of CRC. A two-tailed *P* value less than 0.05 was considered statistically significant. Statistical analysis was performed with SPSS for Windows V.13.0.

RESULTS

The 1298 patients were divided into study group and control group. Three hundred and thirty-two patients (25.6%) with chronic HBV infection included in study group were further divided into 3 subgroups according to their natural history of HBV infection^[14]. Chronic hepatitis B (CHB) was identified in 37 patients (2.9%) according to the presence of HBsAg and HBeAg or HBV-DNA which are markers of active viral replication. Inactive HBsAg carriers (IC), identified in 108 patients (8.3%), were characterized

Table 1 Baseline characteristics of patients included in this study *n* (%)

Characteristic	Study group	Control group	<i>P</i>
No. of patients	332 (100)	966 (100)	
Gender			NS
Male	196 (59.0)	550 (55.0)	
Female	136 (41.0)	416 (45.0)	
Age (yr)			NS
Median	53	60	
Range	16-81	16-87	
Depth of tumor invasion ¹	299 (100)	857 (100)	NS
T1	21 (6.3)	40 (4.1)	
T2	38 (11.4)	165 (17.1)	
T3	87 (26.2)	264 (27.3)	
T4	153 (46.1)	388 (40.2)	
Lymph-node metastasis ¹	295 (100)	843 (100)	NS
N0	160 (48.2)	490 (58.1)	
N1	87 (26.2)	216 (25.6)	
N2	48 (14.5)	137 (14.2)	
Chronic liver dysfunction ²	39 (11.7)	65 (6.7)	< 0.05
Albumin (g/dL)	39.4 ± 5.5	39.4 ± 11.1	NS
Total bilirubin (mg/dL)	13.0 ± 5.8	12.7 ± 6.7	NS
LDH (IU/L)	191.5 ± 141.7	197.5 ± 164.1	NS
ALP (IU/L)	71.7 ± 35.5	75.6 ± 49.7	NS
GGT (IU/L)	34.7 ± 54.9	35.4 ± 55.9	NS

¹Comparison was made only in 1156 cases after primary tumor resection, including 299 cases in study group and 857 cases in control group; ²Chronic hepatitis or liver cirrhosis was clinically diagnosed according to the findings in serum chemistry, ultrasonography and computed tomography. LDH: Lactate dehydrogenase; GGT: γ -glutamyl transferase; ALP: Alkaline phosphatase; NS: Not significant.

by the presence of HBsAg and anti-HBe and the absence of HBeAg or HBV-DNA. Resolved hepatitis B (RHB) observed in 187 patients (14.4%) was characterized by negative HBsAg and the presence of anti-HBc \pm anti-HBs. Nine hundred and sixty-four patients (74.6%) were included in control group. No significant difference was found in sex, age, depth of tumor invasion, lymph-node metastasis, lactate dehydrogenase, γ -glutamyl transpeptidase, alkaline phosphatase, albumin, and total bilirubin between the two groups (Table 1). However, the liver function was significantly worse in study group than in control group.

Follow-up

The mean follow-up time of patients was 6 mo after operation. The median interval time of patients was 6 mo after operation. The median follow-up time of patients was 57.2 mo (range 0-110.4 mo) after operation.

Liver and extrahepatic metastasis

Liver metastasis occurred in 319 patients including synchronous liver metastasis in 193 cases and metachronous liver metastasis in 127 cases. Of the 193 patients, 39 had synchronous liver metastases. Of the 127 patients, 18 had metachronous liver metastasis. Synchronous or metachronous extrahepatic metastasis occurred in 270 patients was defined as distant metastasis but not as liver metastasis. The incidence of recurrence or metastasis to the distant sites is summarized in Table 2. The incidence of liver and extrahepatic metastasis was comparable between the two

Table 2 Synchronous and metachronous metastasis in two groups *n* (%)

Sites of metastasis	Study group (<i>n</i> = 332)	Control group (<i>n</i> = 966)	<i>P</i> value	HR (95% CI)
Liver			< 0.01	0.50 (0.38-0.66)
Yes	47 (14.2)	272 (28.2)		
No	285 (85.8)	694 (71.8)		
Extrahepatic			< 0.01	1.88 (1.52-2.33)
Yes	106 (31.9)	164 (17.0)		
No	226 (68.1)	802 (83.0)		

HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 3 Synchronous and metachronous metastasis in chronic hepatitis B and control groups *n* (%)

Metastatic sites	CHB group (<i>n</i> = 37)	Control group (<i>n</i> = 966)	<i>P</i> value	HR (95% CI)
Liver			< 0.01	0.29 (0.12-0.72)
Yes	3 (8.1)	272 (28.2)		
No	34 (91.9)	694 (71.8)		
Extrahepatic			< 0.01	2.55 (1.77-3.67)
Yes	16 (43.2)	164 (17.0)		
No	21 (56.8)	802 (83.0)		

CHB: Chronic hepatitis B; HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 4 Synchronous and metachronous metastasis in inactive carriers and control group *n* (%)

Metastatic sites	IC group (<i>n</i> = 108)	Control group (<i>n</i> = 966)	<i>P</i> value	HR (95% CI)
Liver			< 0.01	0.36 (0.22-0.59)
Yes	11 (10.2)	272 (28.2)		
No	97 (89.8)	694 (71.8)		
Extrahepatic			< 0.01	2.24 (1.66-3.01)
Yes	41 (38.0)	164 (17.0)		
No	67 (62.0)	802 (83.0)		

IC: Inactive carriers; HR: Hazard ratio; 95% CI: 95% confidence interval.

groups. The incidence of liver metastasis was significantly lower in study group than in control group (14.2% *vs* 28.2%, *P* < 0.01). The Mantel-Haenszel χ^2 analysis showed that HBV infection significantly decreased the risk of liver metastasis (HR: 0.50, 95% CI: 0.38-0.66). The incidence of extrahepatic metastasis was significantly higher in study group than in control group (31.9% *vs* 17.0%, *P* < 0.01). No difference was found in liver metastasis between the two groups.

The liver metastasis rate in patients with CHB, IC and RHB is listed Tables 3-5. CHB, IC and RHB decreased the risk of liver metastasis and increased the risk of extrahepatic metastasis. The HR was the lowest in patients with CHB (HR: 0.29, 95% CI: 0.12-0.72).

The number, size and surgical resection rate of metastatic lesions are listed in Table 6. The number of liver metastatic lesions was significantly less in study group than in control group with a higher surgical resection rate

Table 5 Synchronous and metachronous metastasis in resolved hepatitis B and control groups *n* (%)

Metastatic sites	RHB group (<i>n</i> = 187)	Control group (<i>n</i> = 966)	<i>P</i> value	HR (95% CI)
Liver			< 0.01	0.63 (0.46-0.85)
Yes	33 (17.6)	272 (28.2)		
No	154 (82.4)	694 (71.8)		
Extrahepatic			< 0.01	1.54 (1.16-2.04)
Yes	49 (26.2)	164 (17.0)		
No	138 (73.8)	802 (83.0)		

RHB: Resolved hepatitis B; HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 6 Clinical features of liver metastatic lesions in two groups *n* (%)

Metastatic lesion	Study group	Control group	<i>P</i> value
Number	47	272	< 0.05
Single	17 (36.2)	73 (26.8)	
Multiple	30 (63.8)	199 (74.2)	
Size (cm)	3.6 ± 2.0	3.9 ± 1.3	NS
Resected	14 (29.8)	43 (15.8)	< 0.05

Size of liver metastatic lesions is expressed as mean ± SD. NS: Not significant.

(*P* < 0.05). No significant difference was found in size of liver metastatic lesions between the two groups.

Survival rate

The 5-year survival rate was 57.0% and 58.2%, respectively, for the patients in two groups. No significant difference was found in OS and DFS rate between the two groups (Figure 1A and B).

DISCUSSION

In the current study, the risk of liver metastasis was significantly lower in study group than in control group (HR: 0.50, 95% CI: 0.38-0.66, *P* < 0.05). A significant difference was found in extrahepatic metastasis rate and no significant difference was found in survival rate between the two groups, suggesting that HBV infection may have a significant effect on liver metastasis of CRC. It was reported that the liver metastasis rate is low in patients with other malignancies due to HBV infection^[7,15].

In this study, the liver metastasis rate of CRC was lower in CHB, IC and RHB subgroups than in control group (*P* < 0.05). CHB was characterized by positive HBeAg while the serum HBV DNA level and normal aminotransferase level were very low or undetectable in IC. RHB results from previous HBV infection without further virological, biochemical or histological evidence of active virus infection or disease^[14,16]. Thus, it is reasonable to postulate that HBV infection with or without virus replication, may affect liver metastasis of CRC. In this study, CHB most significantly decreased the risk of liver metastasis of CRC followed by RHB.

Hepatic resection remains the only curative therapy for liver metastasis of CRC. In this study, the 5-year survival

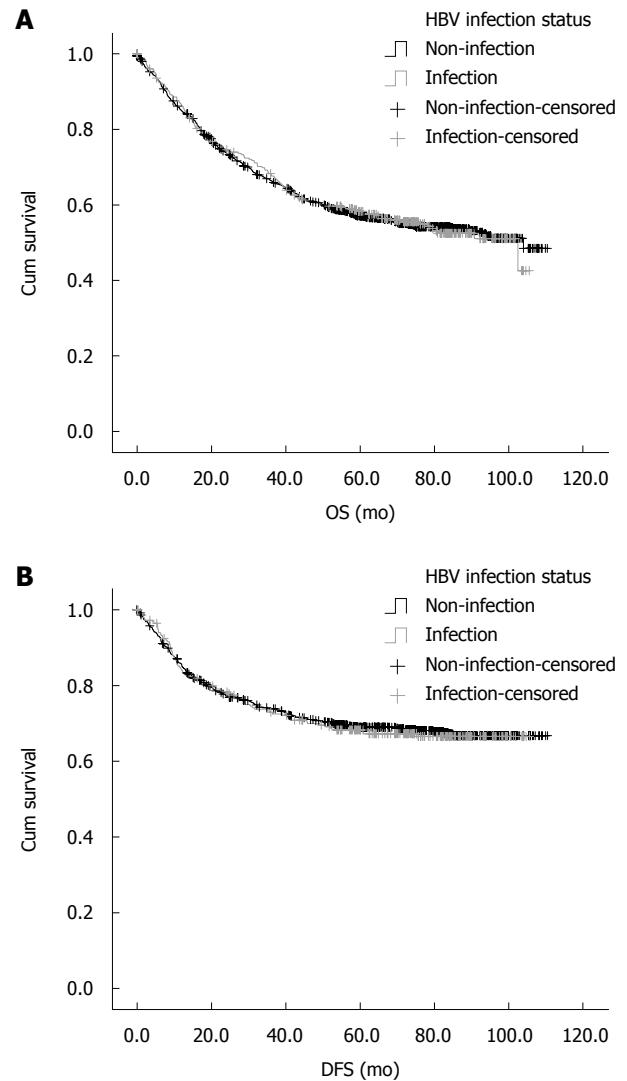


Figure 1 Overall survival rate (A) and disease-free survival rate (B) for patients in two groups after operation. HBV: Hepatitis B virus; OS: Overall survival; DFS: Disease-free survival.

rate of CRC patients was 25%-40% after operation, which is consistent with the reported findings^[17,18]. The number of liver metastatic lesions was much less in study group than in control group, leading to a higher surgical resection rate of liver metastatic lesions, indicating that HBV infection plays an important role in the pathogenesis of liver metastasis of CRC. However, no difference was found in overall survival and disease-free survival rate between the two groups, suggesting that liver metastasis of CRC results from the difference in extrahepatic metastasis.

Whether changes in liver-associated immunity contribute to the impediment of CRC colonization in patients infected with HBV remains unclear. The liver has a rich diversity of innate immune cells, particularly lymphocytes including natural killer cells, which respond to altered expression of self-antigens and lyse neoplastic target cells in the absence of additional activating stimuli^[19]. A large number of phagocytic and antigen-presenting cells including liver sinusoidal endothelial cells, Kupffer cells and dendritic cells play an important role in local innate immunity of the liver^[20]. Furthermore, it was reported

that HBV replication enhances the cytotoxicity of immunocytes during chronic HBV infection. Cytotoxic T lymphocytes (CTL) and Kupffer cells are essential for the immune response during HBV infection. HBV replication activates the specific lytic pathways of cell injury by CTL and Kupffer cells^[21]. A previous study showed that the hepatic microenvironment in patients with HBV-positive metastatic liver cancer can greatly change their gene expression profiles, and the two significant clusters in the profile revealed notable changes-associated with gene products involved in immune function. In fact, over 30% of the genes in these clusters are related to this process^[22]. Another study on tumor and stroma interaction suggested that the propensity of metastatic liver cancer is inherent to the tumor cells and affected by the local environment of metastatic sites^[23].

In conclusion, activation of liver-associated immunity due to HBV infection reduces the incidence of liver metastasis in CRC patients.

ACKNOWLEDGMENTS

The authors thank all medical staff at Department of Colorectal, Gastric and Pancreatic Surgery, Sun Yat-Sen University Cancer Center, for their help.

COMMENTS

Background

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in Western countries. Metastatic liver disease more frequently develops metachronous metastasis following treatment of CRC. It was reported that hepatitis B virus (HBV) infection finally reduces the risk of intrahepatic metastasis in hepatocellular carcinoma (HCC) patients with a higher survival rate and therefore can be considered an important prognostic factor for HCC patients. Rare reports are available on the relation between HBV infection and hepatic metastasis of CRC.

Research frontiers

The authors designed a cohort study to observe the relation between HBV infection and liver metastasis of CRC.

Applications

The major points summarized in the article can be applied in further studies on the correlation between liver metastasis and colorectal cancer.

Peer review

In this manuscript, the authors evaluated the effect of HBV infection on liver metastases in patients with colorectal cancer. Some discussions should be added and survival curves should be reconsidered.

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Clinical significance of K-ras and BRAF mutations in Chinese colorectal cancer patients

Hong Shen, Ying Yuan, Han-Guang Hu, Xian Zhong, Xiao-Xian Ye, Mo-Dan Li, Wei-Jia Fang, Shu Zheng

Hong Shen, Ying Yuan, Han-Guang Hu, Xiao-Xian Ye, Mo-Dan Li, Department of Oncology, Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China

Xian Zhong, Department of Internal Medicine, Bin Jiang Hospital, Hangzhou 310052, Zhejiang Province, China

Wei-Jia Fang, Department of Medical Oncology, First Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310003, Zhejiang Province, China

Shu Zheng, Key Laboratory of Cancer Prevention and Intervention of Ministry of Education, Cancer Institute, Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China

Author contributions: Shen H supervised the study, acquired and analyzed the data, and drafted the manuscript; Yuan Y co-supervised the field activities and designed the analysis strategy; Hu HG and Zhong X reviewed and edited the manuscript; Ye XX, Li MD and Fang WJ interpreted the data; Zheng S designed the study, co-supervised the field activities, and revised manuscript critically for important intellectual content.

Supported by The Department of Education of Zhejiang Province of China, grant No. Y200804314; the Zhejiang Provincial Natural Science Foundation, grant No. R2090353; the Department of Science and Technology of Zhejiang Province, grant No. 2008C33039; and the Chinese Ministry of Health, grant No. N20100148

Correspondence to: Shu Zheng, Professor, Key Laboratory of Cancer Prevention and Intervention of Ministry of Education, Cancer Institute, Second Affiliated Hospital, Zhejiang University College of Medicine, 88 Jiefang Road, Hangzhou 310009, China. zhengshu@zju.edu.cn

Telephone: +86-571-87784501 Fax: +86-571-87214404

Received: November 25, 2010 Revised: January 4, 2011

Accepted: January 11, 2011

Published online: February 14, 2011

METHODS: Genomic DNA was isolated from frozen tissues. Pyrosequencing analysis was conducted to detect mutations in the *K-ras* (codons 12, 13, and 61) and *BRAF* genes (codon 600). Statistical analysis was carried out using SPSS-15.0 software.

RESULTS: Among the 118 colorectal cancer patients, we detected 41 (34.7%) mutations in the *K-ras* gene. Mutation frequencies at codon 12 and codon 13 were 23.7% (28/118) and 10.2% (12/118), respectively. Only one patient harbored a point mutation at codon 61 (0.8%, 1/118). Gender was the only factor that showed an obvious relationship with *K-ras* gene mutation (female 44.7% vs male 28.2%, $P = 0.037$). Other clinicopathological features, such as age, location of the tumor, tumor differentiation, Tumor, Node and Metastases classification, and the Union for International Cancer Control staging, showed no positive relationship with *K-ras* gene mutations. No significant correlation was observed between the presence of K-ras mutations (codons 12, 13, and 61) and the survival of the patients. BRAF mutations were rare, and only two patients (1.7%) harbored a detectable mutation at codon 600.

CONCLUSION: *K-ras* gene mutation is a common event in our 118 Chinese CRC patients, with an obvious relationship with gender. However, it seems not to be an independent prognostic factor in CRC patients. The *BRAF* gene is rarely mutated in Chinese CRC patients.

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Key words: *K-ras*; *BRAF*; Colorectal cancer; Mutation

Peer reviewer: Daniel S Straus, PhD, Professor, Biomedical Sciences Division, University of California, Riverside, CA 9252, United States

Shen H, Yuan Y, Hu HG, Zhong X, Ye XX, Li MD, Fang WJ, Zheng S. Clinical significance of K-ras and BRAF mutations in Chinese colorectal cancer patients. *World J Gastroenterol*

Abstract

AIM: To identify and assess mutations in the *K-ras* and *BRAF* genes in a cohort of Chinese patients with colorectal cancer (CRC) for their association with various clinicopathological parameters and prognosis.

2011; 17(6): 809-816 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i6/809.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i6.809>

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world. In recent years, the morbidity and mortality of colorectal cancer has risen in the Chinese population. The development of CRC is a multistep process, which can arise due to the cumulative effect of mutations in various proto-oncogenes, tumor suppressor genes, and also from epigenetic changes in DNA. Recent evidence suggests that the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase signaling pathway, which mediates cellular responses to growth factors and regulates the elements of the cell cycle, apoptosis and differentiation^[1], plays a critical role in the pathogenesis of colorectal cancer. Both the *K-ras* and *BRAF* genes encode proteins that act in the ERK signaling pathway. The *K-ras* proto-oncogene encodes a 21 kDa RAS protein, a member of a highly conserved family of GTPases involved in signal transduction processes. Mutations in the *K-ras* gene render the protein constitutively active in signaling by eliminating the GTPase activity. More recently, mutations in the *K-ras* gene have proved to be predictors of response to epidermal growth factor receptor-targeted therapies, such as cetuximab and panitumumab, for patients with metastatic colorectal cancer.

In human CRC, mutations in the *K-ras* gene are very frequent (20%-50%), whereas mutations of the *BRAF* gene, a downstream molecule of *K-ras*, occur in only 9%-11% of patients with sporadic diseases. Mutations in the *K-ras* and *BRAF* genes are frequently found to be mutually exclusive in colorectal cancer^[2,3]. Both genes harbor the majority of mutations in distinct hotspots in the *BRAF* gene at codons 463-468^[4] and 600^[3,4], and in the *K-ras* gene at codons 12, 13^[5], and, more infrequently, at codon 61^[6]. Approximately 90% of the activating mutations in the *K-ras* gene are scored at codon 12 (wild-type: GGT) and codon 13 (wild-type: GGC) in exon 1, while only 5% are located at codon 61 (wild-type: CAA) in exon 2^[5,7,8]. Some studies have been conducted about the relationship between *K-ras* gene mutation and various clinicopathological characteristics, but no consistent results were obtained. The prognostic significance of *K-ras* gene mutations is still controversial. As for the *BRAF* gene, very few papers regarding its prognostic significance are available in Western countries or China.

For the detection of mutations in the *K-ras* and *BRAF* genes, various techniques have been described, including temporal temperature gradient electrophoresis^[9], denaturing gradient gel electrophoresis^[10], restriction endonuclease-mediated selective polymerase chain reaction (PCR)^[11], and direct sequencing of a PCR product^[12]. For all non-sequencing methods, it is difficult to independently confirm the existence of any mutations that are identified. In addition, our previous work showed that direct sequenc-

ing of a PCR product was not a sensitive method for the detection of *K-ras* gene mutations^[12]. Pyrosequencing has emerged as a sensitive and rapid sequencing method for single-nucleotide polymorphism (SNP)/mutation analysis, which overcomes the above limitations^[13]. It is a non-electrophoretic, real-time sequencing technic, and a sequence-by-synthesis method that relies on the lumino-metric detection of pyrophosphate released upon nucleotide incorporation *via* a four-enzyme mixture reaction cascade^[14,15]. It can analyze multiple samples in a short time, which makes it attractive for clinical use. So far, few papers have reported the use of pyrosequencing for the detection of *K-ras* gene mutations in colorectal cancer patients. Pyrosequencing studies on the *BRAF* gene are even fewer.

In this paper, we detected mutations in the *K-ras* and *BRAF* genes from 118 Chinese CRC patients using pyrosequencing. Correlations with various clinicopathological characteristics and the prognosis of patients were further analyzed.

MATERIALS AND METHODS

Patients and specimens

Tumor specimens used in this study were obtained from 118 CRC patients who received a radical resection operation in the 2nd Affiliated Hospital of Zhejiang University College of Medicine from February 2001 to January 2005. All patients were followed up by the Cancer Research Institute until September 2010, and the data concerning cancer recurrence and patient survival were collected. Tumor stage was classified according to the 7th edition of the Tumor, Node and Metastases (TNM) classification of the Union for International Cancer Control (UICC) staging. The clinicopathological data of all patients are shown in Table 1. Data and tissue collection was approved by the Ethics Committee of Zhejiang University College of Medicine, following the ethical guidelines of the 1975 Declaration of Helsinki.

DNA preparation and pyrosequencing

Tumor tissues were collected from the Zhejiang University Cancer Institute tissue bank. All the tissue samples were confirmed independently by two gastrointestinal pathologists. Genomic DNA was extracted with the QIAamp DNA Mini-Kit (QIAGEN, Mississauga, ON), according to the manufacturer's recommendations. The primers for the amplification and pyrosequencing assay of *K-ras* and *BRAF* gene are listed in Table 2.

PCR was performed using 100 ng genomic DNA as template. Each mixture contained 10 pmol of each primer. The reactions were performed in 1 × reaction buffer, 0.2 μmol/L dNTPs, 2 mmol/L MgCl₂, and 1.25 U Blend Taq polymerase (ToYoBo) in a total volume of 50 μL. The amplification reactions were as follows: an initial denaturing cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 25 s, and 72°C for 30 s; and a final extension cycle at 72°C for 2 min.

The PCR products were directly subjected to the se-

Table 1 Characteristics of colorectal cancer patients in this study

Terms	n (%)
No. of patients	118
Median age (yr)	61
Gender	
Male	71 (60.2)
Female	47 (39.8)
Colorectal segment	
Cecum	5 (4.2)
Ascending colon	23 (19.5)
Transversal colon	8 (6.8)
Descending colon	5 (4.2)
Sigmoid	25 (21.2)
Rectum	52 (44.1)
UICC stage	
I	18 (15.3)
II	48 (40.7)
II A	32 (27.1)
II B	16 (13.6)
III	37 (31.4)
III A	5 (4.2)
III B	25 (21.2)
III C	7 (5.9)
IV	15 (12.7)

UICC: Union for International Cancer Control.

Table 2 Primers designed for amplification and pyrosequencing assay of *K-ras* and *BRAF* genes

Primer sequence	Product (bp)
<i>K-ras</i> gene Forward: codon 12 5'-GCAGTCAACTGGAATTTTCATG-3' & 13 Reverse: (exon 1) 5'-biotin-GAAACCCAAGGTACATTTCAGA-3' Pyrosequencing assay: 5'-TGIGGTAGTTGGAGCT-3'	431
<i>K-ras</i> gene Forward: codon 61 5'-ATCCAGACTGTGTTTCTCCCTTC-3' (exon 2) Reverse: 5'-biotin-ACTGCTCTAATCCCCAAGAACT-3' Pyrosequencing assay: 5'-TATTCACGACACAGCAGGT-3'	378
<i>BRAF</i> gene Forward: codon 600 5'-ACAAGCCTTCAAAAATGAAGTAG-3' (exon 15) Reverse: 5'-biotin-ATCCAGACAACTGTTCAAACTGA-3' Pyrosequencing assay: 5'-GGTGATTTTGGTCTAACTACA-3'	362

quencing analysis using the pyrosequencing PyroMark ID system (PSQ 96 MA, Biotage AB, Sweden). For pyrosequencing, ssDNA was prepared from 40 μ L biotinylated PCR product using streptavidin-coated sepharose, and 0.5 mmol/L sequencing primer was used for analysis (Table 2). Sequencing was performed with the SNP Reagent Kit (Biotage AB, Sweden) according to the manufacturer's instructions.

Statistical analysis

The Mann-Whitney *t* test, the Kruskal-Wallis test, and Fisher's test were used to evaluate the associations between

Table 3 Mutations of K-ras codons 12, 13, and 61 DNA detected by pyrosequencing assay

	Wild type (AA)	Point mutation (AA)	No. of mutations (%)
K-ras codon 12	GGT (Gly)	AGT (Ser)	2 (1.7)
	GGT (Gly)	GAT (Asp)	16 (13.6)
	GGT (Gly)	GCT (Ala)	2 (1.7)
	GGT (Gly)	GTT (Val)	8 (6.8)
K-ras codon 13	GGC (Gly)	GAC (Asp)	12 (10.2)
K-ras codon 61	CAA (Gln)	CAT (His)	1 (0.8)

AA: Amino acid; Gly: Glycine; Gln: Glutamine; Ser: Serine; Asp: Aspartic acid; Ala: Alanine; Val: Valine; His: Histidine.

the K-ras wild-type/mutation type and the clinicopathological variables of patients [Mann-Whitney *t* test for dichotomous variables (gender, metastasis *vs* no metastasis); Kruskal-Wallis test for no dichotomous variables (age, tumor region, differentiation and TNM stage). Kaplan-Meier survival analysis was performed to evaluate the relationship between K-ras wild-type/mutation type and survival of CRC patients. Calculations were carried out using the SPSS-15.0 software (SPSS Inc., Chicago, IL). *P* value less than 0.05 was regarded as statistically significant. All statistical tests were two-sided.

RESULTS

Mutation characteristics of K-ras gene

A total of 41 mutations of *K-ras* gene were detected in the 118 patients, with a mutation rate of 34.7% (41/118). And 23.7% (28/118) of mutations were at codon 12 and 10.2% (12/118) at codon 13. Only one patient harbored a point mutation at codon 61 (0.8%, 1/118). The mutations in the *K-ras* gene are summarized in Table 3. Figure 1 shows an example of pyrosequencing analysis of mutations located at codon 12, 13, and 61. Compared with the wild-type sequence of codons 12, 13, and 61 (GGT/GGC/CAA), one mutation was detected at codon 12 (Figure 1A) (GGT > GAT), one at codon 13 (Figure 1B) (GGC > GAC), and one at codon 61 (Figure 1A) (CAA > CAT).

The distribution of all the detected mutations is shown in Figure 2. The most frequently observed mutations were G-A transitions (30/41, 73.2%), followed by G-T transversions (8/41, 19.5%), two G-C transversions (2/41, 4.9%), and one A-T transversion (1/41, 2.4%). A total of 28 mutations were detected at codon 12 (wild-type GGT), representing four different mutational types. Two G-A transitions (2/28, 7.1%) were located at the first nucleotide of codon 12, resulting in an amino acid change from glycine (Gly) to serine, while the other base substitutions were all located at the second nucleotide of codon 12. The mutated GAT leading to an amino acid change from Gly to aspartic acid (Asp) (16/28, 57.1%) was the most frequently observed mutation. Eight patients were found to harbor a GTT mutation leading to an amino acid change from Gly to valine (Val) (8/28, 28.6%), while the other two patients harbored a GCT mutation (2/28, 7.1%), changing Gly to alanine. Twelve patients (12/118, 10.2%)

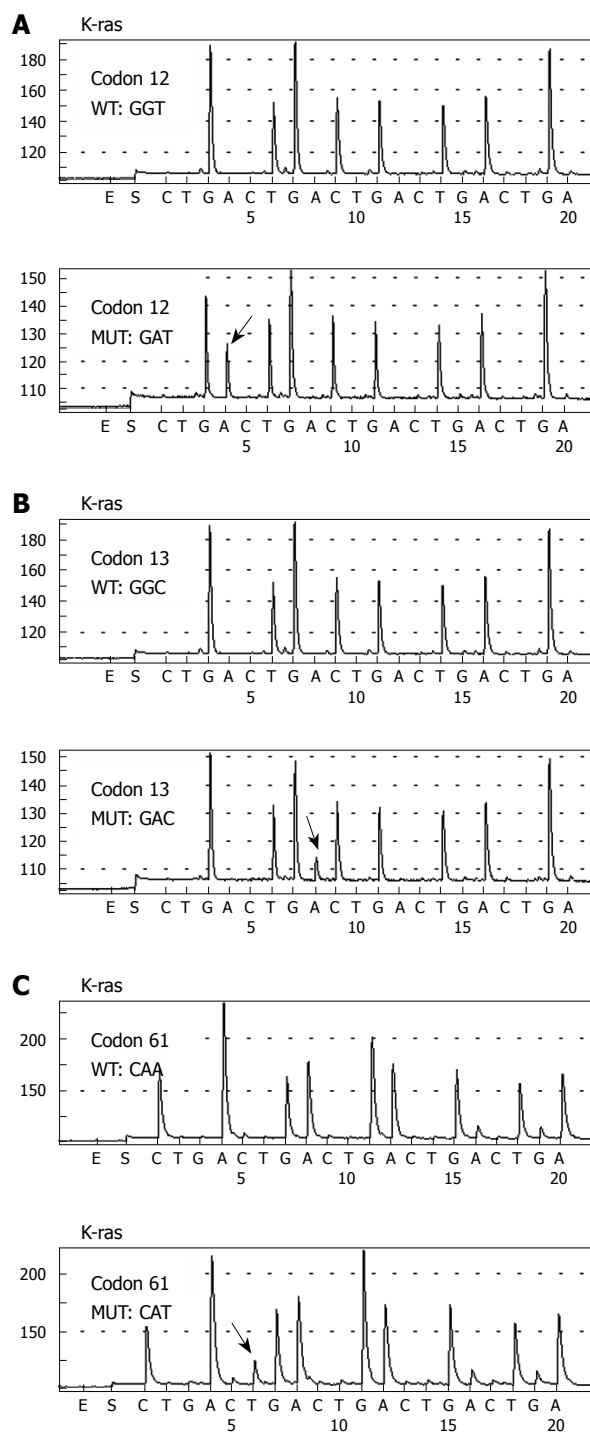


Figure 1 Pyrosequencing analysis of K-ras codons 12, 13, and 61 DNA sequences in colorectal cancer patients. The highlighted arrow shows the nucleotide change at the mutation site.

had detectable mutations at codon 13. The G-A transition was the only mutational type found, resulting in an amino acid change from Gly to Asp. Among all the 118 CRC patients, only one (1/118, 0.8%) had a detectable point mutation (A-T transversion) at codon 61, which resulted in an amino acid change from glutamine to histidine.

Correlation between K-ras gene mutations and clinicopathological features

As shown in Table 4, differences in the categorical vari-

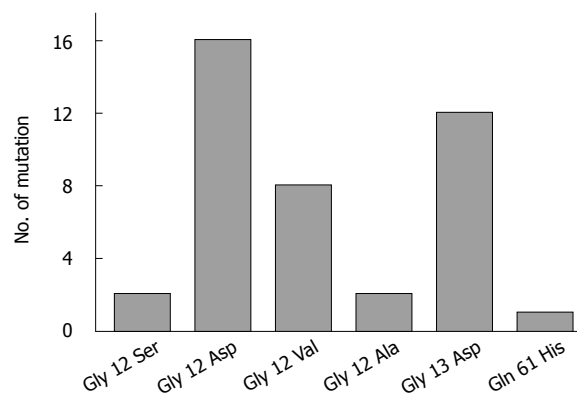


Figure 2 Distribution chart of six different K-ras mutations in 118 colorectal cancer patients. Gly: Glycine; Gln: Glutamine; Ser: Serine; Asp: Aspartic acid; Val: Valine; Ala: Alanine; His: Histidine.

Table 4 Correlation between K-ras mutations and clinicopathological factors in colorectal cancer *n* (%)

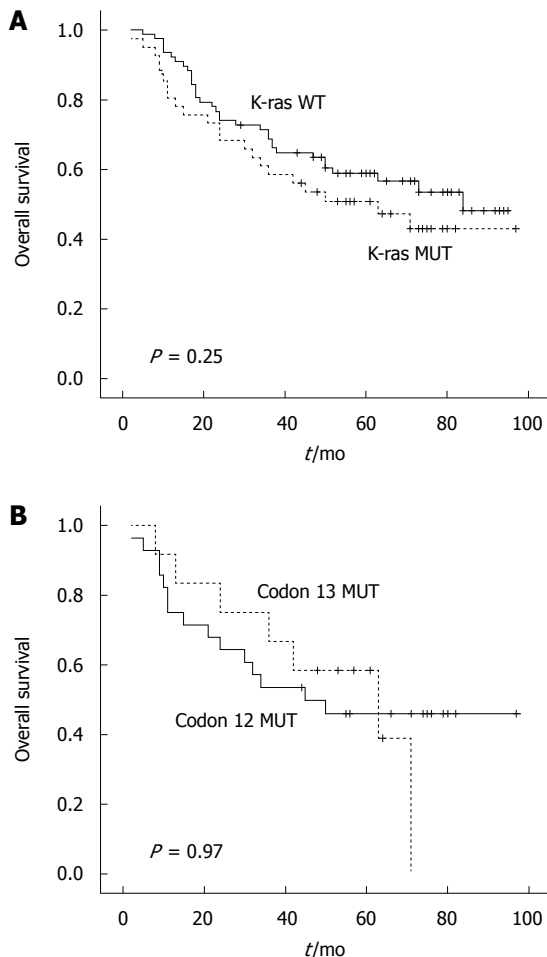
Terms	All	Wild type	Mutation type	P value
No. of patients	118	77 (65.3)	41 (34.7)	
Gender				0.037
Male	71	51 (71.8)	20 (28.2)	
Female	47	26 (55.3)	21 (44.7)	
Median age (yr)	61.0	64.0	60.0	0.728
Males	65.0	65.0	65.5	
Females	60.0	60.5	58.0	
Colorectal segment				0.559
Cecum	5	1 (20.0)	4 (80.0)	
Ascending colon	23	17 (73.9)	6 (26.1)	
Transversal colon	8	4 (50.0)	4 (50.0)	
Descending colon	5	4 (80.0)	1 (20.0)	
Sigmoid	25	16 (64.0)	9 (36.0)	
Rectum	52	35 (67.3)	17 (32.7)	
Differentiation				0.761
Poor	17	12 (70.6)	5 (29.4)	
Moderate	42	25 (59.5)	17 (40.5)	
Well	59	40 (67.8)	19 (32.2)	
UICC classification				0.631
I	18	9 (50.0)	9 (50.0)	
II	48	37 (77.1)	11 (22.9)	
III	37	23 (62.2)	14 (37.8)	
IV	15	8 (53.3)	7 (46.7)	
Bowel wall invasion (pT)				0.120
pT1	2	1 (50.0)	1 (50.0)	
pT2	21	11 (52.4)	10 (47.6)	
pT3	65	43 (66.2)	22 (33.8)	
pT4	30	22 (73.3)	8 (26.7)	
Lymph node metastasis (pN)				0.585
pN0	69	47 (68.1)	22 (31.9)	
pN1-2	49	31 (63.3)	18 (36.7)	
Distant metastasis (pM)				0.301
pM0	103	70 (68.0)	33 (32.0)	
pM1	15	8 (53.3)	7 (46.7)	

UICC: Union for International Cancer Control.

ables, including age, gender, anatomical location of the tumor, tumor differentiation, TNM classification and UICC staging, between patients with and without K-ras mutations were evaluated for significance with χ^2 tests. Gender was the only variable that showed a significant relationship with K-ras gene mutation status. The prevalence of

Table 5 Relationship in K-ras mutation between gender and tumor location

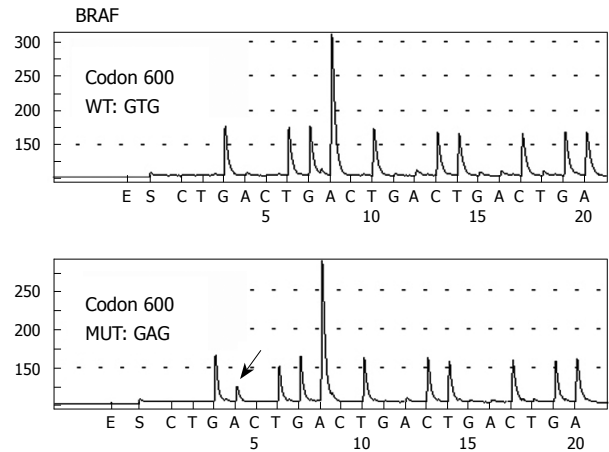
	No. of K-ras mutated patients (%)		Total No. of patients
	Colon	Rectum	
Male	12 (16.9)	8 (11.2)	71
Female	5 (10.6)	16 (34)	47

**Figure 3** Kaplan-Meier survival curve in colorectal cancer patients with regard to K-ras gene codon mutations.

gene mutations was higher in female patients than in male patients (44.7% *vs* 28.2%, $P = 0.037$). The K-ras mutation frequency in the rectum was higher in female than in male patients (34.0% *vs* 11.2%, $P < 0.05$). Male patients had a higher mutation rate in the colon than female patients (16.9% *vs* 10.6%, $P < 0.05$) (Table 5).

Correlation of K-ras gene mutations with patient survival

As shown in Figure 3, patients with the wild-type K-ras gene had a median survival of 84.0 mo, which was a little longer than the patients with a mutated K-ras gene, whose median survival was 63.0 mo. However, this difference was not statistically significant (Figure 3A) ($P = 0.25$). The patients with a mutation at codon 12 and codon 13 had a median survival of 45.0 mo and 63.0 mo, respectively. The patient harboring a mutation at codon 61 had a rather long survival

**Figure 4** Pyrosequencing analysis of BRAF gene codon 600 DNA sequences in colorectal cancer patients. The highlighted arrow shows the nucleotide change at the mutation site.

of 73.0 mo. There were no significant correlations between the presence of K-ras mutations at codons 12, 13 and 61) and patient survival (Figure 3B).

Mutation characteristics of BRAF gene

Of the 118 CRC patients analyzed, only two patients (1.7%, 2/118) harbored a detectable mutation at codon 600 in exon 15 of the BRAF gene (Figure 4). Both mutations were T > A transitions (GTG > GAG) at codon 600, which resulted in an amino acid change from Val to glutamic acid (Glu). Both patients had a wild-type K-ras gene. One patient was a 70-year-old man with a moderately differentiated adenocarcinoma in the ascending colon at pathological stage of T3N1M0. The patient survived for 13 mo after the operation, but died of other diseases without evidence of tumor recurrence and metastasis. The other patient was a 56-year-old man. He was operated on for a well-differentiated adenocarcinoma in the transverse colon also at pathological stage of T3N1M0. Sixty-three months after operation, the patient died of tumor recurrence.

DISCUSSION

In this study, we detected various mutations of the K-ras and BRAF gene in 118 Chinese CRC patients using pyrosequencing. Mutations in the K-ras gene occurred very frequently (20%-50%) in CRC. It was reported in Western countries that approximately 90% of the activating K-ras mutations were found at codon 12 and codon 13 of exon 1, and about 5% at codon 61 located in exon 2. In domestic reports, with a limited number of samples, there was a similar mutation rate at codon 12 and 13, and a rather lower rate at codon 61 (0%-4.8%).

In our previous studies published in the 1990s, K-ras gene mutations were detected in 12 out of 35 (12/35, 34.3%) CRC patients by the PCR-RFLP method^[14]. Eleven out of the 12 detected mutations (11/35, 31.4%) were located at codon 12, while the other one was at codon 61 (1/35, 2.9%). None of the patients had a mutation at codon 13. Mutations were also found in the pericancer-

ous mucosa in some cases^[17], indicating that the *K-ras* gene might play an important role in the early stage of colorectal carcinogenesis. In the present study, a similar mutation rate was observed. Among the 118 patients in this study, the rates of mutated (41/118, 34.7%) and non-mutated (77/118, 65.3%) *K-ras* genes were similar to those reported by other countries. The *K-ras* mutation rates at codon 12, codon 13 and codon 61 were 23.7% (28/118), 10.2% (12/118) and 0.8% (1/118), respectively. Among all 41 mutations detected in *K-ras* gene in this study, 68.3% (28/41) were located at codon 12, 29.3% (12/41) at codon 13, and 2.4% (1/41) at codon 61. Similar to the previously reported data, G-A transitions were the most frequently found type of *K-ras* gene mutations in our study, followed by G-T transversions. Among the four different mutations detected at codon 12, Gly12Asp was the most frequent point mutation, accounting for about 40% of all mutations detected in the *K-ras* gene and 60% of all mutations at codon 12. All 12 point mutations detected at codon 13 were Gly13Asp. This base substitution accounted for 30% of all mutations detected in the *K-ras* gene. The distribution of the six different mutations (Figure 1) among the mutated patients was in concordance with the published data^[18]. These point mutations resulting in amino acid substitution would activate RAS proteins, produce an alteration in the transduction of signals in the RAS pathway and ultimately lead to increased mitogenic signaling.

It is widely accepted that mutations in the *K-ras* gene are early events in colorectal carcinogenesis. As described by Vogelstein *et al.*^[5], a *K-ras* gene mutation might happen in the progression from adenoma to carcinoma. Some studies have investigated the relationship between *K-ras* mutation and various clinicopathological parameters, but the results remain controversial. For example, it was reported by Zlobec *et al.*^[19] that *K-ras* mutations were associated with neither clinicopathological parameters, such as gender, age, tumor location, histological type, tumor T and N stage, tumor grade and vascular invasion nor survival time of patients. Naguib *et al.*^[20] reported a mutation rate of 22% in the *K-ras* gene (codon 12 and 13), and a positive relationship with more advanced Dukes' stage and microsatellite stable status. In the present study, we did not find any significant correlations between the presence of *K-ras* mutations at codons 12, 13, and 61 or mutation type and various clinicopathological features, such as age, anatomical location of the tumor, tumor differentiation, TNM classification, and UICC staging. Gender was the only variable that showed an obvious relationship with *K-ras* gene mutation, and suggested that female patients had a higher prevalence of gene mutation than male patients (44.7% *vs* 28.2%, $P = 0.037$). Breivik *et al.*^[7] demonstrated that *K-ras* mutations were much less frequent in colon samples from male patients compared with female patients at codon 12 and 13 in Western populations. However, we found that male patients had a higher mutation rate in colon samples than female patients in the Chinese population (16.9% *vs* 10.6%, $P < 0.05$). Ethnicity, environment, and lifestyle differences may explain the difference in *K-ras* mutation frequency in the colon between Chinese

and Western population. However, a larger population of Chinese patients is needed to confirm our findings.

Some studies have indicated the importance of *K-ras* alterations in predicting long-term outcome, while others have failed to show such a relationship. The collaborative RASCAL study reported a correlation between the specific *K-ras* mutation Gly12Val and poor prognosis, especially in Dukes' C CRC patients^[21]. The Gly12Val mutation at codon 12 reported by Al-Mulla *et al.*^[22] and the G > A mutation at codon 13 reported by Samowitz *et al.*^[18] both might be related to the poor survival of the patients. On the other hand, Tortola *et al.*^[23] and Dix *et al.*^[24] failed to prove a positive relationship between *K-ras* mutation and shorter survival. In our study, prognostic analysis for *K-ras* mutations showed that none of the *K-ras* mutations were predictive of patient survival. Patients with a wild-type *K-ras* gene had a median survival of 84.0 mo, which was slightly longer than patients with a mutated *K-ras* gene (63.0 mo); however, this difference was not statistically significant ($P = 0.25$). There was also no significant difference in survival between patients harboring a mutation at codon 12 and those at codon 13 (45.0 mo *vs* 63.0 mo, $P = 0.97$). The shorter survival in the patients reported by Al-Mulla *et al.*^[22] (Gly12Val mutations at codon 12) and by Samowitz *et al.*^[18] (G > A mutation at codon 13), was not observed in our study. A further study with a larger number of samples will hopefully confirm the results in this study.

The *K-ras*/BRAF/ERK signaling pathway plays an important role in colorectal carcinogenesis. Encoding a downstream molecule of *K-ras*, the *BRAF* gene (codon 600) is mutated in 12%-15.6% (45/374) of colorectal carcinomas^[19,20]. It was reported by Zlobec *et al.*^[19] that *BRAF* gene mutations are strongly associated with right-sided tumor location, higher tumor grade, absence of peritumoral lymphocytic inflammation, and microsatellite instability (MSI-H)^[19]. It was also reported that a mutated *BRAF* gene is an adverse prognostic factor in right-sided colon cancer patients independent of MSI status, and in patients with lymph node-negative disease^[19]. More recently, Richman^[25] showed that mutations in either KRAS or BRAF are factors for poor prognosis and the overall survival (OS), and have minimal impact on progression-free survival (PFS). The mutation status of either gene does not affect the impact of irinotecan or oxaliplatin on PFS or OS. To our knowledge, this is the first paper reporting mutations in the *BRAF* gene in a large population of Chinese CRC patients. In our study, only two mutations (1.7%, 2/118) at codon 600 in exon 15 of the *BRAF* gene were detected in 118 Chinese CRC patients. Both patients had a wild-type *K-ras* gene. The mutation rate was very low compared with that reported in Western studies. Both mutations were T to A transversions (GTG > GAG), resulting in an amino acid change from Val to Glu. Both patients were male, with a Dukes' C (T3N1M0) carcinoma at the right-side of the colon. Due to the limited mutations detected, it was impossible to analyze the correlation of *BRAF* gene mutations with various clinicopathological features or prognosis. There might be a few explanations for the low incidence of *BRAF* gene mutations in our study. First, we only ana-

lyzed mutations at codon 600 in exon 15 of the *BRAF* gene. Therefore, mutations at other sites (e.g. codons 463-468) might affect the result. Second, ethnic differences might exist in CRC between Chinese and Western populations. Liao *et al.*^[26] found that the *BRAF* mutation frequency was only 4.9% in 61 Chinese colorectal tissues, which is also lower than that in Western populations. Wójcik *et al.*^[27] screened for mutations in exons 11 and 15 of the *BRAF* gene in 163 resected adenocarcinomas in a Polish population and only six (3.7%) tumors had a missense point mutation (G469A, D594G, G596R, K601N, and two V600E). Future studies containing a larger number of Chinese samples are expected to further clarify the result.

In conclusion, the mutation of the *K-ras* gene was a common event in our 118 Chinese CRC patients, with an obvious relationship with gender. Female patients had a higher prevalence of gene mutation than male patients. *K-ras* gene mutation seemed not to be an independent prognostic factor in CRC patients. The *BRAF* gene was rarely mutated in Chinese CRC patients.

ACKNOWLEDGMENTS

The authors are grateful to Hang Zhang for her kind help in collecting the tumor samples and for the clinical information concerning the colorectal cancer patients. The authors would like to thank Wei-Ting Ge for his help with the DNA sequencing data analysis.

COMMENTS

Background

In recent years, the morbidity and mortality of colorectal cancer (CRC) has risen in the Chinese population. The *K-ras* and *BRAF* genes encode proteins that act in the extracellular signal-regulated kinase signaling pathway, which mediates cellular responses to growth factors and regulates the elements of the cell cycle, apoptosis and differentiation. Both *K-ras* and *BRAF* are prone to mutations in sporadic CRC. Mutations in *K-ras* could lead to constitutive activation of this pathway, resulting in cancer progression. Several recent studies have shown a strong correlation between *K-ras* and *BRAF* mutations and response to panitumumab and cetuximab.

Research frontiers

Results were in consistent among studies of the relationship between *K-ras* gene mutations and various clinicopathological characteristics. The prognostic significance of *K-ras* gene mutations is also controversial. With regard to the prognostic significance of the *BRAF* gene, less information is available in both Western and Chinese populations.

Innovations and breakthroughs

Recent reports have highlighted the importance of *K-ras* and *BRAF* gene mutations, the clinicopathological characteristics of CRC and its response to epidermal growth factor receptor-targeted therapies. Pyrosequencing is a powerful, sensitive and rapid sequencing method for single-nucleotide polymorphism (SNP)/mutation analysis. This is the first study to report mutations of *K-ras* and *BRAF* genes in a large population of Chinese CRC patients using pyrosequencing.

Applications

Using pyrosequencing technology, the authors found that *K-ras* gene mutations were common in Chinese CRC patients, with an obvious relationship with gender. The *BRAF* gene was rarely mutated in Chinese CRC patients.

Terminology

Pyrosequencing: Pyrosequencing is a method of determining the order of nucleotides in DNA based on the "sequencing by synthesis" principle. It relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. It is a powerful, sensitive and rapid sequencing method and can be used for DNA SNP/mutation analysis.

Peer review

This paper presents new results on the frequency of *K-ras* and *BRAF* mutations in colorectal carcinomas of Chinese patients.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-7375, United States

Giuliana Decorti, MD, PhD, Department of Life Sciences, University of Trieste, Via L. Giorgieri n° 7, Trieste 34127, Italy

Bernardo Frider, Dr., MD, Professor, Department of Hepatology, Hospital General de Agudos Cosme Argerich, Alte Brown 240, Buenos Aires 1155, Argentina

Ralph Graeser, PhD, Group Leader, Molecular and Cellular Biology, ProQinase GmbH, Breisacher Str. 117, Freiburg, 79106, Germany

Ki-Baik Hahm, MD, PhD, Professor, Gachon Graduate School of Medicine, Department of Gastroenterology, Lee Gil Ya Cancer and Diabetes Institute, Lab of Translational Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon, 406-840, South Korea

Ming-Chih Hou, MD, Professor of Medicine and Director, Department of Internal Medicine, Natinal Yang-Ming University, Taipei 11217, Taiwan, China

Selin Kapan, Dr., Associate Professor of General Surgery, Dr. Sadi Konuk Training and Research Hospital, Department of General Surgery, Kucukcekmece, Istanbul 34150, Turkey

Serdar Karakose, Dr., Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Kemal Kismet, MD, Dr., 4th General Surgery Department, Ankara Training and Research Hospital, Ankara, 06430, Turkey

Weekitt Kittisupamongkol, MD, Hua Chiew Hospital, 665 Bumrungruang Road, Bangkok 10100, Thailand

Michael Kremer, MD, Skipper Bowles Center for Alcohol Studies, CB# 7178, 3011 Thurston-Bowles Building, University of North Carolina, Chapel Hill, NC 27599, United States

Michelle Lai, MD, MPH, Instructor in Medicine, Harvard University, Department of Medicine, Division of Gastroenterology/Hepatology, Beth Israel Deaconess Medical Center, 110 Francis Street, Suite 4A, Boston, MA 02215, United States

Paulino Martínez Hernández Magro, Dr., Department of Colon and Rectal Surgery, Hospital San José de Celaya, Eje Vial Norponiente No 200-509, Colonia Villas de la Hacienda, 38010 Celaya, México

Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

Sebastian Mueller, MD, PhD, Professor of Medicine, Department of Internal Medicine, Salem Medical Center, and Center for Alcohol Research, University of Heidelberg, Zeppelinstraße 11 - 33, Heidelberg, 69121, Germany

Shotaro Nakamura, MD, Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Dietmar Öfner, MD, MAS, MSc, Professor, Head, Department of Surgery, Paracelsus Private Medical University Salzburg, Müllner Hauptstrasse 48, Salzburg, 5020, Austria



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 04-05, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 07-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYs, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclu-

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

CSSN

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by

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SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights

When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

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Title page

Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, e.g. Telephone: +86-10-59080039 Fax: +86-10-85381893

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Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67 , $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

Instructions to authors

uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:..., B:..., C:..., D:..., E:..., F:..., G: ...*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. ^a $P < 0.05$, ^b $P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, ^c $P < 0.05$ and ^d $P < 0.01$ are used. A third series of P values can be expressed as ^e $P < 0.05$ and ^f $P < 0.01$. Other notes in tables or under illustrations should be expressed as ¹F, ²F, ³F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability^[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references^[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Please provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at <http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed> and <http://www.crossref.org/SimpleTextQuery/>, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date,

volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

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Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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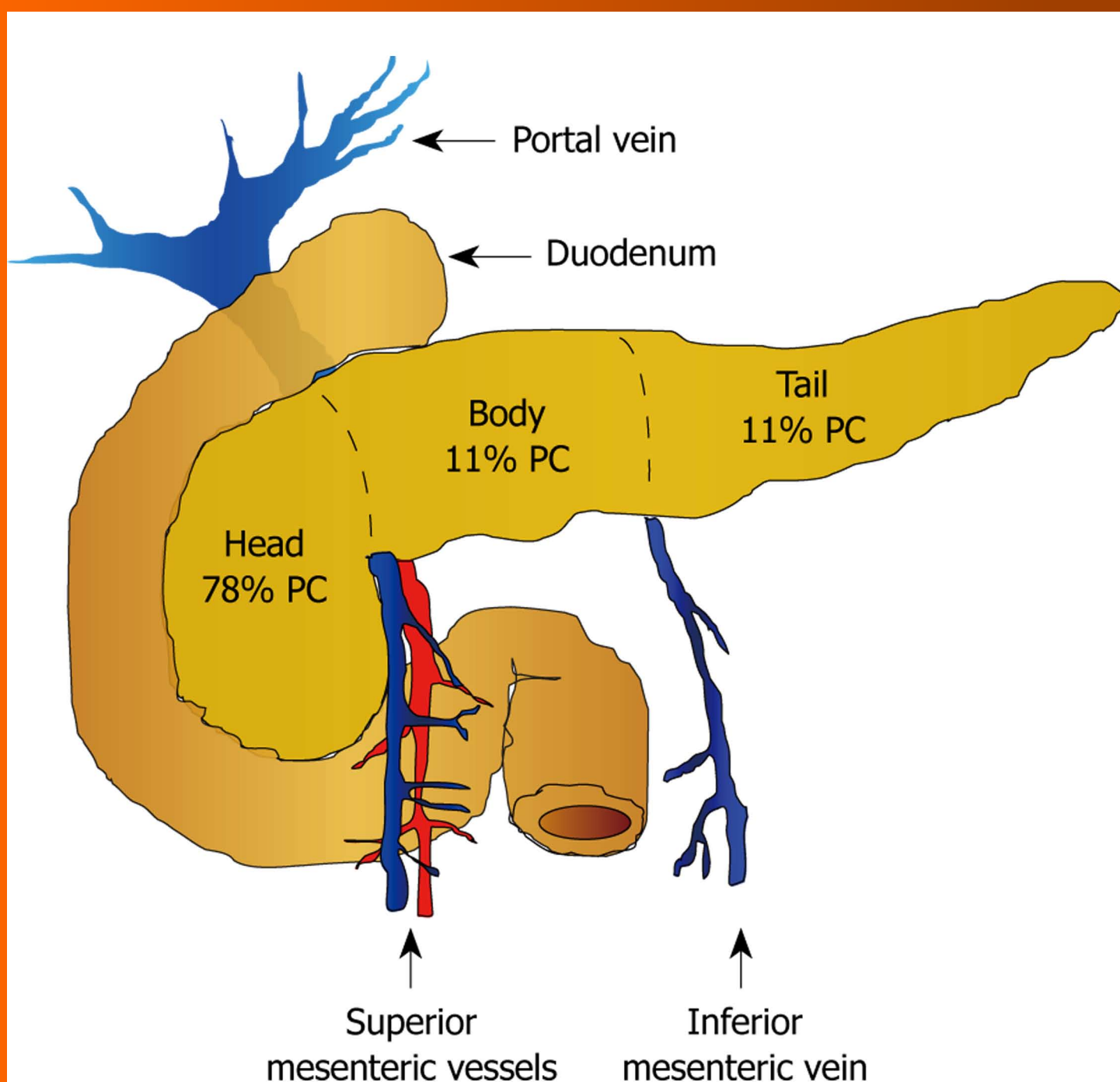
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World J Gastroenterol 2011; 17(7): 867-897
<http://www.wjgnet.com/1007-9327/full/v17/i7/867.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
February 21, 2011

SERIAL PUBLICATION NUMBER
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

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Fax: +86-10-8538-1893
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MicroRNAs in pancreatic ductal adenocarcinoma

Jong Y Park, James Helm, Domenico Coppola, Donghwa Kim, Mokenge Malafa, Seung Joon Kim

Jong Y Park, Division of Cancer Prevention and Control, Moffitt Cancer Center, FL 33612, United States
 James Helm, Mokenge Malafa, Gastrointestinal Tumor Program, Moffitt Cancer Center, FL 33612, United States
 Domenico Coppola, Department of Anatomic Pathology, Moffitt Cancer Center, FL 33612, United States
 Donghwa Kim, Department of Molecular Oncology, Moffitt Cancer Center, FL 33612, United States
 Seung Joon Kim, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, Seoul 137-040, South Korea

Author contributions: Park JY drafted the initial concept, wrote, reviewed and finalized the manuscript; Helm J provided clinical information, participated in writing, and revised the manuscript; Coppola D provided clinical information, participated in writing, and revised the manuscript; Kim D provided valuable advice for study design and drew the figure; Malafa M provided clinical information, participated in writing, and revised the manuscript; Kim SJ designed the manuscript format, collected the references and wrote the manuscript.

Supported by Moffitt Faculty Support Fund

Correspondence to: Seung Joon Kim, MD, PhD, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, Seoul 137-040, South Korea. cmcksj@catholic.ac.kr

Telephone: +82-2-22586063 Fax: +82-2-5993589

Received: August 14, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: February 21, 2011

lated miRNA expression is involved in carcinogenesis at many sites, including the pancreas. Aberrant expression of miRNAs may upregulate the expression of oncogenes or downregulate the expression of tumor suppressor genes, as well as play a role in other mechanisms of carcinogenesis. The purpose of this review is to summarize our knowledge of deregulated miRNA expression in pancreatic cancer and discuss the implication for potential translation of this knowledge into clinical practice.

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Key words: MicroRNAs; Pancreatic cancer

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Park JY, Helm J, Coppola D, Kim D, Malafa M, Kim SJ. MicroRNAs in pancreatic ductal adenocarcinoma. *World J Gastroenterol* 2011; 17(7): 817-827 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/817.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.817>

Abstract

Ductal adenocarcinoma of the pancreas is a lethal cancer for which the only chance of long-term survival belongs to the patient with localized disease in whom a potentially curative resection can be done. Therefore, biomarkers for early detection and new therapeutic strategies are urgently needed. miRNAs are a recently discovered class of small endogenous non-coding RNAs of about 22 nucleotides that have gained attention for their role in downregulation of mRNA expression at the post-transcriptional level. miRNAs regulate proteins involved in critical cellular processes such as differentiation, proliferation, and apoptosis. Evidence suggests that deregulated

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States, with 36 800 estimated deaths in 2010, with the great majority being due to ductal adenocarcinomas^[1]. Due to the asymptomatic onset of pancreatic cancer, most patients are in advanced or metastatic condition at the time of diagnosis, resulting in poor prognosis. Most patients found to have pancreatic cancer die within 12 mo, and few survive 5 years after diagnosis. The poor prognosis of these patients is due to its late clinical presentation with symptoms, early and aggressive local invasion, and high metastatic potential^[2]. Advances in chemo-radiation therapy have been slow over the last few decades, and the overall prognosis in pancreatic cancer has remained essentially unchanged. The only chance of long-term survival with pancreatic adenocarcinoma belongs to

patients with localized disease in whom a potentially curative resection can be performed. Earlier diagnosis and better treatments are urgently needed to improve the survival rate of pancreatic cancer.

Histologically, the pancreas is divided largely into the exocrine and endocrine pancreas, the former consists of ducts and acini, and the latter constitutes the islets that have a hormone secretory function. The most common type of pancreatic cancer, representing about 85% of all pancreatic cancer types^[3], arises from the epithelial lining of the exocrine pancreatic duct. Therefore, in this review, we mainly focus on miRNA expression in pancreatic ductal adenocarcinoma (PDAC).

Pancreatic cancer originates from the sequential accumulation of multiple genetic alterations^[4]. In the past several decades, significant progress in the identification and characterization of cancer-related gene abnormalities has been made. However, this progress has not yet been effectively translated into new reliable biomarkers that lead to the earlier diagnosis or more effective treatment of this deadly disease. Specific miRNAs affecting tumor suppressor genes or oncogenes may be critical biomarkers that lead to early detection, or potential drug targets for pancreatic cancer.

Although regulation of oncogenes and tumor suppressor genes, by genetic and epigenetic changes has been regarded as being important in the development of pancreatic cancer^[5-8], the exact molecular mechanisms of carcinogenesis and of pancreatic cancer progression remain unknown. Gene silencing is frequently caused by epigenetic changes, such as DNA methylation or altered miRNA expression rather than by genetic events such as mutation or deletion. miRNA binding at the 3' untranslated region (UTR) in tumor suppressor genes is an epigenetic change that may contribute to carcinogenesis and cancer progression. Although relatively few genetic mutations have been identified in PDAC, aberrant miRNA expression has been found in both pancreatic tumor tissues and cell lines.

BIOGENESIS, FUNCTION AND TARGETS OF miRNAs

miRNAs are about 22-nucleotide non-protein-coding RNA molecules that regulate gene function in various gene silencing pathways. These molecules are phylogenetically conserved and play important roles in cell survival, proliferation, differentiation, apoptosis and angiogenesis^[9,10]. miRNA expression patterns differ, depending upon cell, tissue, and disease types, and changes in these expression patterns have been implicated as an important player in carcinogenesis.

The miRNA, lin-4, was first discovered in 1993 as a small non-coding RNA that regulates *Caenorhabditis elegans* development by negative regulation of lin-14 protein expression^[11]. In 2000, the second miRNA, let-7, was identified from *C. elegans* and confirmed as a 21-nucleotide small RNA^[12]. Since the discovery of lin-4 and let-7, many more miRNAs have been identified using various experimental

and computational methods^[13]. In the most recent database (miRBase 15 release), over 15000 mature miRNAs are identified in 133 species^[14]. Although they do not encode proteins, miRNAs are transcribed by RNA polymerase II as independent units in the nucleus (Figure 1). The primary transcript (pri-miRNA) is processed by the nuclear RNase III Drosha and its cofactor DGCR8/Pasha to generate precursor miRNA (pre-miRNA), a 60-70-nucleotide RNA that has a stem loop structure^[15-17]. Pre-miRNA is rapidly exported to the cytoplasm by exportin 5 in a Ran-GTP-dependent manner, where it is further processed by a second RNase III, dicer, which cuts off the terminal loop and generates a mature about 22-nucleotide miRNA. Mature miRNA is initially part of an imperfect double-stranded RNA duplex called miRNA/miRNA*. This double-stranded RNA duplex binds to a protein (Argonaute 2) as a part of the RNA induced silencing complex (RISC), while the strand of the duplex that is complementary miRNA* is released. The RISC, containing its miRNA, binds to the target mRNA and triggers either mRNA degradation or inhibition of translation, depending on the degree of complementarity between miRNA and its target^[18-21].

Each miRNA regulates multiple target genes. In fact, bioinformatics predict that miRNAs may regulate about 50% of all human genes^[22]. Therefore, precise identification of miRNA targets is critical to advance our understanding of the role of miRNA regulation in carcinogenesis. Accurate identification of physiologically active miRNA targets is now a considerable impediment to the functional characterization of individual miRNAs.

miRNAs negatively regulate their target mRNAs primarily through base-pairing interactions, which leads to either mRNA degradation or translational inhibition depending upon the degree of match between the "seed sequence" (positions 2-7 at the 5' side) of miRNA and 3'UTR of mRNA (Figure 1). When the seed sequence perfectly or partially matches with target 3' UTR of mRNA, then it may lead to degradation of the mRNA or inhibit translation^[18-21]. Based upon publicly available algorithms, each miRNA has several hundred potential target mRNAs. Recent reports have further indicated that secondary structures of mRNA contribute to target recognition sites, due to the fact that there is energetic cost to free base-pairing interactions for accessible targets^[23-25]. Kertesz *et al.*^[26] have shown that target site accessibility is as important as sequence match in the seed sequence region, and that effective miRNA binding requires unpairing of local regions that flank the target, as well as that the target region is unpaired in thermodynamic equilibrium. Thus, simultaneous profiling of miRNA and mRNA, as well as protein expression, has recently been shown to be a timely strategy to achieve the required precision in the identification of functional miRNA targets^[27-30].

In summary, miRNAs regulate their targets by direct mRNA cleavage or translational inhibition. miRNAs are coded by genes and are transcribed by RNA polymerase II. They have their own regulatory elements and appear as transcriptional units containing either unique or multiple

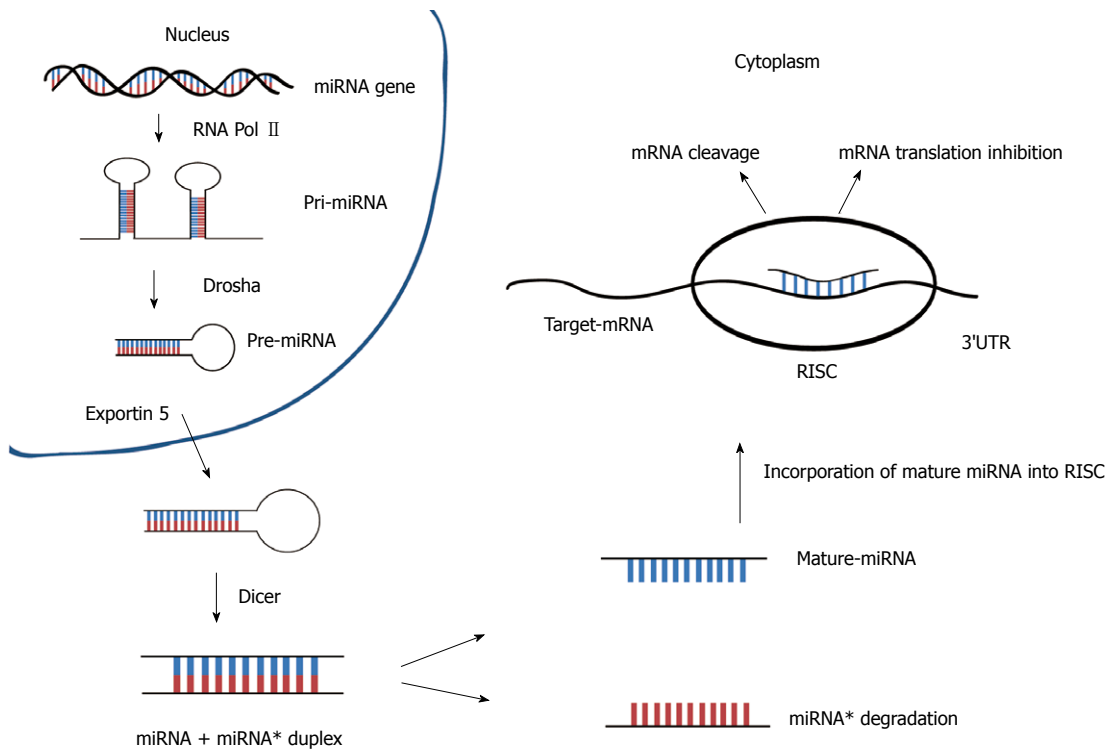


Figure 1 Schematic representation of miRNA biogenesis. miRNA genes are transcribed by RNA polymerase II (RNA pol II) into long transcripts called primary miRNAs (pri-miRNA) that contain multiple stem-loop/hairpin structures as independent units in the nucleus. pri-miRNA is processed by the nuclear RNase III Drosha and its cofactor DGCR8/Pasha to generate precursor miRNA (pre-miRNA). The pre-miRNA is rapidly exported to the cytoplasm by exportin 5, where it is further processed by a second RNase III, Dicer, that cuts off the terminal loop and generates a mature about 22-nucleotide miRNA. The mature miRNA is an imperfect double-stranded RNA duplex called miRNA/miRNA*. The double-stranded RNA duplex binds to a protein (Argonaute 2) as a part of the RNA induced silencing complex (RISC), while one of the strands of the duplex, which is complementary miRNA*, is released. The RISC, which contains its miRNA, binds to the target mRNA and triggers either mRNA degradation or inhibition of translation, depending on the degree of complementarity between miRNA and its target.

miRNAs (polycistronic). Circumstantial evidence linking miRNAs and carcinogenesis has been observed in over 50% of miRNA genes, which are located within regions of loss of heterozygosity, amplification, fragile sites, viral integration sites, and other cancer-associated genomic regions. Recent high-throughput methodologies have shown deregulated miRNA expression in an increasing number of human cancers, including pancreatic cancer. Differences in miRNA expression patterns have been found to distinguish tumors of different developmental origin, even better than traditional mRNA expression profiling^[31].

miRNA AND HUMAN CANCER

The first evidence of miRNA involvement in human cancer came from a study that characterized chromosome 13q14 in chronic lymphocytic leukemia (CLL)^[32]. Calin *et al.*^[32] have shown that miR-15 and miR-16 are deleted or downregulated in about 70% of CLL cases. The tumor suppressive role of miR-15a and miR-16-1 has been supported further by the discovery that expression of both miRNAs inversely correlates with expression of the anti-apoptotic BCL2 protein^[33]. BCL2 expression is inhibited by miR-15a and miR-16-1 and these repressions induce apoptosis in leukemic cells. These data suggest a model whereby somatic deletions of miR-15a and miR-16-1 aid leukemogenesis by allowing tumors to escape apoptosis.

Since this first report of aberrant miRNA expression in CLL, deregulation of a number of miRNAs has been found in other human cancers. While some miRNAs, including miR-125b and miR-145 in breast cancer, and let-7 in lung cancer, are reduced, others such as miR-21 and miR-155 in breast cancer, miR-155 in lung cancer, the precursor of miR-155 in Burkitt lymphoma, miR-17-92 cluster and miR-155 in B-cell lymphoma, are overexpressed^[31,34-39]. These studies also have shown that miRNA expression signatures correlate well with specific clinical cancer characteristics, and could be used to differentiate normal and cancerous tissues, as well as subtypes of malignancy^[40-43].

Deregulation of miRNA in cancer could be caused by: (1) chromosomal regional gain, loss or translocation; (2) aberrant expression and activation of transcriptional factors; (3) epigenetic alterations; and (4) changes in miRNA processing^[44]. As described above, the association between chromosomal abnormality and miRNA expression in CLL is due to downregulation of the miR16-1/15a cluster in chromosome 13q14.3^[32]. In contrast, upregulation of miR-155 in tumor appears to be due to transcriptional regulation and aberrant miRNA processing^[36,45]. miR-155 is encoded in non-coding DNA known as BIC (B-cell integration cluster), located at chromosome 21q21.3, where neither amplification nor loss of heterozygosity is observed. Several studies have shown that miR-155 is in-

Table 1 miRNA deregulation in human pancreatic cancer

miRNA	Lee <i>et al</i> ^[49]	Szafranska <i>et al</i> ^[50]	Bloomston <i>et al</i> ^[51]	Zhang <i>et al</i> ^[52]	Other	Outcome
let-7					↓ ^[53]	
let-7d	↑ ¹					
let-7f-1	↑					
miR-10a			↑		↑ ^[54]	
miR-10b			↑			
miR-15b	↑			↑		
miR-16-1	↑					
miR-18a		↑				
miR-21	↑		↑		↑ ^[55, 56]	Poor ^[55]
miR-23a			↑			
miR-23b			↑			
miR-24-1,2	↑					
miR-29c		↓				
miR-31		↑				
miR-92-1	↑					
miR-93		↑				
miR-95				↑		
miR-96		↓				
miR-99			↑			
miR-100	↑		↑			
miR-100-1/2			↑			
miR-103-2			↑			
miR-107	↑		↑			
miR-125a			↑			
miR-125b-1	↑		↑			
miR-130b		↓				
miR-139	↓					
miR-141		↓				
miR-142-P	↓					
miR-143		↑	↑			
miR-145		↑				
miR-146			↑			
miR-146a		↑				
miR-148a		↓	↓			
miR-148b		↓	↓			
miR-150		↑				
miR-155	↑	↑	↑			Poor ^[57]
miR-181a	↑		↑			
miR-181b			↑			
miR-181b-1			↑			
miR-181b-2			↑			
miR-181c	↑		↑			
miR-181d			↑			
miR-186				↑		
miR-190				↑		
miR-196a		↑		↑		miR-196a-2; Poor ^[51]
miR-196b		↑				
miR-199a-1			↑			
miR-199a-2			↑			
miR-200b				↑		
miR-203		↑				Poor ^[57]
miR-205		↑	↑			
miR-210		↑	↑			Poor ^[57]
miR-212	↑					
miR-213			↑			
miR-216		↓				
miR-217		↓				
miR-220			↑			
miR-221	↑	↑	↑	↑		
miR-222		↑	↑	↑		Poor ^[57]
miR-223		↑	↑			
miR-224		↑				
miR-301	↑					
miR-345	↓					

miR-375		↓	↓
miR-376a	↑		
miR-424	↑		

¹Arrows indicate increased (↑) or decreased (↓) expression of the specified miRNA.

duced at the transcriptional level by transforming growth factor β /Smad, nuclear factor- κ B and activator protein-1 family transcription factors through direct interaction with the miR-155/BIC promoter^[46-48]. Further studies have shown that miR-155 processing also regulates mature miR-155 expression levels^[36,45], suggesting that overexpression of miR-155 in cancer is due to transcriptional activation and miRNA processing.

miRNA EXPRESSION PROFILE IN NORMAL PANCREATIC TISSUE AND PANCREATIC TUMOR

miRNA expression profiles in pancreatic tumor tissues are different from those identified in normal pancreas or in chronic pancreatitis. Most miRNA expression profile analyses show that miRNAs are deregulated in tumor tissues as compared to normal pancreas, and that the expression pattern is tissue specific. Several studies focusing on miRNA expression profiles in pancreatic tissues have identified a number of differentially expressed miRNAs. Table 1 summarizes the aberrantly expressed miRNAs in human pancreatic cancer and their association with patient survival.

Szafranska *et al*^[50] have performed the first comprehensive miRNA expression profile study in tissues from normal pancreas ($n = 7$), chronic pancreatitis ($n = 7$), PDAC ($n = 10$) and 33 human tissues of different non-pancreatic origin, to identify miRNA candidates with a potential for future clinical application from a pool of 377 known and novel miRNAs. The authors have found that two miRNAs, miR-216 and miR-217, are pancreas-specific. These results were in agreement with those of two previous studies^[58,59]. Furthermore, both miR-216 and miR-217 are absent or only minimally expressed in pancreatic carcinoma tissues and cell lines. Therefore, miR-216 and miR-217 are potential biomarkers. Based upon clustering analysis, the three pancreatic tissues types can be classified according to their respective miRNA expression profiles. Among 26 miRNAs that have been identified as most prominently deregulated in PDAC, only miR-217 and miR-196a have been found to discriminate between normal pancreas, chronic pancreatitis and tumor tissues. These miRNAs are also potential biomarkers.

Recently, expression of 201 miRNA precursors (representing 222 miRNAs) was profiled in pancreatic adenocarcinoma, paired with benign tissue, normal pancreas, chronic pancreatitis and pancreatic cancer cell lines with the real-time PCR miRNA array^[49]. These three cell types could be classified by the clustering algorithm. One hundred miRNA precursors have been identified as aberrantly

expressed miRNAs including known ones in other cancers and novel ones in pancreatic tumor. A list of the top 20 aberrantly expressed miRNA precursors has been proposed as a signature for pancreatic adenocarcinoma.

Bloomston *et al.*^[51] have identified a large global expression pattern of miRNAs that can differentiate PDAC from chronic pancreatitis with 93% accuracy. Among several deregulated miRNAs in the pancreatic cancers, most notably, miR-21 and miR-155 are uniquely overexpressed in pancreatic tumor, as compared to tissues from normal pancreas and chronic pancreatitis. Both miR-21 and miR-155 have been suggested to play an important role in functioning as a proto-oncogene and have been shown to be overexpressed in several cancers. These authors have performed an miRNA microarray profiling with about 1100 miRNA probes, which included 326 human miRNAs, using microdissected pancreatic tumor tissues.

Zhang *et al.*^[52] have evaluated 95 miRNAs, selected from pancreatic cancer profiling, and correlated them to their potential biological functions related to cancer biology, cell development, and apoptosis. Among them, eight miRNAs (miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95) are differentially expressed in most pancreatic cancer tissues and cell lines. All of these eight genes are significantly unregulated, from 3- to 2018-fold, in pancreatic tumors as compared with normal control samples.

In summary, these profiling data may provide novel insights into the miRNA-driven mechanisms involved in pancreatic carcinogenesis, and offer new potential targets for early detection and therapeutic strategies in pancreatic cancer.

miRNAs AS BIOMARKERS FOR PANCREATIC CANCER DIAGNOSIS

Development of biomarkers for pancreatic cancer is especially critical because most patients with this disease remain asymptomatic until the disease progresses to become locally advanced or develops distant metastases. Therefore, most of these patients are surgically inoperable at the time of diagnosis. Sensitive and specific biomarkers for pancreatic cancer are urgently needed to offer better therapeutic options and survival outcome.

Over the years, a number of protein- and DNA-based biomarkers have been proposed as markers of early detection for pancreatic cancer. However, most of these markers fail to have clinical potential, and they have not influenced patients' survival. Since the first discovery of miRNAs by Lee *et al.*^[11] in 1993, many researchers have investigated expression profiles, biological functions and targets of miRNAs in carcinogenesis and tumor progression, with the purpose of translating the results to clinical settings.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) of the pancreas is not likely to be used routinely for screening for PDAC because of its invasive na-

ture. However, this procedure has recently emerged as a specific and minimally invasive modality for preoperative diagnosis and staging of pancreatic cancer. Furthermore, EUS-FNA may also be useful for screening high-risk individuals, as well as for the prognosis and predicting the response to treatment in cases in which the tumor is inoperable^[60-62]. Szafranska *et al.*^[63] have identified potential miRNA markers in EUS-FNA biopsies of pancreatic tissue. The combination of expression pattern of miR-196a and miR-217 can differentiate PDAC cases from healthy controls and chronic pancreatitis in the FNA samples. Furthermore, miR-196a expression is likely specific to PDAC cells and is positively associated with the progression of PDAC.

Carcinogenesis in PDAC develops with a multistep progression from morphologically distinct non-invasive precursor lesions within exocrine pancreatic ducts^[64]. These precursors include the intraductal papillary mucinous neoplasms (IPMNs), the mucinous cystic neoplasms, and pancreatic intraepithelial neoplasia (PanIN). Two studies have been carried out to detect expression patterns of miRNA in IPMNs and PanIN. IPMNs are grossly visible, non-invasive, mucin-producing precursors of pancreatic cancer within the main pancreatic duct or one of its branches^[65,66]. In contrast, PanINs are non-invasive, microscopic epithelial neoplasms, arising within smaller pancreatic ducts, < 5 mm in diameter, and characterized by cytological and architectural atypia^[65,67]. Habbe *et al.*^[68] have reported significant overexpression of 10 miRNAs in IPMNs ($n = 15$). miR-155 and miR-21 show the highest relative fold-changes in the precursor lesions. These results have been validated by *in situ* hybridization analysis. miR-155 and miR-21 are upregulated in most IPMNs [83% (53/64) and 81% (52/64)] as compared to normal ducts [7% (4/54) and 2% (1/54)]. With these promising data, the potential use of these miRNAs as biomarkers has been evaluated in pancreatic juices. A total of 15 pancreatic juice samples from 10 patients with IPMNs, and five with other pancreatobiliary disorders obtained at the time of surgical resection were measured for relative levels of miR-155 and miR-21 by quantitative real-time RT-PCR. Upregulation of both miR-155 and miR-21 in the subset of IPMN-associated pancreatic juices was observed, as compared with control samples. These results indicate that aberrant miRNA expression occurs early in the precursor lesion during the multiple stages of pancreatic cancer development, and miRNA profiles may be assessed with more accessible clinical samples, such as pancreatic juice, and could be used as a diagnostic tool.

du Rieu *et al.*^[69] have investigated miRNAs in PanIN tissues from a conditional Kras (G12D) mouse model ($n = 29$) and from human origin ($n = 38$). Expression of miR-21, miR-205 and miR-200 has been found to be positively associated with PanIN progression in the Kras (G12D) mouse model. In the human tissues, expression of miR-21, miR-221, miR-222 and let-7a increases with PanIN grade. The authors, using *in situ* hybridization analysis, have observed that miR-21 expression is concen-

Table 2 miRNAs and their targets involved in human pancreatic cancer

miRNA	Function	Targets	Related cellular events	Ref.
let-7	Suppress	RAS ^[71]	Inhibit cell proliferation, KRAS expression, and mitogen-activated protein kinase activation	[53]
let-7, miR-200	Suppress		Reverse EMT	[72]
Let-7a	Suppress	RAS	Attenuate KRAS expression and radiosensitize tumor cell	[73]
miR-10a	Oncogenic	HOXB1, 3	Promote metastatic behavior	[54]
miR-21	Oncogenic		Induce cell proliferation, invasion, chemoresistance	[56]
miR-21	Oncogenic		Potentially associated with cell proliferation	[74]
miR-200c	Suppress		Potentially associated with G0/G1 arrest and increased apoptotic rate	
miR-21, miR-221	Oncogenic	PTEN, RECK, CDKN1B	Arrest cell cycle, induce apoptosis, and sensitize the effects of gemcitabine with inhibition of miR-21 or -221	[75]
miR-22	Suppress	SP1, ESR1	Potentially inhibit tumorigenesis	[76]
miR-34	Suppress	BCL2, NOTCH1/2	Inhibit clonogenic cell growth and invasion, induce apoptosis and G1 and G2/M arrest in cell cycle, sensitize to chemotherapy and radiation, and potentially inhibit pancreatic cancer stem cells	[77]
miR-107	Suppress	CDK6	Induce in vitro cell growth downregulation	[78]
miR-155	Oncogenic	TP53INP1	Inhibit apoptosis	[79]
miR-194, miR-200b, miR-200c, miR-429	Oncogenic	EP300	Potentially promote metastatic behavior	[80]
miR-224, miR-486	Oncogenic	CD40	Potentially associated with invasion and metastasis	[81]

BCL2: B-cell CLL/lymphoma 2; CD40: CD40 molecule; CDK6: Cyclin-dependent kinase 6; CDKN1B: Cyclin-dependent kinase inhibitor 1B; EP300: E1A binding protein p300; ESR1: Estrogen receptor 1; HOXB1, 3: Homeobox B1, 3; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NOTCH1/2: Notch 1/2; PTEN: Phosphatase and tensin homolog; RECK: Reversion-inducing-cysteine-rich protein with kazal motifs; SP1: Sp1 transcription factor; TP53INP1: Tumor protein p53 inducible nuclear protein 1; EMT: Epithelial-to-mesenchymal transition.

trated in the dysplastic ductal epithelial cells. Using PDAC-derived cell lines, they also have noted that miR-21 expression is regulated by Kras (G12D) and epidermal growth factor receptor (EGFR).

Wang *et al.*^[70] have studied plasma samples from patients with PDAC and have found that four miRNAs (miR-21, miR-210, miR-155 and miR-196a) are able to differentiate pancreatic cancer patients from healthy controls, with moderate accuracy (sensitivity: 64%, and specificity: 89%). In summary, these studies suggest a potential value of miRNAs in the clinical setting as a potential diagnostic tool for PDAC.

miRNAS AS ONCOGENES AND TUMOR SUPPRESSORS

miRNAs are functionally classified into oncogenes or tumor suppressors based upon their targets, thus binding to oncogenes or tumor suppressor genes. Therefore, oncogenic miRNAs are upregulated in tumors, whereas tumor suppressor miRNAs are downregulated. The functions and targets of a handful of miRNAs have been investigated in pancreatic cancer (Table 2).

Torrisani *et al.*^[53] have reported that tumor suppressor let-7 miRNA is expressed in normal acinar pancreatic cells, but is extensively downregulated in PDAC samples, as compared with adjacent non-involved tissues. Transfection of pancreatic cancer cell lines with let-7 miRNA inhibits cell proliferation, Kras expression, and mitogen-activated protein kinase activation. This study has demonstrated that intracellular restoration of let-7 miRNA reverts neoplastic characteristics of PDAC, suggesting that let-7 miRNA functions as a tumor suppressor in pan-

creatic cancer. In addition, the results of this study suggest let-7 miRNA as a replacement therapy for pancreatic cancer.

miRNAS AS THERAPEUTIC TARGETS IN PANCREATIC CANCER

Most epithelial tumors, including pancreatic cancer, are believed to progress toward loss of epithelial differentiation and acquisition of a mesenchymal phenotype that leads to enhanced cancer cell invasion and migration^[82,83]. The aggressiveness of pancreatic cancer is, in part, due to its drug resistance characteristics, which are also associated with the epithelial-to-mesenchymal transition (EMT). Several studies have shown that the events leading to EMT are regulated by miRNAs^[84-89]. Li *et al.*^[72] have investigated the effects of let-7 and miR-200 on the morphological changes of EMT in gemcitabine-resistant pancreatic cancer cells (GRPCCs). They have found that: (1) the expression of miR-200 and let-7 is significantly downregulated in GRPCCs, which have EMT characteristics; and (2) transfection of GRPCCs with miR-200 rescues the epithelial phenotype by upregulating the epithelial marker E-cadherin and downregulating the mesenchymal markers ZEB1 and vimentin. These authors also have demonstrated that tumor cell sensitivity to gemcitabine is increased after re-expression of miR-200b. These results suggest that EMT could be regulated by miRNAs, and provide a potential strategy for treatment.

RAS mutations are frequent in human tumors and are known to be one of the responsible factors for radiation-induced cell death^[90,91]. Using transfection of Lin28 siRNA into pancreatic cancer cells harboring Kras mutation,

Oh *et al*^[73] have shown that upregulation with let-7a results in attenuated expression of Kras and increased radiosensitization of pancreatic cancer cells. This suggests that miRNA could be used as a valuable therapeutic option in radioresistant tumors that have Kras mutations.

The main reason for poor survival in pancreatic cancer is the presence of metastasis at the time of diagnosis. Weiss *et al*^[54] have shown that miR-10a expression promoted metastasis, and repression of miR-10a inhibited invasion and metastasis in xenotransplantation experiments using zebrafish embryos. They have further identified tumor suppressors HOXB1 and HOXB3 as targets of miR-10a, and have reported that retinoic acid receptor antagonists inhibit miR-10a expression and suppress metastasis. These data suggest new therapeutic applications for miRNA in patients with metastatic pancreatic cancer.

Several studies have reported significant overexpression of miR-21 in pancreatic tumors^[49,51], suggesting the potential role of miR-21 in pancreatic cancer. Moriyama *et al*^[50] have confirmed that miR-21 is overexpressed in pancreatic cancer cells. They also have observed that miR-21 contributes to cell proliferation, invasion, and chemoresistance. They also have found that mRNA expression of invasion-related genes, matrix metalloproteinase (MMP)-2 and MMP-9, and vascular endothelial growth factor is positively correlated with miR-21 expression. The above studies show that miR-21 functions as an oncogene, and that it is involved in pancreatic cancer chemoresistance. Therefore, miR-21 could be a target for a therapeutic strategy for patients with chemoresistant pancreatic cancer.

Zhang *et al*^[74] have found that pancreatic cancer cells treated with trichostatin A (TSA), one of the common histone deacetylase inhibitors^[92,93], are arrested in G0/G1 phase, and exhibit an increased in apoptotic rate. The treatment also induces downregulation of miR-21 and upregulation of miR-200c. The data support the oncogenic function of miR-21, and the tumor suppressor function of miR-200, suggesting that epigenetic regulation of miRNAs with histone deacetylase inhibitor could be used as a therapeutic option in pancreatic cancer.

It has been shown that antisense oligonucleotides (ASOs) can inhibit upregulated miRNAs in tumors^[94]. Park *et al*^[75] have investigated miR-21 and miR-221 biological function using ASOs in pancreatic cancer. ASOs for miR-21 and miR-221 both reduce proliferation of pancreatic cancer cell lines, increase apoptosis by 3-6-fold, and induced G1 arrest. ASOs also increase the levels of the miR-21 targets PTEN and RECK, and the miR-221 target, CDKN1B, at the protein level. The authors have found that ASO targeting of miR-21 and miR-221 sensitizes tumor cells to the effects of gemcitabine, and that ASO-gemcitabine combination treatments generate synergistic antiproliferative effects in pancreatic cancer cells. These results imply that targeting miRNAs with ASOs could be a potential new therapeutic strategy for pancreatic cancer.

In vitro and *in vivo* studies have reported the anticancer activity, with low toxicity, of curcumin (diferuloylmethane)^[95,96], a naturally occurring flavonoid from the rhizome of *Curcuma longa*^[97,98]. Sun *et al*^[76] have investigated whether

curcumin affects the expression profiles of miRNAs in pancreatic cancer, and have reported overexpression of miR-22 and downregulation of miR-199a* in pancreatic cancer cells treated with curcumin. The predicted target genes of miRNA-22 are Sp1 transcription factor (SP1) and estrogen receptor 1 (ESR1). The expression of these genes (SP1 and ESR1), which are involved in cell growth, metastasis and apoptosis, is suppressed by upregulation of miR-22. Thus, Sun *et al* have suggested that one of the important anticancer mechanisms of curcumin is modulation of miRNA expression, such as miR-22.

Some cancer stem cells are involved in tumor initiation, self-renewal and survival^[99], and miRNAs have been shown to have critical roles in cancer stem cell differentiation. Ji *et al*^[77], using cell sorting of CD44⁺/CD133⁺, have examined the roles of miR-34 in p53-mutant human pancreatic cancer cell lines, to find a potential link between stem cells and pancreatic cancer. These authors have observed that miR-34 upregulation results in significant inhibition of clonogenic growth and cell invasion, induction of apoptosis, G1 and G2/M cell cycle arrest, and sensitization of the cells to chemotherapy and radiation. They also have detected an 87% reduction in tumor initiating cells (or cancer stem cells), which was mediated by downregulation of its downstream targets BCL2 and NOTCH. This study has shown that restoration of miR-34 could have significant promise as a novel molecular therapy for human pancreatic cancer *via* inhibiting pancreatic cancer stem cell differentiation.

Aberrations in epigenetic regulation are common in human cancers, and tumor suppressor genes are frequently silenced by this mechanism in nearly all malignancies^[100,101]. Recent studies have shown that subsets of miRNAs are also silenced by the same mechanism^[102,103]. For example, Lee *et al*^[78] have shown that miR-107 is silenced by promoter DNA methylation in pancreatic tumors. These authors treated human pancreatic cancer cell lines with the demethylating agent, 5-aza-2'-deoxycytidine or the histone deacetylase inhibitor, TSA, or with a combination of the two, and identified the upregulation of 14 miRNAs, including miR-107. Retroviral expression of miR-107 in pancreatic cancer cells downregulates *in vitro* cell growth by repressing cyclin-dependent kinase 6, a putative miR-107 target. This study shows that epigenetic mechanisms of miRNA may be involved in pancreatic carcinogenesis.

Tumor protein p53 inducible nuclear protein 1 (TP53-INP1) is a pro-apoptotic stress-induced gene. TP53 is able to activate TP53INP1 transcription as a target^[104,105]. However, overexpression of TP53INP1 induces cell cycle arrest and apoptosis *in vitro*, independently from TP53. Gironella *et al*^[79] have reported that TP53INP1 is expressed in normal tissues but is markedly downregulated or lost in early stages of pancreatic cancer development. TP53INP1 repression by transfection of miR-155 causes loss or significant decrease in expression of TP53INP1. These data suggest that TP53INP1 is an additional potential target of miR-155.

Several studies have suggested that EP300 may func-

tion as a tumor suppressor. This gene is located on chromosome 22q; a region known for its frequent loss of heterozygosity in different cancers, including pancreatic cancer^[106-109]. Mees *et al.*^[80] have classified 16 human PDAC cell lines into three hierarchical groups according to their metastatic potential, and have profiled their mRNA and miRNA expression. The highly metastatic PDAC cell lines, when compared to the non-metastatic cell lines, have shown decreased mRNA and protein expression of EP300, which is related to significant upregulation of EP300-targeting miRNAs (miR-194, miR-200b, miR-200c and miR-429). Using the same 16 human PDAC cell lines, these authors have found markedly reduced expression of CD40 protein, which is involved in the host antitumor immune response^[110,111]. CD40-targeting miR-224 and miR-486 are upregulated in the highly invasive and metastatic PDAC^[81]. These results show that miRNAs are involved in regulating the metastatic behavior of PDAC, and in modulating metastasis-specific tumor suppressor genes. Targeting of these miRNAs may have potential therapeutic value in PDAC.

miRNAS AS CLINICAL ASPECTS IN PANCREATIC CANCER

Most tumors show deregulation of miRNAs for the initiation and progression of human cancer, therefore, many researchers have been trying to exploit these miRNAs for therapeutic applications, and to develop novel therapies for human cancer^[112-115]. Thus, oncogenic miRNAs can be suppressed with ASOs to their precursor or mature forms^[94,116], and tumor suppressor miRNAs can be up-regulated^[53,72].

Numerous miRNA studies have demonstrated that miRNA-directed targeting therapy has therapeutic potential in human cancer. Recent studies have further demonstrated synergistic effects when miRNA-directed therapy is used in combination with conventional chemotherapy or radiotherapy for pancreatic cancer^[73,75]. However, currently, there is no miRNA that is used in the clinical setting for treatment of cancer patients. Significant work needs to be done before miRNA-directed therapeutic strategies can be applied. However, current data have shown encouraging preliminary results to support their clinical applications in human cancer.

Several investigators have attempted to utilize miRNA expression profiles as a diagnostic tool to differentiate tumors from normal tissues^[43,117,118], and as predictors of clinical outcome. However, there have not been sufficient studies that have investigated the correlation between alterations in miRNA expression and patient outcome in PDAC.

A few miRNA expression patterns have been investigated to predict prognostic outcome from specimens of patients with pancreatic cancer^[51,55,57]. Bloomston *et al.*^[51] have analyzed the association between survival of patients and miRNA expression patterns. In the subgroup analysis of patients with lymph-node positive disease, a

panel of six miRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187 and miR-30a-3p) was able to differentiate between long-term survivors and short-term survivors who died within 2 years. Furthermore, high expression of miR-196a-2 is associated with poor outcome; patients with high miR-196a-2 expression have a shorter median survival of 14.3 mo when compared with patients with low miR-196a-2 expression, who have a median survival of 26.5 mo.

Dillhoff *et al.*^[55] have performed *in situ* hybridization after microdissection and tissue microarray analysis of 80 resected pancreatic cancer specimens, and found 79% of the pancreatic cancer samples, 27% of the chronic pancreatitis samples, and 8% of the normal pancreatic samples had positive miR-21 expression. Among the subset of patients with node-negative disease, high miR-21 expression resulted in poorer survival than in patients with low miR-21 expression (median: 27.7 mo *vs* 15.2 mo, *P* = 0.037), although miR-21 expression did not correlate with tumor size, differentiation, nodal status, or T stage.

Greither *et al.*^[57] have measured the levels of miR-155, miR-203, miR-210, miR-216, miR-217 and miR-222, which are known to be differentially expressed in pancreatic tumors. From 56 microdissected PDACs, they found that elevated levels of miR-155, miR-203, miR-210 and miR-222 were associated with poorer overall survival rates. They further noted that higher expression of all four miRNAs had a 6.2-fold increased risk of tumor-related death as compared to cases in which the expression of these miRNAs was low.

CONCLUSION

Since the discovery of miRNAs, growing evidence has confirmed a link between miRNAs and malignant diseases, and has identified their functions and targets that affect the complex process of carcinogenesis. Like other malignant tumors, PDAC has its unique miRNA expression patterns, which are different from those of other human tumors, and are able to differentiate normal pancreas from benign inflammatory pancreatic tissues and pancreatic cancer. At present, several important oncogenic and tumor suppressor miRNAs, and their molecular targets, have been identified in PDAC. More importantly, this information will lead to new development of prognostic, diagnostic, and treatment strategies. However, additional studies are required to find ways to utilize miRNAs as a therapeutic target in the clinical setting.

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Current trends in staging rectal cancer

Abdus Samee, Chelliah Ramachandran Selvasekar

Abdus Samee, Department of Surgery, Princess Royal Hospital, Telford, Shropshire, United Kingdom

Chelliah Ramachandran Selvasekar, Department of Surgery, Mid Cheshire Hospitals NHS Foundation Trust, Crewe, United Kingdom

Author contributions: Selvasekar CR designed the study; Samee A and Selvasekar CR were literature search and drafting the article; Samee A and Selvasekar CR revised the critically.

Correspondence to: Chelliah Ramachandran Selvasekar, MD, FRCS, Consultant Colorectal Surgeon, Department of Surgery, Mid Cheshire Hospitals NHS Foundation Trust, Crewe, United Kingdom. crselvasekar@aol.com

Telephone: +44-1270-612046 Fax: +44-1270-612494

Received: August 30, 2010 Revised: November 12, 2010

Accepted: November 19, 2010

Published online: February 21, 2011

Abstract

Management of rectal cancer has evolved over the years. In this condition preoperative investigations assist in deciding the optimal treatment. The relation of the tumor edge to the circumferential margin (CRM) is an important factor in deciding the need for neoadjuvant treatment and determines the prognosis. Those with threatened or involved margins are offered long course chemoradiation to enable R₀ surgical resection. Endoanal ultrasound (EUS) is useful for tumor (T) staging; hence EUS is a useful imaging modality for early rectal cancer. Magnetic resonance imaging (MRI) is useful for assessing the mesorectum and the mesorectal fascia which has useful prognostic significance and for early identification of local recurrence. Computerized tomography (CT) of the chest, abdomen and pelvis is used to rule out distant metastasis. Identification of the malignant nodes using EUS, CT and MRI is based on the size, morphology and internal characteristics but has drawbacks. Most of the common imaging techniques are suboptimal for imaging following chemoradiation as they struggle to differentiate fibrotic changes and tumor. In this situation, EUS and MRI may provide complementary information to decide further treatment. Functional imaging using positron emission

tomography (PET) is useful, particularly PET/CT fusion scans to identify areas of the functionally hot spots. In the current state, imaging has enabled the multidisciplinary team of surgeons, oncologists, radiologists and pathologists to decide on the patient centered management of rectal cancer. In future, functional imaging may play an active role in identifying patients with lymph node metastasis and those with residual and recurrent disease following neoadjuvant chemoradiotherapy.

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Key words: Rectal cancer; Staging; Investigations; Magnetic resonance imaging; Ultrasound; Endoanal ultrasound; Positron emission tomography; Computerized tomography

Peer reviewer: Christopher Mantyh, MD, Associate Professor, Department of Surgery, Duke University Medical Center, Box 3117, Durham, NC 2771, United States

Samee A, Selvasekar CR. Current trends in staging rectal cancer. *World J Gastroenterol* 2011; 17(7): 828-834 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/828.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.828>

INTRODUCTION

Nearly one million patients are diagnosed with colorectal cancers (CRC) annually in the world^[1]. The incidence of CRC is highest in the western world where it is the second commonest cause of cancer death and fourth commonest cause of death from cancer worldwide^[2]. In the western world there is a life time risk of CRC of 5%. Overall the 5 year survival has improved in the UK (55% in males and 51% in females) but to a lesser extent than in the USA and Europe^[3].

Around 30%-40% of colorectal cancer is defined to arise from the rectum which is defined as the distal margin of tumor within 15 cm of the anal verge^[4,5].

Colonoscopy and biopsy is considered as the gold

standard investigation to confirm the diagnosis of rectal cancer and to exclude synchronous lesions. Patients are then staged to assess the extent of local disease and to identify the distant spread.

Traditional rectal cancer surgery is associated with high rates of local recurrence of 5%-20%^[9]. However, with the combination of high quality surgery using total mesorectal excision^[7] along with use of neoadjuvant and adjuvant treatment there has been a significant reduction in local recurrence and improved survival^[8]. The surgeon aims to achieve a microscopic tumor free (R₀) resection. Despite this, there is a risk of local failure. Careful preoperative assessment of the pelvis identifies high risk patients in whom the resection margins are either involved or within 1 mm of the mesorectal fascia. Involvement or threatened CRM (tumors within 1 mm of the mesorectal fascia) have a reduced chance of obtaining complete clearance. Thus, the status of the CRM has become more important than the TNM staging. In Europe and the UK, patients with involved CRM/threatened CRM are considered for long course chemoradiation prior to surgery.

IMPORTANCE OF PREOPERATIVE STAGING IN RECTAL CANCER

Accurate pre-operative staging of rectal cancer is crucial in planning the surgical treatment and is the strongest predictor for recurrence^[9]. The staging helps us to formulate a structured multidisciplinary management care plan and assess the prognosis. It is also used to compare the results of hospitals offering rectal cancer treatment and to define the role of different treatment modalities.

Preoperative staging of rectal cancer can be divided into either local or distant staging. Local staging incorporates the assessment of mural wall invasion, circumferential resection margin involvement, and the nodal status for metastasis. Distant staging assesses for evidence of metastatic disease.

Rectal cancer is palpable in 40%-80% of cases^[10]. Digital rectal examination helps in documentation of the size, location, distance from the anal verge, and fixity. Lesions felt by digital rectal examination can be visualized using a rigid proctoscope. The procedure allows an accurate localization and assessment of the tumor including fixity. Biopsies can be carried out where necessary. Rectal examination using proctoscopy may be considered as an important tool for newly diagnosed rectal cancers. Painful local perineal and anal conditions such as fissures or abscesses can restrict the use of this excellent tool. A trial comparing the use of CT virtual proctoscopy with rectal ultrasound examination in determining the stage of rectal cancer is being conducted in the USA and its results are awaited (<http://clinicaltrials.gov/ct2/show/NCT00585728>).

Currently, several modalities exist for the preoperative staging of rectal cancer. A combination of modalities involving use of computed tomography (CT), magnetic resonance imaging (MRI), and/or endorectal ultrasonography (EUS) is used to precisely assess the extent of spread of rectal cancer. The choice of investigations performed,

however, is influenced by local expertise, guidelines and availability. Imaging in rectal cancer plays a crucial role in optimizing radiotherapy target definition to avoid adjacent vital structures^[11]. EUS and MRI of the pelvis are used to assess the local spread while CT is the main modality to assess systemic spread. PET is indicated when there is clinical, biochemical or radiological suspicion of local recurrence or systemic disease.

Computerized tomography and computerized tomography colonography or virtual colonoscopy

CT scan of the entire chest, abdomen and pelvis is used for the detection of metastatic disease. CT is widely available and has faster acquisition times. However, it is not considered as the investigation of choice when it comes to assessing the layers of the rectal wall; hence it is not useful for local staging in rectal cancer and certainly is poor at evaluating superficial rectal cancers. The accuracy of CT to assess the tumor has been reported to be between 80%-95% in patients with advanced local disease^[12]. The accuracy, however, decreased to around 63% when a broader spectrum of tumor sizes was analyzed. Sensitivity to pick up nodal disease has been found to be between 55%-70%^[13]. In a meta-analysis involving 5000 patients, CT showed an accuracy for T staging of 73% and for nodal staging of 22%-73%^[14].

The use of contrast enhanced multidetector CT colonography has improved the staging accuracy^[15], by achieving superior spatial resolution and visualizing pictures in a variety of planes. However, its role in staging remains to be determined and currently it is used mainly to assess the distant metastatic disease (Figure 1A and B).

Virtual colonoscopy or CT colonogram (CTC) has been reported to be safer than colonoscopy^[16] while being more sensitive than barium enema, and appears to be more acceptable to patients than either of the other tests^[17]. The procedure can be performed by technicians thus saving clinicians time. In principle the data could be analysed by computer-assistance thus accelerating diagnosis time^[18]. The results of the SIGGAR trial evaluating CTC versus colonoscopy or barium enema in symptomatic elderly patients are awaited^[19]. CTC is the best radiological imaging for assessing the colon and rectum and at the same time identifies nodal disease and distant metastasis. The diagnosis of rectal cancer still needs to be confirmed by colonoscopy and biopsy.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) is routinely used for preoperative staging of rectal cancer as it provides an accurate assessment of the tumor and the surrounding mesorectal fascia. It identifies patients at risk of local recurrence and those likely to benefit from neoadjuvant therapy. When compared with CT and ultrasound, MRI is more reliable for the evaluation of the extent of locoregional disease, planning radiation therapy, assessing postoperative changes and pelvic recurrence. The evaluation of nodal metastases remains a challenge with MRI (Figure 2).

Earlier MRI studies used body coils which lacked the

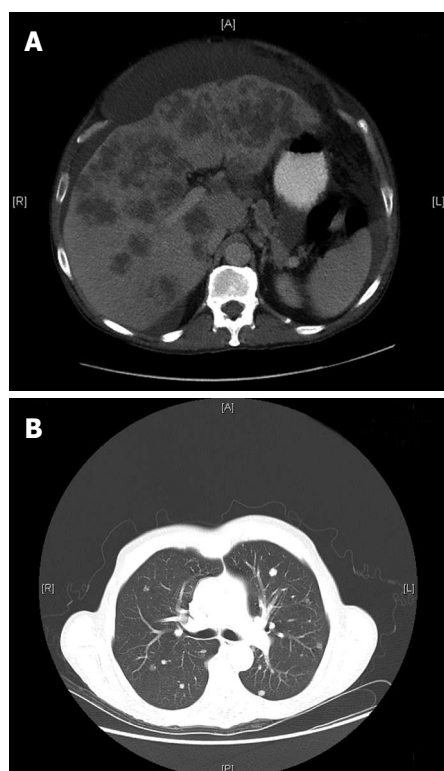


Figure 1 Computerized tomography. A: Computerized tomography (CT) abdomen showing a patient with rectal cancer having liver metastasis and ascites; B: CT Chest showing a patient with rectal cancer having lung metastasis.

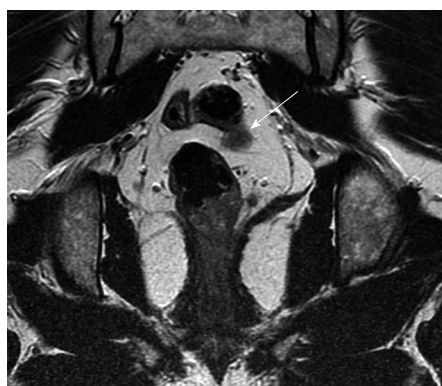


Figure 2 Coronal magnetic resonance imaging (arrow) showing possible lymph node or early vascular involvement.

resolution to differentiate the different layers of the rectal wall and added no advantage to conventional CT^[20]. Subsequent use of phased-array coils permitted reliable identification of the mesorectal fascia which is crucial in the management of rectal cancer^[21]. Initial studies suggested a histological clearance of at least 10 mm could be accurately predicted when the radiological clearance from the mesorectal fascia and critical structure was at least 5 mm^[22]. Subsequent single centre study showed 92% accuracy in prediction of CRM involvement when the CRM cutoff of 1 mm was used and this is now confirmed from the multicentre European MERCURY study^[21,23]. In Europe, MRI is now routinely used in the preoperative investigation for rectal cancer. Techniques for obtaining optimal

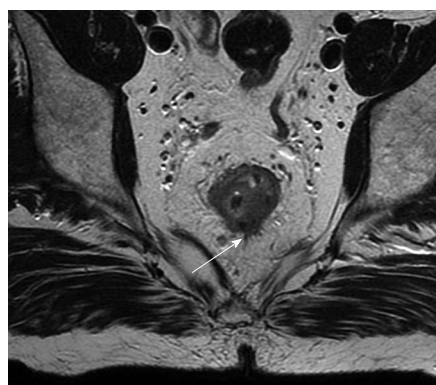


Figure 3 Magnetic resonance imaging (arrow) showing possible extension beyond the muscularis propria, radiologically staged as early T₃.

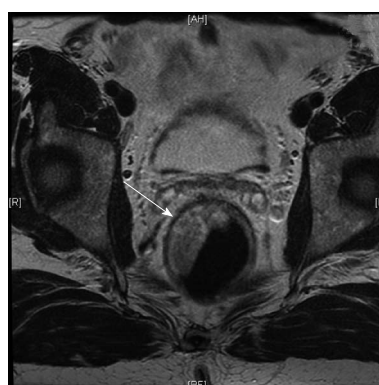


Figure 4 Coronal T2 W magnetic resonance imaging (arrow) showing the intact muscularis propria in a patient with rectal cancer. Radiologically staged as T₁ or T₂.

MRI images are described in the literature^[24]. An axial picture enables identification of the distance of the CRM to the tumor. Coronal sections are useful in low rectal tumors to identify the relation to anal sphincter complex, pelvic floor, and pelvic side wall^[25]. High signal intensity of the tumor on T2 w images suggest the presence of mucinous carcinoma which has poor prognosis compared to non-mucinous carcinoma^[26]. The standard phased array MRI produces good quality images with good contrast resolution and a relatively large field of view. Routine use of intravenous contrast does not appear to improve the accuracy^[27]. MRI cannot differentiate between T₂ and early T₃ lesions; a nodular or rounded advancing margin at the interface between muscularis propria and perirectal fat is suggestive of T₃ (Figure 3). Sometimes spiculations in the perirectal fat are considered as T₃ when in fact they are T₂ with desmoplastic reaction^[22,28]. MRI certainly cannot differentiate between a T₁ and T₂ cancer (Figure 4). Another area of drawback is restaging following long course chemoradiotherapy. Studies by Chen and Hoffmann found T staging accuracy was 52% and 54% when compared to histology^[29]. This is due to the inability to distinguish fibrosis from tumor with MRI similar to EUS. In low anterior tumors where the mesorectal fascia is close to the muscularis propria early T₃ can still infiltrate the mesorectal fascia^[24]. Extramural vascular invasion is known to be an independent predictor of local recurrence^[30,31]. The presence of a tubular structure in

proximity to a T₃ tumor or nodules with an irregular margin probably represents vascular invasion^[21,32]. Recently there has been interest in the use of functional imaging such as diffusion weighted MRI imaging (DWI) and CT/PET to distinguish fibrosis from tumor^[33].

MRI has been found to be useful in more advanced disease by providing clearer definition of the mesorectum and mesorectal fascia and seems to be a promising tool in assessing the locally advanced disease. With the advent of endorectal coils, the T staging accuracy has been reported to be between 70%-90%^[34]. However, this technique has its limitations specially when evaluating the surrounding tissue, owing to signal attenuation at a short distance from the coil. Patient's compliance, limited availability and cost also contribute to its less wide application. Obstructing or nearly obstructing lesions can be difficult to negotiate as are high rectal cancers leading to failed/improper coil insertion in approximately 40% of patients^[34].

Nodal accuracy has also been found to be variable although use of superparamagnetic iron oxide particles appears to be promising^[35] as evidenced by studies in head, neck and urological cancers.

Ultrasound

Abdominal ultrasound (USS) is used to evaluate liver for metastasis, ascites, adenopathy, and for omental cake. The false negative rate is reported to be around 8%^[36]. The technique, although inexpensive and widely available, is operator dependent. Intraoperative USS is rarely used apart from when synchronous rectal and liver resections are planned. Rapid advancement in imaging modalities has made USS a less favoured imaging modality in rectal cancer staging^[37].

Endorectal ultrasound

Endorectal ultrasound (EUS) is sensitive for early rectal cancers (T₁ and T₂ lesions) with an accuracy of 69%-97%^[38-43] and is useful in the surveillance following post transanal surgery. The standard technique involves a transanal probe enclosed in a water filled balloon introduced into the rectum to allow radial visualization of the rectum. High resolution allows the assessment of the rectal wall but the assessment of the mesorectal fascia is not possible and the assessment of the lymph nodes can be an issue and overstating has been a concern. Peritumor inflammation and artifacts due to faeces may lead to an ultrasound appearance which can be misinterpreted as tumor. These drawbacks can be exaggerated between the muscle layer and the surrounding fat which makes T₂ and T₃ lesions difficult to distinguish^[44]. The accuracy of the T stage evaluation varies from 62%-92%^[45]. In a meta-analysis of 11 studies it has been shown that sensitivities for superficial tumors are better than advanced lesions^[46]. A 20 year (1984-2004) systematic review looking at studies with a minimum of 50 patients, evaluating the use of endorectal ultrasound and magnetic resonance imaging (MRI) in the local staging of rectal cancer, have found a complementary role for these imaging modalities in the assessment of tumor depth. Ultrasound was found to be highly accurate in early lesions (T_{1,2}, 40%-100%; T_{3,4}, 25%-100%, overall 82%).

The review also found a similar accuracy in the assessment of nodal metastases^[47]. Two meta-analyses in literature have shown that the sensitivity is affected by T stage^[48]. A meta-analysis including 84 studies found EUS to be slightly superior in assessing the local involvement such as lymph nodes, however, no significant differences were noted when compared to other imaging modalities such as MRI. The results suggest that none of the current imaging modalities enable reliable detection of metastatic nodal disease^[49].

EUS however, has its limitations as it cannot reliably distinguish an irregular outer rectal wall due to peritumoral inflammation or transmural tumor extension. Obstructing lesions may be difficult to scan especially with rigid probes leading to suboptimal staging. The scanning, although less expensive and portable, is operator dependent and has a steep learning curve. Bulky, high, stenotic, advanced (T₃) lesions or post-neoadjuvant therapy downstaged tumors can be a challenge^[50-52].

EUS nodal staging accuracy is around 75%^[53]. Morphologic characteristics suggestive of malignant involvement include hypoechoic appearance, round shape, peritumoral location, and size > 5 mm^[45,46,51-53]. The loco-regional tumor assessment using three-dimensional EUS consists of transverse, coronal and sagittal scan and has been found to be superior to CT and two-dimensional EUS. The 3D-reconstructed image shows tumor protrusion infiltrating into adjacent structures, thus, allowing for improved T and N staging^[54]. Further, EUS-guided fine-needle aspiration can be carried out at the same time from the lesion or suspiciously looking lymph nodes.

Positron emission tomography

The principle of positron emission tomography (PET) is based on the differential metabolic profile of tumors compared to normal tissue. Fluoro-deoxy-glucose (FDG) is the most common PET tracer used. Due to increased metabolic activity, and change in the tumor biology, tumors preferentially show an increased uptake which results in radiolabelling^[55]. Although selective, FDG accumulates in areas of infection, inflammation, in organs of increased metabolic activity such as brain, myocardium, liver or kidneys leading to false positive results^[55]. FDG uptake is also influenced by the presence of mucin. PET is useful in identifying non-mucinous tumors compared to mucinous tumors. FDG/PET is mainly useful in the assessment of local recurrence and metastatic disease when conventional imaging is not helpful^[56,57]. Currently it is not used as a primary staging modality in rectal cancers. Interpretation of PET without anatomic correlation poses difficulties hence PET-CT fusion scans where the pictures of both investigations are fused using software is used. This offers a detailed anatomical and functional imaging and is gaining rapid popularity and acceptance. The combination provides additional value to localize the hot spots. There are some technical limitations with this combination imaging and with the false positive rates due to other disease and physiological processes. The role of PET CT fusion scan has not changed compared to PET scans.

However, a recent study has found preoperative PET

changed the management in 17% of patients^[58] with improved staging accuracy in combination with CT^[56]. Another study carried by Gearhart in 37 patients reported an altered management plan for 27% of patients using FDG-PET/CT imaging modality for low rectal cancer^[59].

Staging accuracy post-neoadjuvant therapy

With the increasing use of pre-operative neoadjuvant therapy, rectal tumor re-staging is increasingly performed prior to curative resection.

A reduction in staging accuracy has been noted which may be as a result of effects of neoadjuvant treatment due to post-radiation edema, inflammation, fibrosis, and necrosis^[60].

A recent study of 29 patients undergoing neoadjuvant therapy and pretreatment and post-treatment staging with CT, MRI, and PET showed that PET was 100% sensitive in predicting response to therapy (compared with 54% for CT and 71% for MRI). Corresponding specificity for predicting tumor response to treatment was 60%, 80%, and 67% for PET, CT, and MRI, respectively^[61], thus suggesting a further possible role of PET in predicting response to neoadjuvant therapy.

Tumor re-staging following post-neoadjuvant therapy remains problematic and it is hoped that a combination of imaging technique (CT, MRI, and EUS) and functional (PET) imaging may improve staging accuracy.

Suggested investigations for tumor staging of rectal cancer

On review of the literature, phased array MRI and EUS should be considered as the initial modalities to stage the local tumor. A fixed, locally advanced rectal cancer may be imaged better by MRI (Figure 5), whereas EUS is more appropriate for an early mobile rectal tumor (T₁-T₂ lesions). MRI has been shown to be highly accurate in predicting a clear circumferential resection margin in patients undergoing TME. Although both MRI and EUS provide a comparable overall T- and N-staging, use of these modalities is limited by issues such as availability, costs and technical expertise. CT scanning, although still the current standard for distant staging, may not be an effective tool to stage the local disease. A combination of CT and PET offering a detailed anatomical and functional imaging, however, seem to be promising and gaining popularity and acceptance for recurrent rectal cancers.

Suggested investigation for nodal staging of rectal cancer

The accuracy of MRI, CT and EUS for identifying malignant nodes is poor. Current criteria are based on size, shape and morphology. Any node of 1 cm and over is taken as significant^[62]. The enlarged lymph node can be as a result of the inflammatory process but normal size nodes can have micrometastases. Brown *et al*^[54] found 58% of positive malignant nodes were less than 5 mm. Morphological characteristics such as round shape, irregular borders and heterogenous signal intensity suggest nodal involvement^[63].

Nodal accuracy has also been found to be variable, although use of superparamagnetic iron oxide particles

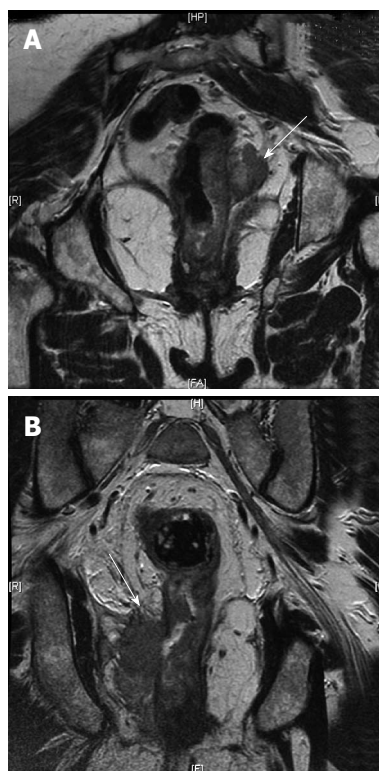


Figure 5 Magnetic resonance imaging. A: Magnetic resonance imaging (MRI) (arrow) showing the rectal cancer involving the circumferential resection margin; B: MRI (arrow) showing the rectal cancer invading the ischioanal fat on the right (T₄).

(SPIO) seem to be promising as evidenced by studies in head, neck and urological cancers. The technique involves use of a contrast media containing SPIO which accumulates in normal lymph nodes, whereas due to defective phagocytosis, the uptake is poor or absent in malignant nodes. Hence by using T2 weighted imaging, these nodes can be identified. Initial studies are promising but further research is needed^[35].

CHOOSING THE CORRECT MANAGEMENT BASED ON STAGING IN THE ELDERLY

Over the age of 80, there is 10% mortality with rectal cancer surgery^[64]. Studies from Brazil have shown a complete pathological response with chemoradiation^[65] and it is well known that the elderly respond better to radiotherapy. Hence in a selected group of patients, imaging with EUS and MRI can identify patients who can be treated with neoadjuvant treatment and those with a complete radiological response can be followed by active surveillance with an intensive imaging protocol to identify those who recur to be considered for standard salvage surgical treatment or for local excision, thereby avoiding the risks associated with major rectal cancer surgery and possibly avoiding the need for permanent stoma and enabling organ preservation. This is possible only with high quality imaging techniques to assess the loco-regional disease.

CONCLUSION

Imaging in rectal cancer helps in deciding the treatment and determining the prognosis. The newer techniques help in superior image resolution, three-dimensional viewing, with decreased image acquisition times, minimal bowel preparation, and sometimes with functional qualities. This may be important following neo-adjuvant treatment. The most accurate method of rectal wall staging of rectal cancer is endorectal ultrasound and MRI but accurate staging of mesorectal fascia and lymph nodes is by phased array MRI. The management of rectal cancer is based on the proximity of the tumor to the mesorectal fascia. Hence the phased array MRI is the best overall technique for local staging of rectal cancer. Neoadjuvant treatment is not without risks; hence careful staging is important in obtaining good oncological and functional results and improving patient experience in the management of rectal cancer. In symptomatic patients local excision is beneficial in only 5% and this is the group which benefits most from EUS. With the introduction of colorectal screening it is felt nearly 50% of cancers may be of early stage disease which can be identified by EUS and managed by organ preserving intervention. Hence the role of EUS is likely to increase as part of the staging investigations in future and all these investigations are complementary in the management of rectal cancer.

ACKNOWLEDGMENTS

The authors would like to thank Dr. M Tee, Consultant Radiologist, Mid Cheshire Hospitals NHS Foundation Trust for providing the images used in this manuscript.

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Imran Hassan, MD, Assistant Professor, Series Editor

Management of stage IV rectal cancer: Palliative options

Sean M Ronnekleiv-Kelly, Gregory D Kennedy

Sean M Ronnekleiv-Kelly, Gregory D Kennedy, Department of Surgery, Section of Colon and Rectal Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, G4/701A CSC, Madison, WI 53792, United States
Author contributions: Ronnekleiv-Kelly SM and Kennedy GD wrote the manuscript.

Correspondence to: Gregory D Kennedy, MD, PhD, Department of Surgery, Section of Colon and Rectal Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, G4/701A CSC, Madison, WI 53792, United States. kennedyg@surgery.wisc.edu

Telephone: +1-608-2632521 Fax: +1-608-2637652

Received: August 30, 2010 Revised: January 4, 2011

Accepted: January 11, 2011

Published online: February 21, 2011

Ronnekleiv-Kelly SM, Kennedy GD. Management of stage IV rectal cancer: Palliative options. *World J Gastroenterol* 2011; 17(7): 835-847 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/835.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.835>

INTRODUCTION

In 2009, there were approximately 41 000 new cases of rectal cancer in the United States^[1]. In general, 70%-80% of those presenting patients have resectable disease and are treated curatively. Of these patients, nearly 40% develop recurrence, with the majority not being candidates for re-treatment with curative intent^[2]. The goal of curative-intent operations is to remove all disease present. In contrast, the goal of palliative intent operations is to relieve symptoms, and by definition, leave local or metastatic residual disease. Approximately half of patients with rectal cancer may be candidates for palliative therapy at some point during their disease process, either because of locally advanced or metastatic disease at the time of presentation, or the late development of metastases^[3].

Palliative treatment strategies for advanced stage rectal cancer should be individualized to patients according to their symptoms. Chemotherapy for metastatic disease is the current recommendation for asymptomatic patients^[4]. Symptomatic patients can present particularly difficult challenges and can be treated with chemotherapy or combined chemoradiation therapy in conjunction with a procedure, if necessary, to relieve their symptoms. Local interventions can often effectively treat symptoms and increase quality of life. Options include extirpative resection, diversion procedures, endoscopic stenting, and laser or argon photocoagulation. The choice of treatment is partially dependent upon the patient's symptoms, age, comorbid conditions, and extent of disease.

Although the most appropriate treatment option is not always evident, a careful multidisciplinary approach with the surgeon playing the central role of determining when

Abstract

Approximately 30% of patients with rectal cancer present with metastatic disease. Many of these patients have symptoms of bleeding or obstruction. Several treatment options are available to deal with the various complications that may afflict these patients. Endorectal stenting, laser ablation, and operative resection are a few of the options available to the patient with a malignant large bowel obstruction. A thorough understanding of treatment options will ensure the patient is offered the most effective therapy with the least amount of associated morbidity. In this review, we describe various options for palliation of symptoms in patients with metastatic rectal cancer. Additionally, we briefly discuss treatment for asymptomatic patients with metastatic disease.

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Key words: Palliative therapy; Rectal cancer; Malignant bleeding; Malignant obstruction; Endorectal stenting; Laser ablation

Peer reviewer: Patricia Sylla, MD, General and Colorectal Surgery, Massachusetts General Hospital, WACC 460, 15 Parkman Street, Boston, MA 02114, United States

aggressive operative intervention is warranted can ensure the most appropriate treatment strategy is devised. The goals in palliation should include the alleviation of symptoms, enhancing quality of life and improving comfort^[5]. Herein, we review the current relevant literature on various treatment strategies as they are related to the palliative treatment of rectal cancer.

EVALUATION

Rectal cancer is defined as a malignant lesion within 15 cm of the anal verge as seen by rigid proctoscopy^[6-8]. Subsequent to histological confirmation of diagnosis *via* tumor biopsy, initial work-up of the extent of disease guides subsequent treatment^[4,9]. Proper staging is essential as decisions regarding neoadjuvant versus adjuvant therapy and operative versus palliative surgical intent will be based on clinical stage. The patient should undergo proctoscopy to determine distance from anal verge, as well as colonoscopy to interrogate the entire colon for synchronous lesions. Cross-sectional imaging of the chest, abdomen and pelvis in conjunction with endoscopic ultrasound (EUS) can assess depth of tumor penetration or invasion of local structures, lymph node status, and presence of metastatic disease^[9,10]. Although EUS has appropriate sensitivity and specificity for differentiating muscularis propria invasion (94% and 86%), as well as perirectal tissue invasion (90% and 75%), magnetic resonance imaging (MRI) has proven to be an important adjunct for accurate staging of rectal cancer as well^[9,11,12]. MRI has been found to have an 85% diagnostic accuracy for T-stage with 57%-85% accuracy for correctly identifying spread to lymph nodes; furthermore, the relationship to mesorectal fascia in conjunction with detection of adjacent organ invasion is superior utilizing MRI versus EUS^[13-18]. In addition to imaging, a preoperative carcinoembryonic antigen level combined with basic laboratory values, comprehensive history and complete physical examination to assess performance status and comorbidity play important roles in the preoperative workup, because these factor significantly for choice of intervention^[19].

When the pretreatment evaluation has determined a patient to no longer be appropriate for curative intent due to the presence of distant metastases or local invasion precluding a margin-negative resection, quality of life and symptom relief must become the main focus. In general, findings indicative of unresectability are utilized to predict the ability to achieve resection with negative margins. In those situations presented in Table 1, negative margins are obtained in 6%-36% of cases and surgical extirpation can result in significant postoperative disability^[20]. However, resectability of the disease should be assessed by an experienced surgeon. In a study by Mathis *et al.*^[21], patients who were initially deemed locally unresectable, secondary to advanced primary colon and rectal cancer, were treated with aggressive multimodal therapy and found to have median survival of 3.7 years. Conversely, decision stratification must be influenced by expected survival in those patients evaluated properly and determined not to be candidates for aggressive resection. Consideration of

Table 1 Contraindications to resective operative intervention

Sciatic nerve pain
Bilateral ureteral obstruction
Extensive fixation to lateral pelvic side wall (CT/MRI or trial dissection)
Sacral involvement above S2 (resection produces spinal instability or post-operative complications)
Bilateral lymphedema or bilateral venous thrombosis (indicating encasement of major vascular structures)
Multiple peritoneal metastasis or metastasis fixed to or invading vital structures

CT: Computed tomography; MRI: Magnetic resonance imaging.

operative interventions is more appropriately included in the conversation of palliative treatment for patients with expected outcomes exceeding 6 mo^[19,22-25].

Approximately 50% of patients either present with distant metastases or develop distant metastases after primary treatment. Those that cannot be treated curatively should have care guided by patient wishes, functional status, expected life duration, and extent of disease and debilitating symptoms. In a study by Law *et al.*^[26], the most common presenting symptoms of patients undergoing palliative intervention for colorectal cancer were intestinal obstruction and rectal bleeding. In another study, 42% of patients presenting for palliative treatment were obstructed, 37% of patients experienced rectal bleeding, and 5% were asymptomatic, with the remainder (16%) experiencing pain or rectal discharge^[27]. Taking into consideration the presenting symptoms and the underlying condition of the patient, palliative management can be divided into operative versus non-operative treatment.

CLINICAL SCENARIOS AND MANAGEMENT OPTIONS

Obstruction

Patients with rectal cancer can present with any number of symptoms that prompt evaluation (e.g. bleeding, perforation, abdominal pain, anemia, hematochezia, tenesmus, and malaise) and 10%-25% of patients present with obstructive symptoms^[19,22,26,28]. Such a clinical scenario requires expedient yet thorough evaluation of the patient for resectability and potential for cure, because these patients often necessitate urgent, if not emergency, surgical intervention^[28]. Rosen retrospectively analyzed 116 patients initially presenting with stage IV colorectal cancer and found that 26% presented with obstructive symptoms^[22]. In another study, although the most common symptom precipitating medical evaluation in advanced colorectal cancer was bleeding (24%), Law *et al.*^[26] found that obstruction (23%) in conjunction with change in bowel habits (15%) comprised a significant proportion of patient presentations. Phang *et al.*^[29] found that nearly 10% of patients with rectal cancer presented with a bowel obstruction and required some emergency intervention. In that series, patients who underwent primary resection of the tumor at the time of emergency surgery had

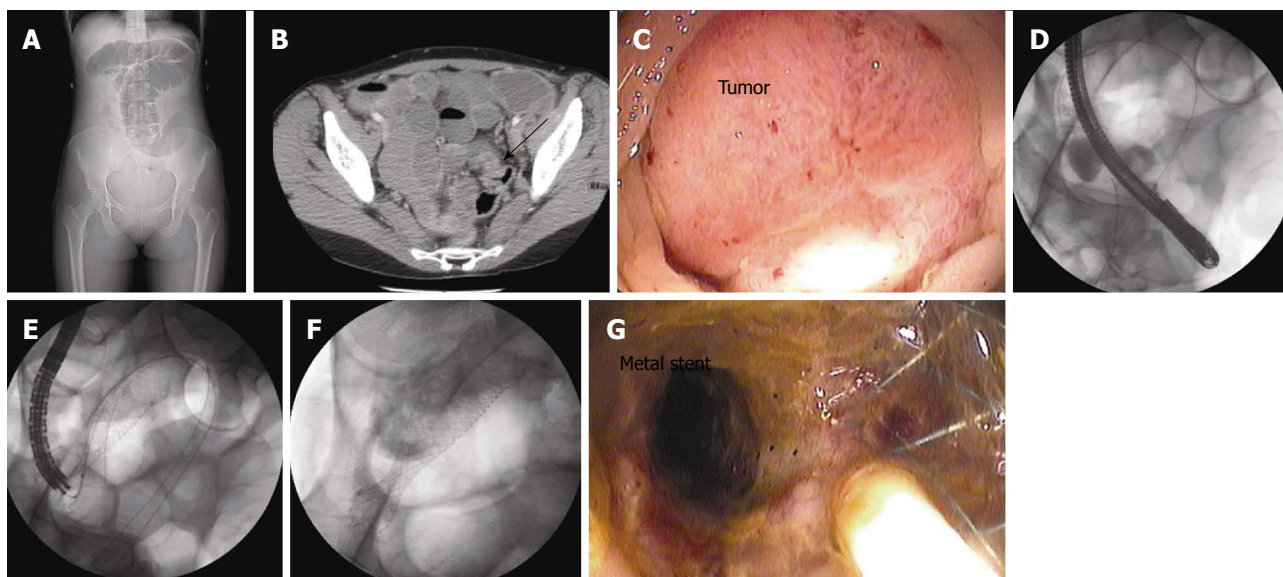


Figure 1 A young patient was diagnosed with an obstructing cancer in the upper rectum. Computed tomography demonstrated findings consistent with peritoneal metastases. She was referred for an endorectal stent to relieve the obstruction. A: Single-view plain radiography demonstrated colonic distension; B: A single-axial section with the arrow demonstrated the tumor; C: Luminal view of the tumor at time of sigmoidoscopy; D: Fluorography during stent placement demonstrated the wire across the tumor; E: Fluoroscopic view demonstrated the endoluminal stent being deployed; F, G: Fluoroscopic and endoscopic views of the stent in place.

worse overall survival and higher local recurrence rates than those patients who had elective surgery. Data such as these support the notion that interventions other than surgical resection should be entertained in those patients with rectal cancer who present in an emergency situation.

Non-operative approach: Self-expanding metallic stents have been widely utilized for maintaining patency in the biliary tree and esophagus. Transition to endorectal stenting was described in case reports in 1995, and since then, its use has increased with development of stents specifically designed for use in the large intestine^[30]. Endorectal stents present one potential option to treat the obstructing rectal cancer. When utilized in this setting, they can be definitive treatment in the patient with widespread disease, or serve as a bridge to elective primary resection and anastomosis in the patient with acute obstruction.

Self-expanding metallic stents (SEMSs) are expandable metallic tubes placed in a collapsed state across the obstructing tumor under fluoroscopic guidance, endoscopic guidance or a combination procedure^[31]. Various stents are utilized and when deployed expand to relieve the obstruction caused by tumor growth. Dedicated colonic stents are generally flared at the ends with a smaller mid-body diameter and differ with respect to length and diameter. Therefore, appropriate stents can be selected based on location and length of lesion as well as severity of obstruction. Examples of available stents include the colonic Z-stent (Wilson-Cook Medical, Winston-Salem, NC, USA) with 25-mm mid-body and 28-mm end diameters and the Ultraflex Precision Colonic Stent (Microvasive, Boston Scientific Corp., Natick, MA, USA) with 25-mm mid-body and 30-mm end diameters. The patient scenario presented in Figure 1 demonstrates a successful stent placement using a combination of fluoroscopic and endoscopic guidance.

Once deployed, the stent eventually becomes incorporated into the tumor and surrounding tissue *via* pressure necrosis, which allows anchoring and prevents migration^[32]. Stent procedures are generally well tolerated with minimal sedation required for placement, which make them an enticing option for palliation of obstruction. In fact, a recent systematic review of 88 studies with 1785 patients who underwent SEMS placement for the relief of malignant colorectal obstruction reported a median success rate of 96.2%, with relief of obstructive symptoms 92% of the time^[31]. When failure did occur, the most common cause was inability to pass a guidewire through the tortuous anatomy. On follow-up, 90.7% of patients in 11 of the studies reporting outcome had a patient stent upon death or at end-point for a mean duration of 106 d^[31]. Studies such as these indicate that stents can be placed successfully in most situations, whether as a bridge to surgery or for definitive palliation.

Unfortunately, few randomized controlled trials have compared effectiveness of SEMSs and surgery for incurable, obstructing rectal cancer. In a non-randomized, prospective study, patients underwent SEMS placement or palliative surgery for obstructing, non-resectable rectal cancer. SEMS was successfully placed in 38/40 patients with mean duration of 269 d^[33]. Although the stent group was statistically older with higher ASA classification, median survival was 296 d in the stent group *vs* 234 d for the surgery group. The length of hospital stay in the stent group was 2 d *vs* 9.5 d in the surgery group. Furthermore, complications requiring intervention occurred in 19% of the stent patients with no postoperative mortality *vs* 32% complication rate in the surgery group with 5% mortality. These results are consistent with the conclusion that surgical intervention confers no significant survival advantages and that SEMSs should be considered a reasonable alternative^[33].

Another series from Germany has found that many patients are relieved of their obstruction and never require further surgery. Hünnerbein *et al.*^[34] has found that 26 of 33 (79%) patients had long-term relief of bowel obstruction. Furthermore, 20 patients died with the stent in place at a mean of 5.3 mo and required no surgical interventions. The findings of this group corroborates those of others indicating the SEMSs are a safe option for the treatment of a malignant large bowel obstruction.

Overall, SEMSs are associated with less risk, shorter hospital stay and less morbidity and mortality than surgical resection or diversion. Although a certain percentage of patients with stent placement may require subsequent surgical intervention, SEMSs appears to have an appropriate role in the therapeutic options for palliation of obstruction. In fact, mortality after surgery for malignant large bowel obstruction in most series is 5%-10%, with one study reporting 18% mortality after surgery for obstructing colon cancer^[22,35-40]. Postoperative complications have been found to range between 20% and 30% in most series, with one study reporting 54% postoperative complications^[22,35-40].

Complications after stent placement such as bleeding, malposition and perforation can occur early after deployment. Late complications after stent placement include stent migration and occlusion. Given the limited life expectancy of the patient population in whom stents are typically placed, long-term complications or failures have been difficult to assess. Long-term complications such as obstruction have been documented to occur in approximately 15% of patients. These complications were successfully treated in all cases with another endoscopic procedure^[34]. Bleeding was a rare complication (< 5% of patients) that was treated with endoscopic electrocoagulation. In this same series, short-term failure occurred in approximately 20% of patients and included stent migration, severe pelvic pain, incomplete stent expansion, and incontinence^[34].

Perforation is an especially morbid complication in that violation of the colon or rectum carries significant consequences for these patients who are often quite debilitated from their primary disease process. This complication can occur as a result of over-expansion in the tumor bed or pressure necrosis in the normal colon. Rates of perforation are approximately 5% and surgical treatment requires a relatively high-risk operative intervention^[31]. Song *et al.*^[41] have found the rate of perforation to be approximately 10%. Although one patient in their series ultimately died as a direct result of the perforation, there were no significant differences in median survival between patients with and without perforation.

Operative approach: Patients with obstruction who require an operative approach can be treated with either resection of the primary tumor or a diverting stoma. Because of the constraints associated with the pelvis and proximity of structures with tumor extension and fixation, complete resection often requires pelvic exenteration or

removal of other organs along with the primary tumor^[19]. These operations tend to be morbid and a less than ideal option in the patient with a limited life expectancy. Therefore, a colostomy is the preferred operation in the patient with an acute malignant obstruction of the large bowel. The sigmoid and transverse colon are the most commonly used conduits for creating a loop colostomy^[42].

Other situations that necessitate operative intervention are those in which a SEMS is contraindicated. For example, the patient with cancer in close proximity to the anal canal (within 3 cm) can have intractable anal pain, tenesmus, and incontinence after placement of a SEMS^[34]. Diverting colostomy can relieve the obstructing symptoms effectively and avoid these intractable symptoms. Additionally, an extended narrowing involving a long segment of the lumen with significant angulation can make SEMS placement impossible and the attempt can be high risk. Difficulty with passing the wire or pre-stent balloon dilation of the stricture may result in perforation, with these difficult obstructions necessitating emergency surgery^[41]. Colostomy formation may be the better alternative in these cases^[42].

A diverting colostomy can be placed using a laparoscopic or open approach. Laparoscopic fecal diversion is an attractive alternative in patients presenting with obstruction. Patients have smaller incisions with less associated pain, shorter hospital stay, quicker onset to return of bowel function, fewer postoperative complications, and the potential to initiate chemotherapy at a shorter interval when compared to open operations^[19,43,44]. However, the laparoscopic approach can be difficult in this setting as the colon is often massively dilated and manipulation of the large organ can be impossible.

A particularly treacherous situation is presented in the setting of emergency decompressive surgery in which mortality approaches 20%, a complication occurs in nearly 50%, with half of patients incurring a permanent stoma^[45]. Furthermore, complications resulting from the stoma are higher in patients undergoing emergency surgery^[46]. In this setting, an expandable rectal stent can be placed as a bridge to surgery or as definitive palliation. In the recent comprehensive review of endorectal stents, patients were able to undergo elective surgery 2-16 d after stent placement. Rates of primary anastomosis for elective surgery after stent placement were twice that of emergency surgery for obstruction with shorter hospital stay, decreased morbidity, and decreased mortality in the elective surgery group^[31].

Negative effects on quality of life and associated complications with a permanent colostomy are other reasons only to approach the obstruction operatively in those patients not amenable to other non-operative approaches^[47]. Complications directly related to the colostomy can occur in up to one-third of patients, and include skin irritation, leakage, prolapse, pain, partial necrosis and retraction^[19,48,49]. In conjunction with these complications, patients are more likely to feel socially restricted as a result of their colostomy when events such as leakage, prolapse

or retraction occur^[50]. Furthermore, many patients are unhappy after the operation, contending that their education was not sufficient to prepare them to deal with the colostomy^[51]. In a study evaluating patient satisfaction after colostomy placement in colorectal cancer, 31% of patients were dissatisfied with the information received regarding the colostomy procedure^[52]. An additional study by Nugent *et al.*^[53] has revealed only 65% of patients felt sufficiently informed regarding what an ostomy entails. Moreover, 20%-35% of patients felt significant impact on quality of life including change in work, travel or social habits; consequently, patients expressed desire to supplement deficiencies with further counseling and follow-up. In fact, it has been shown that intensive preoperative education directed by a nurse with expertise in stoma care improves postoperative outcomes^[54]. Despite these problems, fecal diversion remains an option for relief of symptoms in this patient population, and conversation with the patient to address any concerns may alleviate reservations and improve outcomes.

Primary tumor resection is occasionally indicated and can provide a reasonable quality of life postoperatively in selected patients. The most commonly performed procedures for palliative resection include abdominoperineal resection (APR), Hartmann procedure, low anterior resection (LAR), and exenteration. These operations are less commonly utilized for obstruction due to the expected short duration of survival of the patient. The decision between APR, LAR or Hartmann depends on tumor location and size, comorbidity, and ability to achieve clear margins. When addressing a rectal tumor in which resection does not preclude preservation of sphincter function, intervention would likely include low resection versus Hartmann procedure. An advantage of utilizing LAR is the maintenance bowel continuity and fecal continence. However, if there is poor predicted anal function, or concern for the anastomosis in an irradiated field, the formation of a proximal diverting ostomy negates the advantages of LAR over the Hartmann procedure^[42,55]. With regard to low-lying rectal cancer, an advantage of the Hartmann operation over APR is the avoidance of a perineal wound and associated wound healing complications^[56-58]. The Hartmann operation requires surgical dissection below the tumor for appropriate resection, therefore, studies have reported higher incidence of pelvic abscess than occurs with APR^[57,59]. However, investigating patient outcomes following the Hartmann procedure versus APR for palliation in low-lying rectal cancer (approximately 5-5.5 cm from the anal verge), patients had similar rates of abdominal wound infection, pelvic/abdominal pain and stoma complications, whereas the APR group had a 46% occurrence of perineal wound sepsis and 38% incidence of perineal wound pain^[57]. In contrast, if the rectal cancer involves the anal sphincter, APR is the preferred surgical option^[42].

Pelvic exenteration is considered an extended radical resection in which surrounding organs are removed. This operation should be avoided when the goal of the operation is that of symptom palliation because the operation

is generally fraught with complications and provides little if any improvement in quality of life^[60]. Anterior exenteration includes resection of anterior pelvic organs; posterior exenteration involves a partial sacrectomy when excising the tumor; and complete exenteration is performed when significant invasion of most surrounding structures occurs^[20]. Mortality rate from these procedures when performed for recurrent rectal cancer ranges from 0.6% to 5% at 30 d, with morbidity of 30%-60% and sphincter salvage of 5%-15%^[20]. Therefore, patients who undergo an extended resection may experience prolonged hospital stay as well as higher rates of postoperative complications and re-admissions, while still requiring the formation of a stoma. There have been reports of symptom improvement and enhanced quality of life when performed in symptomatic individuals with unresectable disease^[19]. However, pelvic exenteration is rarely performed for symptom palliation in symptomatic patients with unresectable rectal cancer.

Bleeding

Non-operative approach: Laser ablation is a well established treatment modality for palliation of rectal cancer, in which endoscopy is utilized to deliver focused energy to the rectal lesion^[61]. The most frequently used laser is the neodymium yttrium argon garnet (Nd:YAG) laser, which has the ability to treat bleeding lesions and vaporize tumor tissue. Energy can be delivered to promote coagulative necrosis or vaporization depending on the goal of the treatment, with repeated treatments usually necessary^[61]. Laser ablation has been utilized to palliate obstruction in inoperable rectal carcinoma, especially in cases in which tumor ingrowth causes obstruction, urgency or tenesmus after stent placement. However, laser ablation has been best utilized in cases in which bleeding is the prominent symptom. Coagulation is usually achieved after 2-5 sessions in 80%-90% of patients with complications ranging from 2% to 15%^[61]. In a study by Rao *et al.*^[62], 8/11 patients were treated *via* endoscopic laser ablation for bleeding, with a median symptom-free interval of 10 mo. The average number of treatment episodes was six, with an immediate overall success rate of 91%. Another group that utilized endoscopic diode laser therapy for unresectable rectal cancer found lifelong symptom relief to be achieved in 51/57 patients. Obstruction was relieved in 22/24 patients and bleeding controlled in 29/30^[27].

Complications associated with laser ablation occur in 2%-15% of patients^[61,62]. The majority of complications reported tend to be minor, however, perforation requiring laparotomy occurred in 2/57 patients in a study of laser therapy^[27]. Furthermore, successful palliation becomes less likely to be achieved with improvement in overall survival. Additionally, ablation is relatively ineffective with long-segment or circumferential tumors, or with angulated segments of the rectum. Despite these negative aspects, laser ablation is a relatively low cost, minimally invasive modality for palliation of bleeding that provides acceptable results in high-risk individuals.

Argon plasma coagulation (APC) utilizes electrocautery to ionize argon gas that acts to fulgurate the neoplasm and bleeding vessels. It has been utilized in open surgery to achieve hemostasis in superficial diffuse hemorrhage. This surface coagulation is fairly effective and thus APC has become more widely utilized than laser therapy in many centers for palliation of bleeding^[61]. Because of the minimal depth of penetration (2-3 mm), with concomitant, efficient tissue coagulation, the risk of perforation is decreased compared to that with laser therapy. However, due to its limited penetration, it is not as effective for relieving obstruction. Compared to laser therapy, APC is easier to use, cheaper and more portable, which provides for an attractive option for palliating bleeding in an advanced-stage rectal cancer patient.

Chemotherapy has also been found to provide symptomatic improvement within 1-2 wk of initiating therapy, especially in cases of imminent obstruction or bleeding. In a study by Poultides of 233 patients with synchronous metastatic disease and unresected primary tumor, 217 (93%) never required surgical palliation of their primary tumor, with only 16 patients (7%) requiring emergency surgery for primary tumor obstruction or perforation^[63]. These data indicate that many patients can be treated with systemic therapy alone as preventive palliation, with the caveat that it requires a certain time period to produce desired effect.

In addition to bleeding, patients who present with locally advanced or recurrent disease often experience pelvic pain secondary to involvement of nerve structures within the pelvis, or from involvement of the sacrum. Radiotherapy can provide relief of pain and bleeding in 75% of patients for a median duration of 6-9 mo^[64]. The range of doses studied varied from 20 to 60 Gy. However, radiotherapy has not been shown to confer a survival benefit and is best utilized for palliation of symptoms in patients with short life expectancy (6 mo)^[65]. Outside of palliation for pain and bleeding, external beam radiation plays an integral role in the multimodal treatment of rectal cancer. In patients with locally advanced or recurrent disease, radiation should be utilized as multimodal therapy for potentially resectable disease^[64].

Operative approach: Surgical options for the treatment of bleeding are similar to those for the treatment of obstruction. However, unlike the patient with a large obstructing lesion, the bleeding tumor may be smaller and more amenable to local or transanal excision (TAE) options. Although not a curative operation for locally advanced rectal cancer, TAE for rectal cancer may provide symptomatic relief of bleeding. Transanal endoscopic microsurgery has been successfully used for this indication^[66,67].

Asymptomatic patients

One of the principal concerns when evaluating an asymptomatic patient with metastatic rectal cancer is whether the primary lesion itself will become symptomatic, and necessitate intervention in order to avoid debilitating complications. This concern is what traditionally prompted

surgical resection of primary disease, even in asymptomatic individuals. Proponents state that extirpation of the primary tumor can preclude development of obstruction, perforation or bleeding, thus avoiding a surgical emergency in already compromised patient receiving chemotherapy^[68]. However, patients who are unfit candidates for complete resection do not achieve survival benefit with excision of the primary tumor^[38,69,70]. Additionally, multiple studies have confirmed that asymptomatic or minimally symptomatic patients with incurable colon and rectal cancer have a low risk of developing debilitating symptoms prior to death from progressive disease^[19,38,63,65,71-73]. Tebutt *et al*^[71] have evaluated patients undergoing chemotherapy for metastatic disease, of whom, a subset had undergone resection of the primary, while another cohort initiated chemotherapy immediately after diagnosis. There was no difference in obstruction, peritonitis, gastrointestinal bleed or fistula formation between the two groups. Similarly, in a report by Scoggins *et al*^[38], operative intervention was required in only 9% of patients managed initially without resection (chemotherapy subset), while morbidity and mortality were 30% and 5%, respectively, for asymptomatic patients undergoing initial operation. In a study by Poultides *et al*^[63] which has investigated outcomes in patients with synchronous colorectal metastases treated with chemotherapy, 217 out of 233 (93%) patients never required intervention for perforation, bleeding, obstruction or any other cancer-related complication. From these studies, it is apparent that, for asymptomatic individuals with unresectable metastatic disease, chemotherapy is the appropriate first-line therapy, and surgical resection without removal of all tumor burden will result in delay in starting therapy.

In contrast to systemic treatment alone, certain patients with advanced stage metastatic rectal cancer benefit from combined surgical resection and systemic therapy. The discussion regarding resection of metastatic foci for curative intent is extensive, therefore, it will be briefly reported here. When resection of a primary tumor combined with metastectomy was performed with curative intent, overall 5-year survival rates range from 35% to 58%, which significantly surpassed the 5-year survival attained by non-curative resection or systemic treatment alone^[74-79]. With the development of newer biological agents, combined with more efficacious combination chemotherapy and improvement in surgical techniques that increase the efficacy and safety of resection, the number of potentially curable patients with disease amenable to resection has increased. Patients who present with widespread disease should be evaluated for surgical resectability at 2-mo intervals during cytotoxic chemotherapy^[4]. The purpose of re-evaluation is to ascertain whether response to therapy has reduced the malignant neoplasm to a state in which R0 resection may be achieved^[80]. Coincident with this, expanding criteria for patients amenable to safe resection of rectal cancer metastases has allowed allocation of patients into the "cure" category versus palliative measures. Therefore, understanding which patients should be considered for curative treatment provides an appropriate cohort that should be considered for palliation.

Traditionally, specific characteristics of metastases in colorectal cancer have governed suitability for liver resection. These include ≤ 3 metastases, no evidence of additional extra-hepatic disease, ability to achieve 1-cm resection margin, and small size of metastases (< 5 cm)^[76,78,81-83]. Fortunately, with improvement in medical therapy and surgical proficiency (including imaging modalities, techniques such as portal vein embolization, and adjunct procedures such as radiofrequency ablation), these previous contraindications have become less absolute^[74,76,84]. In a recent study, patients with > 3 hepatic metastases undergoing hepatic resection achieved similar survival as those with < 3 metastases given a microscopically negative resection margin (R0) and sufficient liver remnant^[85]. As a result, the focus has shifted towards potential hepatic function after surgical extirpation instead of quantifying numerically disease pre-resection^[68,76]. Additionally, while hepatic metastasis size > 5 cm has historically predicted poor outcome, tumor size is now only considered a contraindication if attaining a negative margin is impossible (i.e. insufficient remnant liver or proximity to critical structures, which precludes complete resection)^[76,78]. Moreover, despite earlier reports of worse outcome when margins were < 1 cm, the extent of the negative margin has not been shown to confer increased survival (< 1 cm *vs* > 1 cm); rather, only microscopically negative margins are a requisite for survival benefit^[75,82,83]. Furthermore, addressing extra-hepatic disease (specifically pulmonary metastases), plausibility of R0 resection should be the preferential concern dictating tumor resectability. Investigations have demonstrated survival benefit in those patients with both liver and pulmonary metastases that were amenable to margin-negative resection^[86-90]. Similar to evaluation of liver metastases, isolated pulmonary metastases have been extensively investigated with the consensus that resection of pulmonary metastases with microscopically negative margins portends a favorable prognosis compared to chemotherapy alone^[81,86,88,89,91,92]. From these types of data, it is obvious that evaluation of a patient with metastatic disease is complicated and the treatment plan should be jointly developed by a team of well-trained medical, surgical, and radiation oncologists.

Chemotherapy

The presence of synchronous metastases clearly decreases survival. However, those patients who are surgical candidates and can have all sites of disease removed have a better overall prognosis^[93-95]. On the other hand, individuals who do not fall into the category of resectable advanced stage disease, and are also asymptomatic, should have systemic treatment initiated expeditiously after diagnosis.

Since the approval by the FDA in 1962 of 5-fluorouracil (5-FU) for systemic treatment of colorectal cancer, advances in our understanding of the molecular alterations that accrue in malignant colorectal disease have enabled significantly more efficacious chemotherapeutic regimens^[96]. Moreover, specific characterization of the mechanism of action of various cytotoxic agents also has contributed to increasingly potent combination therapies.

Utilized as monotherapy, 5-FU has generated response rates of 10%-15% in patients with advanced colorectal cancer^[97]. Early modifications included addition of folinic acid (leucovorin) which increases the efficacy of 5-FU, as well as varying the method of administration (i.e. bolus *vs* continuous infusion), which demonstrates a higher response rate and increased overall survival (OS) in the continuous infusion group^[96-98]. An oral formulation, capecitabine, also has become available and was approved by the FDA in 2001. A subsequent landmark in the development of a pharmaceutical regimen arose upon inclusion of agents such as irinotecan and oxaliplatin in the armamentarium against colorectal cancer.

Irinotecan, a topoisomerase I inhibitor, had initially demonstrated improved outcomes (overall survival, quality of life) *vs* supportive care alone in patients whose metastatic disease had progressed while on the standard chemotherapeutic regimen of 5-FU and leucovorin^[99]. Additionally, in a similar study comparing irinotecan and 5-FU infusion in patients not responding to or progressing while on first-line 5-FU/leucovorin, patients within the irinotecan arm benefitted from increased progression-free survival (PFS) in conjunction with OS^[100]. Consequently, irinotecan has been evaluated as first-line therapy in combination with bolus 5-FU and leucovorin (IFL), as well as infusional 5-FU and leucovorin (FOLFIRI). Because the addition of this new agent generated favorable results (increased PFS and OS), irinotecan has been incorporated into the armamentarium of primary chemotherapeutic treatments for metastatic disease^[96,101,102].

Concordantly, oxaliplatin, a third-generation platinum complex, has demonstrated efficacy as an antitumor agent in advanced stage colorectal cancer patients with documented progression on standard fluorouracil-based chemotherapy^[103,104]. This again prompted further evaluation of the efficacy of the platinum agent when administered in conjunction with 5-FU/leucovorin. In an equivalent manner to irinotecan, oxaliplatin demonstrated comparably favorable results (longer duration of PFS, higher response rate) when incorporated with leucovorin and 5-FU compared with the latter two agents alone^[105]. Oxaliplatin potentiation of 5-FU cytotoxic activity has resulted in modification of first-line chemotherapy in which folinic acid, fluorouracil, and oxaliplatin (FOLFOX) has emerged as a therapeutic standard for metastatic disease.

In order to determine the best first-line agent for treatment of colorectal cancer, a randomized controlled trial was conducted evaluating FOLFOX and IFL^[106]. This trial demonstrated an improved response to FOLFOX, thereby establishing this regimen as the new gold standard for the treatment of metastatic disease. This postulation was succeeded by the hypothesis that utilization of all three active drugs (5-FU, oxaliplatin, irinotecan), as well as infusional (and not bolus) 5-FU, were the underlying etiologies of increased survival^[96]. A trial comparing first-line FOLFOX6 *vs* FOLFIRI (folinic acid, fluorouracil, irinotecan) followed by FOLFIRI and FOLFOX6 respectively was conducted to determine the appropriate sequence of

combination chemotherapy. OS was comparable in both groups (20.6 mo *vs* 21.5 mo), which was longer than OS in previous studies that have evaluated protocols with only two active drugs (oxaliplatin and 5-FU or irinotecan and 5-FU)^[95]. These results were corroborated by a meta-analysis that has investigated the synergistic impact on survival when implementing therapy with 5-FU, leucovorin, irinotecan and oxaliplatin during the course of treatment^[107].

The improved understanding of the biology of colorectal cancer has led to the development of several new agents that are active against members of the growth factor family. Although several novel agents have been evaluated in a number of diseases, three select therapies have been approved by the FDA for use in metastatic colorectal cancer: bevacizumab (2004), cetuximab (2004), and panitumumab (2006)^[96,108]. FDA approval of cetuximab and panitumumab was contingent upon tumor expression of epidermal growth factor receptor (EGFR) (which occurs in 70%-80% of human colorectal carcinomas), as demonstrated by immunohistochemistry^[96,109,110]. However, differential expression of EGFR *via* immunohistochemistry does not seem to correlate with response to or benefit from anti-EGFR therapy^[110-112]. This finding engendered the question of whether different methods to ascertain EGFR levels in tumors (i.e. fluorescence *in situ* hybridization, RT-PCR) are needed, or whether more specific markers exist that predict response to anti-EGFR treatment. As a result of numerous studies demonstrating association between KRAS mutation status and response to cetuximab/panitumumab therapy (discussed below), current recommendations are for use in colorectal cancer without specified KRAS mutations^[4,113,114].

Cetuximab and panitumumab are high-affinity monoclonal antibodies (chimeric mouse/human IgG1 and human IgG2, respectively) directed against the extracellular ligand binding domain of EGFR. Their efficacy has been demonstrated in both irinotecan-based (FOLFIRI) and oxaliplatin-based (FOLFOX4) treatments^[111,115-117]. When bound by ligand, EGFR activation triggers a cascade of events that propagate growth signals that ultimately promote cell proliferation and survival^[118-120]. Within this signaling cascade lies KRAS, an intracellular G-protein that is mutated in 30%-50% of colorectal cancers; when this genetic aberration occurs in specific codons (12 and 13), the resultant constitutively active protein is no longer dependent upon upstream input from EGFR^[96,109,112,121,122]. The relevance of KRAS mutations becomes apparent for patients treated with anti-EGFR therapy: abolishing the upstream signal does not likely provide any benefit. This principle has been validated by several studies that have evaluated cetuximab treatment in metastatic colorectal cancer, and KRAS mutational status, in which only patients with wild-type KRAS show improved response, PFS and OS^[109,116,120-124]. Furthermore, this disparity in efficacy has also been observed in a study of KRAS mutational status and panitumumab therapy in refractory metastatic colorectal cancer^[112,125]. Moreover, when evaluated as first-line treatment in conjunction with FOLFOX4, panitu-

mumab increased PFS in patients with wild-type KRAS, while those with mutant KRAS suffered a decrease in PFS^[115]. Heinemann has provided an excellent review of the clinical relevance of EGFR and KRAS status with respect to anti-EGFR therapy in patients with metastatic colorectal cancer^[109].

Bevacizumab is a humanized monoclonal antibody directed against soluble vascular endothelial growth factor A (VEGF-A). The biological agent inhibits VEGF-A binding to vascular endothelial growth factor receptor, thus restricting angiogenesis, a process critical to tumor formation, invasion and metastasis^[126-128]. In 2004, a cardinal study investigating the benefit of bevacizumab addition to IFL therapy compared to IFL alone in patients with previously untreated metastatic colorectal cancer demonstrated increased response rate, PFS and OS in the group receiving the anti-angiogenic biological agent^[129]. Additionally, in patients with disease progression after first-line irinotecan-based therapy, bevacizumab supplementation of FOLF-FOX4 generated increased PFS and OS versus FOLFOX4 or bevacizumab alone^[130]. A subsequent study evaluating first-line bevacizumab or placebo combined with FOLF-FOX4 as well as capecitabine/oxaliplatin (XELOX) revealed two important findings. Addition of bevacizumab to oxaliplatin-based therapy increased PFS when used as first-line therapy^[131]. Combination of capecitabine and oxaliplatin was not inferior to FOLFOX4 therapy^[132]. To explore further the clinical effects of targeted therapeutics, in a phase IIIb trial in 2009, patients received oxaliplatin or irinotecan with bevacizumab, leucovorin and 5-FU as initial treatment for advanced systemic disease^[133]. These patients were then randomly assigned to receive panitumumab or placebo. Remarkably, in the oxaliplatin-based group, those that received panitumumab showed decreased PFS and OS compared to the control group, while there was no difference seen with panitumumab addition in the irinotecan-based group^[133]. In confirmation of this detrimental effect of combined anti-VEGF and anti-EGFR therapy, capecitabine, oxaliplatin and bevacizumab were administered as first-line therapy with or without cetuximab in patients with metastatic colorectal cancer, and addition of cetuximab resulted in decreased PFS^[122].

Currently, according to the National Comprehensive Cancer Network guidelines, patients with unresectable, asymptomatic metastatic disease should undergo initial therapy consisting of one of the following: choice of FOLFOX, CapeOX or FOLFIRI, with or without bevacizumab; or FOLFOX or FOLFIRI with or without cetuximab/panitumumab (specifically for disease characterized by wild-type KRAS gene)^[4]. Alternatively, FOLFOX or FOLFIRI alone can be utilized in an attempt to render patients possible candidates for resection^[4]. Additionally, concomitant use of anti-EGFR and anti-VEGF therapy should be avoided^[4]. Patients should be re-evaluated after 2 mo to determine if conversion to resectability has been achieved. Symptomatic improvement is often seen within weeks of initiating chemotherapy, thus negating the need for local intervention^[63]. With these regimens, response

rates of approximately 50% have been achieved, with 50% reduction in bi-dimensional measurements occurring, and another 25% of patients demonstrating a minor response or stabilization^[64]. In addition, chemotherapy is the only modality that has been demonstrated to increase survival in stage IV colorectal cancer, with median OS of 18-20 mo, which indicated that chemotherapy itself is effective for survival benefit and palliation of disease^[4].

CONCLUSION

Approximately 20% of patients presenting with rectal cancer have stage IV disease^[1,134]. Therefore, a thorough knowledge of palliative options is required to optimize quality of life and provide the best chance of long-term survival. Patients undergoing palliative treatment have a relatively short duration of survival (median: 6-9 mo), with dismal 5-year survival rates (0%-5%)^[64]. This is especially true for patients who present symptomatically with obstruction, pain, bleeding and perforation. Patients undergoing chemotherapy for disseminated metastatic colorectal cancer have demonstrated median survival of 15-20 mo with various treatment options^[4]. Therefore, when evaluating patients with metastatic rectal cancer, the patient's age, comorbidity, extent of disease, functional status, tumor characteristics, and symptoms must be taken into account to determine the best possible treatment approach. Given the fact that the majority of patients ultimately succumb to their disease, the constellation of factors must be utilized to provide the most effective relief with the minimum amount of morbidity and mortality. The patient with significant metastatic burden and a relatively unobtrusive primary tumor seems to benefit from the initiation of chemotherapy without further surgical therapy. Complications necessitating surgery are quite rare in this group of patients. However, symptomatic patients with significant burden of disease require a multidisciplinary team consisting of a surgeon, medical oncologist, gastroenterologist, and/or a radiation oncologist, to develop the most efficacious palliative intervention, to achieve the best goal-directed outcome for patients and family members.

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Imran Hassan, MD, Assistant Professor, Series Editor

Neoadjuvant vs adjuvant pelvic radiotherapy for locally advanced rectal cancer: Which is superior?

Sarah Popek, Vassiliki Liana Tsikitis

Sarah Popek, Vassiliki Liana Tsikitis, Department of Surgery, University of Arizona, Tucson, AZ 85724, United States
 Author contributions: All authors contributed equally to this work; all authors analyzed the data and wrote the paper.
 Correspondence to: Vassiliki Liana Tsikitis, MD, Assistant Professor, Department of Surgery, University of Arizona, 1515 N Campbell Ave, Tucson, AZ 85724, United States. Itsikitis@surgery.arizona.edu
 Telephone: +1-520-6266788 Fax: +1-520-6262191
 Received: August 30, 2010 Revised: September 29, 2010
 Accepted: October 6, 2010
 Published online: February 21, 2011

Abstract

The treatment of locally advanced rectal cancer including timing and dosage of radiotherapy, degree of sphincter preservation with neoadjuvant radiotherapy, and short and long term effects of radiotherapy are controversial topics. The MEDLINE, Cochrane Library databases, and meeting proceedings from the American Society of Clinical Oncology, were searched for reports of randomized controlled trials and meta-analyses comparing neoadjuvant and adjuvant radiotherapy with surgery to surgery alone for rectal cancer. Neoadjuvant radiotherapy shows superior results in terms of local control compared to adjuvant radiotherapy. Neither adjuvant or neoadjuvant radiotherapy impacts overall survival. Short course versus long course neoadjuvant radiotherapy remains controversial. There is insufficient data to conclude that neoadjuvant therapy improves rates of sphincter preserving surgery. Radiation significantly impacts anorectal and sexual function and includes both acute and long term toxicity. Data demonstrate that neoadjuvant radiation causes less toxicity compared to adjuvant radiotherapy, and specifically short course neoadjuvant radiation results in less toxicity than long course neoadjuvant radiation. Neoadjuvant radiotherapy is the preferred modality for administering radiation in

locally advanced rectal cancer. There are significant side effects from radiation, including anorectal and sexual dysfunction, which may be less with short course neoadjuvant radiation.

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Key words: Locally advanced rectal cancer; Neoadjuvant radiation; Adjuvant radiation; Rectal neoplasm; Chemoradiotherapy; Neoadjuvant chemoradiotherapy

Peer reviewer: Paul E Sijens, PhD, Associate Professor, Radiology, UMCG, Hanzplein 1, 9713GZ Groningen, The Netherlands

Popek S, Tsikitis VL. Neoadjuvant vs adjuvant pelvic radiotherapy for locally advanced rectal cancer: Which is superior? *World J Gastroenterol* 2011; 17(7): 848-854 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/848.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.848>

INTRODUCTION

Colorectal cancer is the third most frequent cancer in men and women. In 2009, in the United States 40000 new cases of rectal cancer alone were diagnosed^[1]. The past 2 decades have seen many advances in the treatment of patients with rectal cancer. Surgery remains the mainstay. The standard of surgical care now includes total mesorectal excision (TME), which was shown to significantly decrease local recurrence rates^[2]. Evolution of Combined Modality Treatment (CMT) revolutionized care of locally advanced rectal cancer with the most considerable change the introduction of pelvic radiation. Improvements in preoperative staging with endorectal ultrasound and magnetic resonance imaging have allowed experimentation with different regimens of neoadjuvant (preoperative) and adjuvant (postoperative) radiotherapy (RT).

The goals of this review are to provide a critical over-

view of the most relevant clinical trials, and to evaluate the advantages and disadvantages of different RT regimens, in the adjuvant and neoadjuvant setting, for patients with locally advanced rectal cancer (stages II B and C, III A through C).

ADJUVANT RADIATION

RT for rectal cancer was first introduced in the 1980s, in an attempt to decrease rates of local recurrence in patients with locally advanced rectal cancer; at that time, the local recurrence rates after surgical resection were as high as 50%^[3].

One of the first randomized controlled trials (RCTs) to demonstrate success in control of local recurrence with the use of adjuvant therapy was published in 1985 by the Gastrointestinal Tumor Study Group^[4]. That study randomized 227 patients (data from 202 collected) to 4 arms: (1) no adjuvant therapy (the control arm) ($n = 58$); (2) adjuvant RT ($n = 50$); (3) adjuvant chemotherapy ($n = 48$); or (4) adjuvant CMT ($n = 46$). Patients in the CMT arm had significantly decreased local recurrence rates ($P < 0.009$), as compared with the control arm, but the overall survival rates did not significantly differ ($P = 0.07$). That 1985 publication ushered in the era of adjuvant therapy with RT for patients with locally advanced rectal cancer.

In the United States, the first official recommendation for the use of adjuvant chemoradiation in patients with rectal cancer came from the National Institutes of Health (NIH) consensus statement, published in 1990^[5]. The NIH set the standard of care for patients with stage II and III rectal cancer to include adjuvant chemoradiation without specifying the optimal regimen. Subsequently, extensive research has been conducted on the most advantageous timing and dosage of pelvic RT in patients with locally advanced rectal cancer (Table 1). In 1997, the Norwegian Adjuvant Rectal Cancer Project Group published the results of one of the early trials evaluating the chemotherapy dose in adjuvant chemoradiation for patients with locally advanced rectal cancer^[6]. Previous studies had shown improved locoregional control with adjuvant RT, but high toxicity and poor compliance with adjuvant CMT^[4,7]. The Norwegian trial addressed the important issue of clinically significant complications in the setting of adjuvant CMT for rectal cancer. In that trial, 144 patients were randomized to surgery alone or to adjuvant CMT (chemoradiation with long-course RT and short-term 5-fluorouracil (5-FU)-based chemotherapy). The short-term chemotherapy was tolerated by patients without sacrificing the benefits of improved local control. The minimum follow-up time was 4 years. The 5-year recurrence free rates significantly differed (64% in the CMT arm vs 46% in the surgery alone arm, $P = 0.01$), as did the 5-year survival rates (64% in CMT arm vs 50% in surgery alone arm, $P = 0.05$). Further, a meta-analysis in 1988 reviewed all RCTs evaluating adjuvant therapy (8 RT vs surgery alone, 17 chemotherapy vs surgery alone) with the endpoint of overall survival and found only a small improvement in the adjuvant chemo-

therapy arm [odds ratio (OR), 0.83, 95% CI: 0.70-0.98]. No effect on survival was found in the RT arm^[8].

NEOADJUVANT RADIATION

Efforts aimed at improving local control and long term survival stimulated experimentation with adjuvant RT in the 1990s and gave birth to the concept of neoadjuvant RT. Initial reports from small studies suggested that efficacy with neoadjuvant RT was comparable or improved compared to adjuvant RT, and toxicity was less severe. Delineating the veracity of these small studies intrigued investigators over the subsequent decade. Specifically two different regimens of neoadjuvant RT were being assessed: (1) long course RT, used mainly in the United States; and (2) short course RT, used mainly in Europe.

The European Organization for Research and Treatment of Cancer (EORTC) designed a study to evaluate the efficacy and toxicity profile of neoadjuvant RT (long-course). Four hundred and sixty-six patients were enrolled: 175 were ultimately randomized to surgery alone, and 166 randomized to neoadjuvant RT followed by surgery. Patients in the neoadjuvant arm tolerated the treatment adequately, had significantly decreased local recurrence rates (15% vs 30%, $P = 0.003$), but had no improvement in overall survival^[9]. The Swedish Rectal Cancer Trial^[10] was the first major trial to demonstrate significant improvement in local control with short-course RT (25 Gy in 5 consecutive daily fractions) followed by surgery, compared with surgery alone (11% local recurrence rate with short-course RT vs 27% without, $P < 0.001$). In addition, the Swedish trial was the only trial to demonstrate improved 5-year survival rates for patients in the neoadjuvant arm (58% with short-course RT vs 48% without, $P = 0.004$). The patient population included those with stage I rectal cancer as well as locally advanced disease. Note that the results of that trial, published in 1997, preceded surgical standardization to TME; hence, one of its drawbacks was the lack of standardization in surgical technique.

In response, the Dutch colorectal group performed a similar investigation, with the notable exception of standardizing surgery to TME^[11]. Again, patients were randomized to either short course neoadjuvant RT followed by surgery within 1 wk ($n = 695$) or surgery alone ($n = 719$). A significant decrease in local recurrence rates was found at 2 years in the neoadjuvant RT arm (2.4% vs 8.2%, $P < 0.001$), but no difference in overall survival (82% vs 81.8%, $P = 0.84$). An additional variable examined in this study was the import of a positive circumferential margin (CRM). Positive CRM was significantly correlated with an increased risk of local recurrence; and patients with positive CRM received post operative long course RT. The Dutch colorectal group confirmed the findings of the Swedish rectal trial in terms of local control, contradicted findings of improved survival, and raised a new question regarding the role of selective adjuvant RT with positive CRM. That question was addressed with the Medical Research Council (MRC) CR07 trial, whose results were

Table 1 Randomized control trials evaluating timing and dose of radiation therapy

Trial (year results published)	Study design	Patients	Follow-up (mo)	Treatment	Outcome: overall survival	Outcome: local recurrence
Swedish Rectal Cancer Trial (1997) ^[10]	RCT	1168	60	Neoadjuvant short-course RT vs surgery alone	58% vs 48% ($P = 0.004$)	11% vs 27% ($P < 0.001$)
Dutch TME Trial (2001) ^[11]	RCT	1861	24	Neoadjuvant short-course RT (standard TME) vs surgery alone	82% vs 81.8% ($P = 0.84$)	2.4% vs 8.2% ($P < 0.001$)
German Rectal Cancer Study Group (2004) ^[14]	RCT	799	60	Neoadjuvant long-course RT + chemotherapy vs adjuvant long-course RT + chemotherapy	76% vs 74% ($P = 0.80$)	6% vs 13% ($P = 0.006$)
Polish Colorectal Group (2006) ^[16]	RCT	312	48	Neoadjuvant short-course RT vs neoadjuvant long-course RT	67.2% vs 66.2% ($P = 0.96$)	14.2% vs 9% ($P = 0.17$)
MRC-NCIC (2009) ^[17]	RCT	1350	60	Neoadjuvant short-course RT vs selective adjuvant long-course RT + chemotherapy	70% vs 67.9% (HR 0.91, 95% CI: 0.73 to -1.13, $P = 0.40$)	4% vs 11% (HR 0.39, 95% CI: 0.27 to 0.58, $P < 0.0001$)
NSABP R-03 (2009) ^[18]	RCT	267	60	Neoadjuvant long-course RT + chemotherapy vs postoperative long-course RT + chemotherapy	74.5% vs 65.6% ($P = 0.065$)	10.7% vs 10.7% ($P = 0.69$)
Stockholm III (2010) ¹	RCT	303	Ongoing	Neoadjuvant short-course RT + surgery within 1 wk vs neoadjuvant short-course RT + surgery 4 to 8 wk later vs neoadjuvant long-course RT + surgery 4 to 8 wk later		Ongoing

¹Interim results. RCT: Randomized control trial; RT: Radiotherapy; TME: Total mesorectal excision; MRC-NCIC: Medical Rectal Council-National Cancer Institute of Canada; NSABP: National Surgical Adjuvant Breast and Bowel Project; HR: Hazard ratio.

published in 2009 (see below).

As more data became available, two meta-analyses were published in 2000 and 2001 asking two important questions. First, what is the efficacy of neoadjuvant RT in improving survival, and decreasing local recurrence rates^[12] and second, what is superior in improving survival and decreasing local recurrence: adjuvant or neoadjuvant therapy^[13]? Cammà *et al*^[12] addressed the first question; their analysis included 14 RCTs and found that neoadjuvant RT significantly improved the 5-year survival rates (OR, 0.84, 95% CI: 0.72-0.98, $P = 0.03$), the cancer-related mortality rates (OR, 0.71, 95% CI: 0.61-0.82, $P < 0.001$), and the local recurrence rates (OR, 0.49, 95% CI: 0.38-0.62, $P < 0.001$). The Colorectal Cancer Collaborative Group evaluated 22 RCTs (involving a total of 8507 patients) to determine the answer to the second question. The RCTs compared neoadjuvant therapy, adjuvant therapy, or surgery alone and included both short-course and long-course RT. The group found a significant improvement in the yearly local recurrence rate in the neoadjuvant RT arm (a 46% decrease vs surgery alone, $P = 0.00001$) and in the adjuvant RT arm (a 37% decrease vs surgery alone, $P = 0.002$). But the 5-year survival rate (45% with RT vs 42.1% with surgery alone) and the overall survival rate (62% with RT vs 63% with surgery alone, $P = 0.06$) did not significantly differ. Of note, 30 Gy was identified as the biologically active dose of RT.

The issue of neoadjuvant vs adjuvant RT is further clouded by the inclusion of chemotherapy into treatment regimens. In 2004, the German Rectal Cancer Group compared neoadjuvant CMT with adjuvant CMT in patients with locally advanced rectal cancer^[14]. Patients were randomly assigned to 2 arms: (1) neoadjuvant CMT

($n = 421$); and (2) adjuvant CMT ($n = 402$). All patients received long-course RT and 5-FU-based chemotherapy. The 5-year survival rates (76% with neoadjuvant CMT vs 74% with adjuvant CMT, $P = 0.8$) did not significantly differ. But the local recurrence rates significantly improved in the neoadjuvant arm (6% with neoadjuvant CMT vs 13% with adjuvant CMT, $P = 0.006$). The adjuvant arm had higher rates of acute and long-term toxicity (acute: 27% with neoadjuvant CMT vs 40% with adjuvant CMT, $P = 0.001$; long-term: 14% vs 24%, $P = 0.01$). Another important finding was that overstaging of patients resulted in unnecessary administration of neoadjuvant CMT.

In 2005, Law *et al*^[15] contributed to the controversy surrounding overstaging and overtreatment by suggesting that low risk stage II patients do not benefit from neoadjuvant therapy. They reported data on 224 patients with stage II disease who underwent TME surgery without neoadjuvant or adjuvant CMT. They hypothesized that the benefit of treating stage II disease with adjuvant therapy was less than the risk of complications or toxicity from CMT. Median follow up was 43 mo. Five years recurrence rate was reported as 6% which is comparable to previously reported values for patients undergoing neoadjuvant RT and surgery (2.4%-14.2%, Table 1)^[10,11,14,16-18]. Overall survival was reported as 71% which is also similar to data from previous trials for patients undergoing neoadjuvant RT and surgery (58%-82%, Table 1)^[10,11,14,16-18]. They conclude that there is no advantage to treating low risk stage II rectal cancer patients with negative margins with neoadjuvant therapy. There was an emphatic response to this statement from many authors who felt that that not treating stage II patients with neoadjuvant CMT was egregious^[19].

Once short-course neoadjuvant RT was established to

be safe and effective, the next step was to compare its efficacy with that of long-course neoadjuvant RT. In 2006, the Polish Colorectal Study Group randomized 312 patients to either (1) neoadjuvant short-course RT, surgery within 1 wk, and optional adjuvant chemotherapy or (2) neoadjuvant long-course RT, neoadjuvant chemotherapy, and surgery 6 to 8 wk later. Early RT toxicity was higher in the long-course RT arm (18.2% with long-course RT vs 3.2% with short-course RT, $P < 0.001$), but the 5-year survival rates (66% vs 67%, $P = 0.96$) and the local recurrence rates (9% vs 14%, $P = 0.17$) did not significantly differ. The study concluded that short-course and long-course RT had comparable efficacy, but short-course RT remains the standard of care in Poland because of the lower toxicity distribution and higher compliance rates. In 2009, Guckenberger *et al*^[20] introduced a new regimen for short-course RT, administering twice-daily doses of 2.9 Gy for 1 wk (total dose, 29 Gy) to 118 patients. That regimen lowered the single dose and allowed a 6-h tissue recovery period between treatments, but the daily dose was the same as with standard short-course RT (5 Gy daily \times 5 d). The 188 patients had clinical stage II (50%), III (41.5%), and IV (8.5%) rectal cancer; they all received adjuvant 5-FU-based chemotherapy. The median follow-up time was 46 mo. Late toxicity (grade II) occurred in 11% of the patients. The local control rate was 92%. The 5-year survival rate of 67% compared favorably with previously reported rates in randomized trials that also evaluated daily dosing of short-course RT (58%-82%, Table 1)^[10,11,16].

In the United States, the National Surgical Adjuvant Breast and Bowel Project (NSABP) R-03 trial also compared neoadjuvant CMT and adjuvant CMT in patients with locally advanced rectal cancer^[18]; the NSABP R-03 trial was similar to the German rectal cancer group trial published in 2004^[14]. Both arms of the NSABP R-03 trial used long-course RT, and the chemotherapy regimen was 5-FU-based with leucovorin. The study was initially powered for a sample size of 900, but had to close early due to poor accrual. In all, 123 patients were randomized to neoadjuvant CMT and 131 to adjuvant CMT. The surgical technique was not standardized, but rather left to the discretion of the surgeon. Primary endpoints were the disease-free survival and overall survival rates. The overall survival rates (74.5% with neoadjuvant CMT vs 65.6% with adjuvant CMT, $P = 0.065$) and the locoregional recurrence rates [Hazard ratio (HR), 0.86, 95% CI: 0.41-1.81, $P = 0.693$] did not significantly differ - in contrast to the 5-year disease-free survival rates (64.7% vs 53.4%, $P = 0.011$). Of note, the rate of complete pathologic response was 15% in the neoadjuvant CMT group but the rates of sphincter preservation (48% with neoadjuvant CMT vs 39% with adjuvant CMT) did not significantly differ, per the opinion of the operating surgeon. It is difficult to draw conclusions from the NSABP R-03 trial, because it was underpowered and not standardized in operating technique.

In 2009, the MRC and National Cancer Institute of

Canada (NCIC) combined CR07/CTG C016 trial^[17] addressed the issue of selective adjuvant CMT based on operative margins. The trial randomized 1 350 patients to 2 arms: (1) neoadjuvant short-course RT; or (2) initial surgery with selective adjuvant long-course RT and 5-FU-based chemotherapy based on circumferential (CRM) involvement. The surgical technique was not standardized. Median follow-up time was 4 years; the primary outcome measure was local recurrence. In the selective adjuvant arm, 12% of the patients had a positive CRM, 78% of whom then underwent adjuvant RT. In the neoadjuvant arm, a 61% relative risk reduction (HR, 0.39, CI: 0.27-0.58, $P < 0.0001$) was found for local recurrence, and a 24% improvement (HR, 0.76, CI: 0.62-0.93, $P = 0.013$) was found for disease-free survival. But the 2 arms did not significantly differ in overall survival rates. The MRC CR07/NCIC-CTG C016 investigators concluded that neoadjuvant short-course RT was effective therapy in patients with operable rectal cancer.

BENEFITS OF NEOADJUVANT RT

With the advent of neoadjuvant therapy, reliable methods to evaluate its efficacy and to determine the significance of response to treatment have been necessary. Pathologic tumor response has risen to the forefront, although several tumor grading systems are currently in use. Two recent prospective studies evaluated the impact of tumor response on overall survival in patients with locally advanced rectal cancer^[21,22]. Both studies concluded that tumor downstaging was the only variable that significantly and independently correlated with improved survival.

Most significantly the addition of neoadjuvant radiation has resulted in significant downsizing and downstaging of low locally advanced rectal cancers making sphincter preserving procedures feasible and with good oncologic outcomes. Weiser *et al*^[23] performed a retrospective analysis of 148 patients with locally advanced rectal cancer (within 6 cm of the anal verge) who were treated with neoadjuvant CMT (long-course RT) and selective adjuvant chemotherapy. The decision to perform sphincter-preserving surgery was made intraoperatively. The likelihood of sphincter-preserving surgery was associated with significant tumor downstaging. They concluded that neoadjuvant CMT facilitated sphincter-preserving surgery in addition to intersphincteric resection.

However, short course neoadjuvant radiation does not seem to offer the same results. Sauer *et al*^[14] did not find a significant difference in the rates of sphincter-preserving surgery between their neoadjuvant and adjuvant treatment arms. However, they did note that, within the subgroup of patients deemed to require abdominoperineal resection preoperatively ($n = 194$), the number of abdominoperineal resections actually performed was significantly lower in the neoadjuvant arm ($P = 0.004$). Bujko *et al*^[24] specifically looked at whether neoadjuvant short-course RT offered a benefit for sphincter preservation over neoadjuvant CMT in 316 patients and found no significant difference:

61% of patients in the RT arm and 58% in the CMT arm underwent sphincter-preserving surgery ($P = 0.57$). In conclusion, although short-course RT improves local control, no strong evidence exists that it also improves rates of sphincter-preserving surgery indicating short-course neoadjuvant RT does not have a significant effect on preoperative tumor downsizing or downstaging.

A significant benefit of neoadjuvant RT is patient compliance with treatment. Adjuvant RT has been associated with higher rates of treatment interruption. Lebowitz *et al*^[25] assessed for principle factors associated with treatment interruption in 113 RT patients. Patients in the adjuvant arm had a significantly increased chance of RT interruption, as compared with the neoadjuvant RT arm (OR, 14.08, CI: 1.55-127.87). Development of an adverse event was also significantly correlated with RT interruption (OR, 20.66, CI: 1.76-242).

ANORECTAL FUNCTION OUTCOMES

One of the most important variables evaluating quality of life in rectal cancer is anorectal function, specifically bowel function and sexual function^[26]. This is affected by both chemoradiation and surgical technique. The Dutch colorectal group assessed anorectal functional outcomes after short-course preoperative RT and TME and found significant differences between patients who did vs did not undergo RT^[27]. RT patients had higher rates of fecal incontinence (62% with RT vs 38% without, $P < 0.001$), pad wearing as a result of incontinence (56% vs 33%, $P < 0.001$), and anal blood loss (11% vs 3%, $P = 0.004$). RT patients also reported significantly lower satisfaction with bowel function.

A second prospective study randomized 316 patients to (1) short-course neoadjuvant RT or (2) long-course neoadjuvant chemoradiation^[26]. The goal was to evaluate anorectal and sexual dysfunction and quality of life. Early complications were more common in the chemoradiation arm, but no significant differences were found in the degree of anorectal and sexual function or in quality of life.

In addition to bowel and sexual dysfunction, RT patients may experience acute and late RT toxicity, including nausea/vomiting, postoperative hernia, femoral neck fracture, skin problems (nonhealing perineal wounds), ileus, anastomotic stricture, and fistula. The Dutch colorectal group assessed RT toxicity, intraoperative and postoperative complications, and other variables in patients who underwent short-course neoadjuvant RT vs TME alone^[27]. No differences were found in operative time, intraoperative complications, or hospital stay; however, the amount of intraoperative blood loss was higher in the RT arm ($P < 0.001$). Rates of perineal complications were also higher (29% with RT vs 18% with TME alone, $P = 0.008$). But no significant differences were found in the rate of abdominal wound complications (4.0% with RT vs 3.3% with TME alone) or in the overall postoperative mortality rate.

Frykholm *et al*^[28] looked at long-term complications

(minimum follow-up time, 5 years) after either neoadjuvant short-course RT ($n = 255$) or adjuvant long-course RT ($n = 127$), as compared with surgery alone (control group, $n = 82$). Long-term complications (defined as occurring at least 6 mo postoperatively) included recurrent abdominal pain, diarrhea, fecal incontinence, ileus, cystitis, paresthesias, delayed wound healing, and any neurologic dysfunction. The percentage of patients with small bowel obstruction did not significantly differ between the neoadjuvant RT group and control group. In the adjuvant RT group, the risk of developing a small bowel obstruction was significantly higher ($P < 0.01$). Overall, the frequency of complications possibly related to RT in the neoadjuvant group was 20%; in the adjuvant group, 41%. However, in the control group, the percentage of similar complications was 23%. In addition to finding a significant decrease in local recurrence after neoadjuvant short-course RT (13% in the neoadjuvant group vs 22% in the adjuvant group, $P = 0.02$), the cumulative risk of bowel obstruction was significantly higher in the adjuvant group.

Minsky *et al*^[29] also demonstrated significantly lower rates of adverse events and improved compliance in patients treated with neoadjuvant CMT compared to patients treated with adjuvant CMT. Despite receiving higher doses of chemotherapy, the neoadjuvant arm experienced a 13% incidence of acute grade 3 or 4 toxicity compared to a 48% incidence in the adjuvant arm ($P = 0.045$). A meta-analysis by Birgisson *et al*^[30] found that the most common late adverse effects of RT were bowel obstruction, bowel dysfunction (fecal incontinence), and sexual dysfunction. Several different RT regimens were included in the meta-analysis, offering some insight into how complications correlated with dosage. Overall, in the more recent studies which used lower doses and better techniques, the rates of adverse events were lower. Unfortunately, to date, no specific markers have been identified that might help predict which patients have a higher risk of acute RT toxicity. Further work is needed in this important area of ongoing research.

CONCLUSION

Patients with locally advanced rectal cancer clearly benefit, in terms of locoregional control, from both neoadjuvant and adjuvant RT; and patient compliance is better with neoadjuvant RT. No definitive evidence demonstrates the superiority of using short vs long-course RT.

The current standard treatment for patients with locally advanced rectal cancer in the United States consists of neoadjuvant radiation (45 to 55 Gy administered over 5 to 6 wk), followed by neoadjuvant chemotherapy (5-FU-based infusion + leucovorin), surgery 6 to 8 wk after completion of chemotherapy, and additional adjuvant chemotherapy after surgery^[31]. In contrast, the standard regimen in most of Europe is now neoadjuvant short-course RT. The most recent European Rectal Cancer Consensus Conference concluded that neoadjuvant short-course RT (25 Gy administered over 1 wk), especially when combined with

5-FU-based chemotherapy, improved local control for patients with locally advanced rectal cancer^[32].

Several important trials are currently in progress. The next interim analysis from Stockholm III should provide some clues in the debate concerning short-course neoadjuvant RT and timing of surgery. Given the lack of data supporting improved overall survival rates with neoadjuvant or adjuvant RT, treatment failure in patients with stage II and III rectal cancer likely arises from distant metastases.

Current research trials focus on evaluating the impact of chemotherapy regimens on systemic disease in patients with locally advanced rectal cancer. The NSABP R-04 trial (radiation therapy and either capecitabine or fluorouracil with or without oxaliplatin before surgery in treating patients with resectable rectal cancer is designed to compare capecitabine (with or without oxaliplatin) vs 5-FU (with or without oxaliplatin) in patients with operable rectal cancer who undergo neoadjuvant RT. The EORTC is also currently enrolling patients in a similar trial comparing neoadjuvant CMT and adjuvant chemotherapy with (1) capecitabine and oxaliplatin vs (2) capecitabine alone in patients with locally advanced rectal cancer (PETACC-6).

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S- Editor Sun H L- Editor O'Neill M E- Editor Zheng XM



Imran Hassan, MD, Assistant Professor, Series Editor

Sphincter preservation for distal rectal cancer - a goal worth achieving at all costs?

Jürgen Mulsow, Des C Winter

Jürgen Mulsow, Des C Winter, Institute for Clinical Outcomes Research and Education, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland

Author contributions: Mulsow J and Winter DC both contributed to the literature review and drafting of manuscript.

Correspondence to: Des C Winter, Professor, Institute for Clinical Outcomes Research and Education, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland. winterd@indigo.ie

Telephone: +353-1-2695033 Fax: +353-1-2609249

Received: August 30, 2010 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: February 21, 2011

Abstract

To assess the merits of currently available treatment options in the management of patients with low rectal cancer, a review of the medical literature pertaining to the operative and non-operative management of low rectal cancer was performed, with particular emphasis on sphincter preservation, oncological outcome, functional outcome, morbidity, quality of life, and patient preference. Low anterior resection (AR) is technically feasible in an increasing proportion of patients with low rectal cancer. The cost of sphincter preservation is the risk of morbidity and poor functional outcome in a significant proportion of patients. Transanal and endoscopic surgery are attractive options in selected patients that can provide satisfactory oncological outcomes while avoiding the morbidity and functional sequelae of open total mesorectal excision. In complete responders to neo-adjuvant chemoradiotherapy, a non-operative approach may prove to be an option. Abdominoperineal excision (APE) imposes a permanent stoma and is associated with significant incidence of perineal morbidity but avoids the risk of poor functional outcome following AR. Quality of life following AR and APE is comparable. Given the choice, most patients will choose AR over APE, however patients following APE positively appraise this option. In striving toward sphinc-

ter preservation the challenge is not only to achieve the best possible oncological outcome, but also to ensure that patients with low rectal cancer have realistic and accurate expectations of their treatment choice so that the best possible overall outcome can be obtained by each individual.

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Key words: Rectal cancer; Survival; Local recurrence; Morbidity; Anorectal function; Quality of life; Patient preference

Peer reviewer: Hiroki Yamaue, MD, PhD, Department of Surgery, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama 641-8510, Japan

Mulsow J, Winter DC. Sphincter preservation for distal rectal cancer - a goal worth achieving at all costs? *World J Gastroenterol* 2011; 17(7): 855-861 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/855.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.855>

INTRODUCTION

In the management of patients with rectal cancer, sphincter preservation is a priority and regarded a marker of surgical quality. Technical and technological advances have led to an increase in sphincter preserving surgery and a fall in the rate of abdominoperineal excision (APE)^[1]. Furthermore, the recognition of the oncological importance of the circumferential, rather than distal resection margin, has allowed an increasingly aggressive surgical approach. The knowledge that a distal margin of 1 cm will safely allow complete tumor removal affords an ever greater proportion of patients the opportunity of sphincter preserving surgery for low rectal cancer^[2]. In addition, our ever increasing understanding of tumor behaviour gives patients new options in the form of non-operative

treatment (following complete response to neo-adjuvant treatment), or transanal excision in selected circumstances. On the other hand, tumor down-staging following neo-adjuvant chemoradiotherapy has not led to the expected increase in sphincter preserving surgery.

Thus, for patients with low rectal tumors, and for whom APE would formerly have been the only option, a number of sphincter preserving options are now available. However, while it may be technically possible to reconstruct (or avoid radical surgery altogether) an increasing majority of patients with rectal cancer, we should pause to consider the overall merits of this approach and consider the patient's overall outcome (both oncological and functional), while remembering that there remain acceptable non-reconstructive alternatives (APE or low Hartmann's procedure). In doing so, a number of factors must be considered and the 'costs' of sphincter preservation evaluated.

ONCOLOGICAL OUTCOME IN THE TREATMENT OF RECTAL CANCER

The oncological outcome is of paramount importance whether anterior resection (AR), APE, transanal excision, or a non-operative approach is adopted in the treatment of low rectal cancer.

High rates of circumferential resection margin (CRM) positivity (up to 40%) following APE in some series and consequent high local recurrence rates have led to suggestions that the outcome following APE is inherently worse than that following AR. It does appear that rectal tumors in patients who undergo APE are often more locally advanced, more poorly differentiated, and show a lesser response to neo-adjuvant chemoradiotherapy^[3]. However, with meticulous surgery and the avoidance of tumor perforation and margin positivity, results following APE can be similar to those after AR^[4]. Indeed, local recurrence rates in the order of 5% can be achieved following the application of a standardised approach^[5,6].

Undoubtedly the technique of APE has drifted from that originally described by Miles^[7] in which a wide dissection of the rectum was performed to produce a cylindrical specimen. Application of TME principles and evolution in technique have resulted in an APE in which the specimen tapers (Morson's waist) at the level of the pelvic floor with a consequent narrow circumferential resection margin and risk of CRM positivity and tumor perforation. Recourse to originally described principles *via* an extra-levator approach avoids "waisting" of the specimen^[8] and reduces the rate of CRM involvement^[9]. Nonetheless, rates of CRM involvement may still lag behind those seen in AR^[10] and there remains a need to further examine surgical technique in APE and develop a standardised approach with appropriate training if needed.

Inter-sphincteric resection represents the most extreme form of sphincter preserving surgery in which part, or all, of the internal sphincter is resected. This approach may be applied to tumors within 2 cm of the sphincter

complex and is made feasible by the recognition that distal intramural tumor spread beyond 1 cm is uncommon. Thus, inter-sphincteric resection becomes an option for patients with tumors within 2 cm of the sphincter complex, in whom pre-operative continence is intact, and for whom the tumor, at least in its distal part, is confined to the rectal wall. Follow-up suggests that local (6.6%) and distant (8.8%) recurrence rates^[11] are comparable to those in published series of APE. Patients with locally advanced (T3-T4) tumors may become candidates for inter-sphincteric resection if a favourable down-staging response to neo-adjuvant chemoradiotherapy is demonstrated^[3]. Those who are not suitable for inter-sphincteric resection and require APE are likely to self-select as they have locally advanced tumors, that are poorly differentiated and show poor response to neo-adjuvant treatment^[3].

Laparoscopy is increasingly employed as a less invasive approach in the management of rectal cancer. While the initial results from the UK MRC CLASSIC trial highlighted increased rates of margin positivity following laparoscopic rectal cancer surgery (when compared to conventional, open TME)^[12], the long-term oncological outcomes do not appear to be compromised^[13,14]. This study remains the only randomised controlled trial to assess the role of laparoscopy in rectal cancer, however results from prospective series of laparoscopic resection have also demonstrated similar oncological outcomes to those reported following open TME^[15].

Transanal surgery for rectal cancer represents an attractive approach that may allow the morbidity and functional sequelae of total mesorectal excision (TME) to be avoided. Better surgical results with lower margin positivity are achieved following transanal endoscopic microsurgery (TEMS) than conventional transanal (TA) excision (2% *vs* 16%)^[16], however outcomes are generally inferior to those following radical resection with a 3-5 fold increased local recurrence risk^[17]. TEMS appears to be a reasonable option (LR < 5%) in selected patients with favourable pathological features (pT1 Sm1; well or moderately differentiated; < 3 cm diameter; no lymphovascular invasion)^[18]. For tumors with less favourable features, the oncological result following TEMS is inferior to that seen after TME. Difficulty in reliably predicting the T-stage pre-operatively remains an obstacle to patient selection. Likewise, prediction of N-stage is problematic as up to 18% of T1 tumors will have associated nodal disease. However, in patients with adverse pathological features after TEMS, subsequent conversion to radical surgery does not appear to be associated with significantly increased LR rates^[18]. In reality, the decision to adopt a transanal approach is frequently based upon the fitness of the patient.

One-fifth to one-quarter of patients following neo-adjuvant chemoradiotherapy will show a complete pathological response. Predicting those likely to respond and those who have had a complete pathological response remains difficult - up to 40% of patients who appear to have had a complete clinical response have residual disease following

resection^[19]. Conversely, approximately 10% of patients who have an incomplete clinical response will show a complete pathological response^[20]. Observation alone may be a viable alternative in selected patients who show a complete clinical response to neo-adjuvant therapy^[20]. Local recurrence has been reported in 11% of those who had a sustained complete clinical response. These patients appear amenable to salvage therapy without adverse oncological outcome in the event of local recurrence^[21].

There may also be a role for full thickness transanal excision of tumor in selected patients with T3 tumors who show an excellent response to neo-adjuvant chemoradiotherapy and who are deemed unfit for or refuse TME, or who had a perceived complete response to neo-adjuvant treatment. The limited available data point to local recurrence and survival figures that are comparable to those achieved with radical surgery^[22]. This approach requires further validation.

Finally, endoscopic submucosal dissection is an evolving technique that may represent an alternative sphincter preserving approach in the management of rectal tumors. This technique has been reported with low complication rates and in patients in whom complete resection is achieved (approximately 70%) recurrence rates at short-term follow-up are low^[23]. Further studies are required to establish the role of this technique.

FUNCTIONAL OUTCOME AND QUALITY OF LIFE FOLLOWING SURGERY FOR RECTAL CANCER

Functional outcome

Frequency, urgency, and soiling (anterior resection syndrome) are common problems after anterior resection that reflect loss of the capacitance and compliance of the rectal reservoir. Approximately 60% of patients experience some degree of incontinence, while one-third experience frequent symptoms of urgency and frequency. Post-operative studies suggest that anorectal dysfunction after low anterior resection is more a factor of reduced compliance and capacity, than diminished sphincter function^[24,25]. Furthermore, reflexes of the anal sphincter that help to maintain continence are preserved after low anterior resection^[26].

Patients undergoing inter-sphincteric resection have the additional insult of reduced internal sphincter function^[24]. Inter-sphincteric resection is associated with a fall in resting anal canal pressures^[27] and continence when compared to conventional anastomosis, but not with a worsening of stool frequency (typically averaging 2/24 h^[28]) and urgency^[29]. Long-term satisfactory continence rates are achievable in 75% of patients^[11]. Outcomes, particularly in the first post-operative year, can be improved by performing only a partial or subtotal resection of the internal sphincter and through construction of a colonic J-pouch^[27,30,32]. Pre-operative radiotherapy significantly worsens the functional outcome following inter-sphincteric resection^[11].

Following straight anastomosis progressive dilatation

of the neorectum can allow some improvement in compliance^[33] and function over time. Colonic reservoirs (J-pouch or coloplasty) may allow early preservation of function by providing a neorectum functionally comparable to the resected rectum. It is technically possible to create a J-pouch in the majority of patients (95%)^[34]. With optimum pouch size (5 cm)^[35,36] and level of anastomosis (< 8 cm from the anal verge)^[37], there appear to be functional advantages to the creation of a colonic J-pouch. Patients undergoing low anterior resection with J-pouch reconstruction have less stool frequency and urgency when compared to those with a straight anastomosis, however this benefit is not maintained beyond two years^[34]. Surprisingly, this functional gain may not impact positively on quality of life after surgery^[38]. Evidence would suggest that there is no significant advantage to coloplasty over straight anastomosis^[38]. Side-to-end anastomosis using a short side limb may represent an alternative to colonic pouch with the limited available data suggesting comparable functional and surgical outcomes, however further studies are needed^[39-41].

The benefits of the colonic pouch may not be attributable to an increased capacity when compared to straight anastomosis, but rather due to the interruption of normal propulsive motility^[42,43].

Pre- or post-operative irradiation has a significant negative impact on function following anterior resection. In the Dutch TME study, pre-operative radiotherapy was associated with a significant increase in bowel frequency and incontinence (62% *vs* 38% for surgery alone) and this had a significant negative impact on patient satisfaction and daily activity^[44]. Incontinence was worst in patients with lower tumors^[44]. These findings have been replicated in other studies with long-term follow-up showing an approximate doubling of symptoms of faecal incontinence, soiling and bowel frequency when compared to patients treated with surgery alone^[45]. Anorectal manometry has shown irradiated patients to have significantly lower resting and squeeze pressure, while endoanal ultrasound has shown increased scarring of the anal sphincter when compared to non-irradiated patients^[24,45]. Short course pre-operative radiotherapy and pre-operative long-course chemoradiotherapy appear to impact similarly on anorectal function^[46]. The functional outcome following post-operative radiotherapy is worse than following pre-operative treatment with patients experiencing increased frequency of defecation and clustering^[47].

While reduced following pre-operative radiotherapy, the functional result in patients undergoing low anterior resection with colo-anal anastomosis appears to better with a colonic J-pouch rather than straight anastomosis or coloplasty at 24 mo follow-up^[48].

Despite increased tumor down-staging, pre-operative conventionally fractionated radiotherapy does not appear to confer an advantage with respect to sphincter preservation over short-course radiotherapy^[49].

Extended pelvic lymphadenectomy is frequently performed in Japan as an adjunct to TME, and often without neo-adjuvant treatment. This approach does not appear to confer an oncological advantage when compared to

TME alone (with neoadjuvant treatment) and is associated with an increased incidence of urinary and sexual dysfunction^[50-52].

Quality of life

There is an absence of randomised studies comparing outcomes following APE and AR for low rectal tumors (due to presumption that AR is superior). As a result, inferences as to their comparative quality of life outcomes can only be drawn from individual studies. None-the-less, the available data challenges the presumption that a permanent stoma automatically renders an inferior quality of life outcome when compared to that following restorative surgery. A meta-analysis of over 1400 patients from 11 studies showed no difference in general quality of life scores between patients who underwent APE and AR. While APE was associated with better emotional and cognitive function scores and superior future perspectives (patients' understanding of disease stage), vitality and sexual function scored better in patients undergoing AR^[53]. These findings were consistent with those of an earlier meta-analysis^[54], however, their interpretation must be tempered by the poor quality of a number of individual studies, and the limited follow-up duration which fails to allow for the progressive functional improvement patients often experience following AR.

MORBIDITY

The argument in favour of observation (and/or trans-anal excision) in complete responders to neo-adjuvant treatment is the avoidance of the morbidity and functional loss associated with TME, with or without a temporary or permanent stoma. Anorectal dysfunction, sexual dysfunction, difficulty voiding, and urinary incontinence are seen in up to one-third of patients following TME. Furthermore, these problems are exacerbated by pre-operative radiotherapy. Post-operative morbidity following laparoscopic and open rectal resection appears to be similar^[12], while a benefit to the laparoscopic approach with respect to long-term complications such as adhesion small bowel obstruction and incisional hernia remains to be proven^[55]. Laparoscopic resection appears to impact similarly on bladder function when compared to open TME, but may be associated with a worse outcome with regard to male sexual function^[56].

For patients undergoing TME, larger studies have shown overall rates of early morbidity of approximately 40%. This figure increases to almost 50% following pre-operative radiotherapy. Of patients undergoing APE, approximately one-fifth develop perineal wound problems^[57]. The incidence of perineal wound problems rises to 30% following radiotherapy^[57] and doubles following extralevator APE (38%)^[10]. Eleven percent of patients undergoing AR developed clinical anastomotic leaks in the Dutch TME trial. The leak rate was not affected by pre-operative radiotherapy, but was reduced with proximal defunctioning stoma (8% *vs* 16%)^[57]. The mortality rate for non-irradiated patients was 3.3% in the same study.

Again, from the Dutch study we know that approximately 50% of patients undergoing AR will have a de-functioning stoma. It is worth noting that at long-term follow-up (median 48 mo) 21% of patients in one study who had undergone sphincter preserving surgery still had a stoma^[58]. Loop ileostomy closure is associated with 17% morbidity, however the majority (80%) of patients can be managed non-operatively^[59].

PATIENT PREFERENCE

The limited available evidence suggests that a majority (65%) of patients with rectal cancer are willing to defer decision making about their surgery to their surgeon^[60].

What is not known, unlike for breast cancer, is the role that patients with rectal cancer would like to adopt in decision making, and how their given role influences their satisfaction with decision making and outcomes. We do know however that the relative importance that surgeons place on various outcomes such as permanent stoma and incontinence is often not matched by their patients^[61]. Surgeons may in particular underestimate their patients' concerns. Furthermore, surgeon's choices may frequently be at odds with their patient's inherent and perhaps unrecognised true preference^[62]. Patients, for example, express a stronger desire to avoid chemotherapy than to avoid permanent stoma, while doctors express the opposite view.

Multimedia decision aids (incorporating patient values into evidence based data) have been used to assess and quantify the relative importance patients with rectal cancer place on different quality of life outcomes. Patients who have had surgery place greater emphasis on the avoidance of incontinence post-operatively than the avoidance of a permanent stoma^[61].

Trade-off techniques are another useful means of gauging patient's true preferences and will often highlight disparity between patients' preferences and those of their physicians^[62]. Using this technique, the strength of a preference is measured by determining the degree of risk of a particular (poor) outcome that the patient would be willing to accept in order to have the treatment. When patient preferences are assessed using time-trade methods, patients strongly express a desire to avoid a stoma with 65% willing to trade a mean of 34% of their life expectancy to avoid this outcome^[63]. Furthermore, patients expressed a stronger desire to avoid the option of APE and thus permanent stoma than their treating physicians. Again, in patients who have had surgery for rectal cancer, the majority of those without a stoma would be willing to trade frequent (monthly) episodes of incontinence in order to avoid a permanent stoma^[64]. APE patients would however hypothetically trade fewer years of remaining life to be without a stoma, than AR patients would to be without incontinence^[65].

While patients may often be happy to defer decisions as to the type of surgery to their surgeons, the majority of those patients who do choose, would favour AR over APE^[60]. More patients who have had AR would choose

that option again, than patients who have had APE (69% vs 46%)^[60]. Interestingly, at longer term follow-up 80% of patients who had APE indicate that they would choose the same option given the benefit of their experience^[60].

CONCLUSION

Sphincter preservation in rectal cancer - a goal worth achieving at all costs? The answer must be no. While we should strive toward sphincter preserving options, we must recognize the limitations of currently available approaches and accept that sphincter preservation may not be the best overall option for each individual patient.

Oncological outcomes following AR and APE should be equivalent, however there remains room to uniformly improve and standardise approaches and outcomes in APE. If equivalence for oncological outcome is achieved, then functional outcome, quality of life, and ultimately patient preference become of paramount importance in decision making for the treatment of low rectal cancer. Anorectal dysfunction and poor functional outcome are common following AR. The alternative of APE or low Hartmann's procedure imposes a permanent stoma. Quality of life following APE appears to be similar to that following AR. Given the choice, most patients would choose AR over APE. It is doubtful however that patients appreciate fully the functional outcome following AR, and also likely that patients harbour excessively negative misconceptions about life with a permanent stoma. Patients must be informed that function may not be as good as they expect after AR, and also that patients who have undergone APE positively appraise this option at follow-up. The morbidity associated with stoma reversal (following AR), and the significant risk of perineal wound problems following APE must also be considered. Non-radical and even non-operative approaches are increasingly an option in the management of selected patients with low rectal cancer that obviate the morbidity and outcomes following TME. Ultimately we must ensure that patients with low rectal cancer have realistic expectations of their treatment options and that their decisions are truly informed.

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S- Editor Sun H L- Editor O'Neill M E- Editor Ma WH



Imran Hassan, MD, Assistant Professor, Series Editor

Minimally invasive surgery for rectal cancer: Are we there yet?

Bradley J Champagne, Rohit Makhija

Bradley J Champagne, Division of Colorectal Surgery, University Hospitals Case Medical Center, Cleveland, OH 44106, United States

Rohit Makhija, Laparoscopic Colorectal Fellow, University Hospitals Case Medical Center, Cleveland, OH 44106, United States

Author contributions: Champagne BC wrote the paper, performed a literature search, analyzed data; Makhija R performed literature search and contributed to writing the paper.

Correspondence to: Bradley J Champagne, MD, FACS, Associate Professor, Division of Colorectal Surgery, University Hospitals Case Medical Center, Cleveland, OH 44106, United States. brad.champagne@uhhospitals.org

Telephone: +1-216-5267221 Fax: +1-216-8445957

Received: September 26, 2010 Revised: December 16, 2010

Accepted: December 23, 2010

Published online: February 21, 2011

Abstract

Laparoscopic colon surgery for select cancers is slowly evolving as the standard of care but minimally invasive approaches for rectal cancer have been viewed with significant skepticism. This procedure has been performed by select surgeons at specialized centers and concerns over local recurrence, sexual dysfunction and appropriate training measures have further hindered widespread acceptance. Data for laparoscopic rectal resection now supports its continued implementation and widespread usage by experienced surgeons for select patients. The current controversies regarding technical approaches have created ambiguity amongst opinion leaders and are also addressed in this review.

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Key words: Laparoscopic; Rectal cancer; Minimally invasive; Mesorectal excision

Peer reviewer: Dr. Stefan Riss, Department of Surgery, Medical University of Vienna, Vienna, 1090, Austria

Champagne BJ, Makhija R. Minimally invasive surgery for rectal cancer: Are we there yet? *World J Gastroenterol* 2011; 17(7): 862-866 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/862.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.862>

INTRODUCTION

The benefits of laparoscopic colon surgery compared to the open approach are well established^[1-4]. Furthermore, laparotomy has been associated with an increased morbidity when compared to minimally invasive techniques for colorectal disease^[5]. More recently, the implementation of enhanced care programs coupled to laparoscopic resection has also resulted in a significant reduction in length of stay after both colon and rectal resection^[6,7]. Laparoscopic colon surgery for select cancers is slowly evolving as the standard of care but minimally invasive approaches for rectal cancer have been viewed with significant skepticism.

Laparoscopic rectal resection for cancer is performed by select surgeons at specialized centers. The variability in anatomic definitions of the rectum, technique, selection criteria, and need for neoadjuvant therapy amongst this group of surgeons have made parallel comparisons difficult and ambiguous. Concern over local recurrence, sexual dysfunction and appropriate training measures have further hindered widespread acceptance of this approach. This opinion addresses short-term and oncological outcomes for laparoscopic resection of rectal cancer, the aforementioned obstacles, and current controversies regarding technical approaches.

ONCOLOGICAL OUTCOMES

There are many potential endpoints for determining success for laparoscopic rectal resection. Undoubtedly, the

most significant is ensuring oncologic equivalence when compared to the open technique. This variable can primarily be measured by the adequacy of circumferential radial margins, recurrence rates, and both disease free and overall survival. Furthermore, the incidence of sexual dysfunction and other complications after laparoscopic pelvic dissection should approximate that with the open approach.

Circumferential radial margin

A positive circumferential resection margin (CRM) is a known marker for increased risk of future recurrence^[8]. Strict adherence to the principles of “total mesorectal excision” is essential to preserve the mesorectal envelope, obtain an adequate circumferential margin and therefore reduce local recurrence rates. The first randomized trial for laparoscopic rectal resection showed a trend towards increased CRM positivity (6% open *vs* 12% laparoscopic, $P = 0.19$) for anterior resection^[3]. Although this was initially alarming, several surgeons involved were on their learning curve, and preoperative chemoradiotherapy (CRT) was not standardized. Fortunately, three year outcomes showed that the difference in CRM positivity between laparoscopic and open approaches for anterior resection did not influence local recurrence rates. More recently, five year outcomes revealed no difference between groups in survival, disease-free survival, and local and distant recurrence^[9,10]. Wound/port-site recurrence rates in the laparoscopic arm were 2.4% and also unchanged^[10]. Conversion was associated with significantly worse outcomes overall but not disease-free survival.

In the largest retrospective review to date, Ng *et al*^[11] reported 579 laparoscopic rectal resections for cancer with a CRM positivity of 2.14%. These encouraging results were further substantiated by two recent randomized controlled trials that reported CRM positivity rates of 2.9% (open) *vs* 4% (laparoscopic)^[12] and 1.4% (open) and 2.6% (laparoscopic)^[13].

In 2006, the Spanish Association of Surgeons started an audited teaching program to both make known the results of rectal cancer treatment and improve the outcomes by the teaching process. The quality of the pathologic specimens for laparoscopic and open rectal resection patients was scored and the circumferential radial margin was positive if tumor was located 1 mm or less from the surface of the specimen. No differences between groups for the completeness of the mesorectum or distance of the tumor from the CRM were observed^[14]. Although laparoscopic TME amongst this experienced group approximates that for their open resection for select tumors, the results may not be as favorable for low bulky lesions or those in an obese male or narrow pelvis.

Local recurrence

As highlighted above, the five year results of the MRC CLASSIC trial reported similar regional recurrence for laparoscopic *vs* open resection of rectal cancer. Several other studies have also shown acceptable regional recurrence rates. In their retrospective review, Ng and colleagues reported two port site recurrences and a pelvic recurrence rate of 7.4%^[11]. Similarly, ten year outcomes from a pro-

Table 1 Overall survival for laparoscopic rectal resection with minimal 5 yr follow-up

Authors	Survival (laparoscopic)	Survival (open)	Follow-up (yr)
MRC CLASSIC (Jayne <i>et al</i>)	57.9%	58.1%	5
Sartori <i>et al</i>	75.4%	NA	5
Ng <i>et al</i>	63.9%	55.0%	10
Lam <i>et al</i>	64.0%		5
Laurent <i>et al</i>	82.0%	79.0%	5
Ng <i>et al</i>	70.0%	NA	5
Siami <i>et al</i>	80.2%	NA	5
Bianchi <i>et al</i>	81.4%	NA	5
Tsang <i>et al</i>	81.3%	NA	5

NA: Not applicable.

spective randomized trial for the laparoscopic resection of upper rectal cancers demonstrated a regional recurrence rate of 7.1% with no port-site recurrences^[13]. Laurent and colleagues aimed to assess long-term oncologic outcomes after laparoscopic versus open surgery for rectal cancer from in a retrospective comparative study^[15]. 471 patients had rectal excision for invasive rectal carcinoma during the trial period: 238 were treated by laparoscopy and 233 by open procedure. At 5 years, there was no difference of local recurrence (3.9% *vs* 5.5%, $P = 0.371$) between laparoscopic and open surgery^[15].

The multi-institutional series from Japan reported 1057 selected patients with rectal cancer that underwent laparoscopic surgery^[16]. All the data regarding the patient details and operative and postoperative outcome were collected retrospectively. At thirty months recurrence was found in 6.6% of the 1011 curatively treated patients. Specifically, local recurrence occurred in 11 patients (1.0%) and there was no port-site metastasis (Table 1)^[15].

FUNCTIONAL OUTCOMES

Laparoscopic rectal surgery proponents argue that the view in the pelvis is superior compared to the open approach. This magnification theoretically provides better visualization of the pelvic nerves. However, in the first randomized trial for laparoscopic rectal cancer male sexual function, erection and ejaculation were all significantly reduced with laparoscopic surgery. This should be interpreted with caution considering the aforementioned learning curve and that more patients in the laparoscopic group underwent a full TME, as compared to the open group. Bladder function remained similar between groups.

In a prospective evaluation of sexual function Stamopoulos and colleagues^[17] used the international index of erectile function (IIEF) for 56 patients who underwent rectal cancer surgery (38 open *vs* 18 laparoscopic procedures, 38 low anterior *vs* 18 abdominoperineal resections). Rectal cancer resections were associated with a significant reduction in IIEF scores and high rates of sexual dysfunction at 3 and 6 mo. The IIEF and domain scores at different assessment points were comparable between the laparoscopic and open surgery groups^[17].

Morino *et al.*^[18] also analyzed male sexual and urinary function after laparoscopic total mesorectal excision. They found that sexual desire was maintained by 55.6%, ability to engage in intercourse by 57.8%, and ability to achieve orgasm and ejaculation by 37.8% of the patients. The distance of the tumor from the anal verge and adjuvant or neoadjuvant treatments were the significant predictors of poor postoperative sexual function. Seven patients (14%) presented transitory postoperative urinary dysfunction, all of whom were medically treated. Tumor stage and distance from the anal verge were independently associated with the postoperative global international prostatic symptom score (IPSS). No differences were observed in urinary quality of life. The authors concluded that laparoscopic resection did not reproduce or improve on sexual and urinary dysfunction outcomes obtained in the best open TME series^[18].

In another series with investigators well beyond their learning curve, urinary dysfunction was reported by 6 (6%) patients and 6 (6%) patients had sexual dysfunction, manifesting as retrograde ejaculation in four patients and erectile dysfunction in a further two patients. The low rates of sexual dysfunction in this unit may be attributable to pelvic dissection only being undertaken by experienced, dedicated laparoscopic colorectal surgeons. Previous studies reporting poorer functional outcomes have probably included a significant number of patients on the surgeons' learning curve.

CONVERSION

The conversion rate for laparoscopic rectal resection is variable between centers and levels of expertise. The MRC CLASSIC randomized trial had a conversion rate of 32% for rectal cancer^[3], yet a previous experience of only 20 laparoscopic colon and rectal cases was sufficient to participate. A similar conversion rate (30%) was realized by Ng *et al.*^[11] in their ten year experience with laparoscopic rectal resection. After the inception of this trial significant improvements in energy devices, ports, cameras, and stapling devices have occurred that, combined with their experience, would likely decrease their current conversion rate.

Further analysis has shown that factors associated with conversion are BMI, male sex, and locally advanced tumors^[19].

More recently, conversion rates reflect the beneficial impact of extensive experience. Three large retrospective series (2008-2010) have reported conversion rates as low as 5.4%^[11], 15%^[15], and 4.9%^[20]. The multi-center retrospective series from Japan also demonstrated a reasonable conversion rate of 7.3%^[16].

Conversion rates are as dependent on a reasonable inclusion or selection criteria as surgeon experience. Very low bulky tumors, anterior lesions in men with previous intervention for prostate cancer, T4 lesions, reoperative pelvic dissections and morbidly obese patients should be reserved for the open approach in most cases.

DEFINING THE RECTUM

There has been considerable debate as to the exact length

of the rectum, the site of transition from sigmoid to rectum and most importantly the point of reference from where measurements are made. Within the surgical literature, numerous series have reported rectal cancer as being within 15, 16 and even 18 cm from the verge, although several other series use the dentate line as the reference point. Currently, the variability of these definitions not only impacts surgical decision making between centers but also the timing and need for neoadjuvant therapy, which in turn impacts oncologic outcomes and morbidity rates.

There are also significant differences in practice internationally with respect to the selection criteria used for CRT. In the United States, most practitioners adhere to the NCCN guidelines that recommend neoadjuvant CRT for patients with T3 or N1 disease with tumors within 10 cm of the dentate line^[21]. The Mercury study group^[22] has provided evidence that pre-operative MRI can accurately predict surgical resection margins. This report has led to a paradigm shift in the preoperative investigation and treatment of rectal cancer in the UK. With this approach, CRT is predominantly used when the tumor threatens or involves the mesorectal fascia and in all low rectal cancer where there is an inherent increased risk of involving the CRM.

Despite these apparent discrepancies most surgeons and oncologists generally agree that rectal cancer consists of extraperitoneal and intraperitoneal lesions. Tumors at or below the anterior reflection should be grouped together in investigations and are the real subject of this and other discussions surrounding laparoscopic rectal cancer.

TECHNICAL ISSUES

The most important variable being assessed with laparoscopic vs open rectal resection for cancer is the pelvic dissection. Surgeons must analyze their own ability to perform a laparoscopic total mesorectal excision with the same precision achieved by their open technique. Although this fact seems obvious it cannot be understated. Several studies continue to populate the literature describing a "hybrid" technique. With this approach the mobilization of the left colon is performed laparoscopically and the pelvic dissection and transection of the rectum are performed through a Pfannenstiel or lower midline incision. Outcomes with this technique have been favorable and it certainly has inherent advantages but unquestionably it is not laparoscopic rectal surgery. Therefore, although published results substantiate its role, ideally it should not be included in trials or case series for laparoscopic rectal resection and should not be billed or coded as such. If this procedure continues to demonstrate favorable outcomes and has a shorter learning curve it may require its own procedure code in the future.

Internationally, the straight laparoscopic approach with three or four abdominal trocar sites and a left lower quadrant or periumbilical extraction incision is preferred. Outcomes with this approach (outlined in previous section) were initially concerning but have now more consistently been favorable. As discussed above, the protracted opera-

tive times and concerns over both local recurrence and sexual function have been diminished with increased operative experience. This may be the most technically demanding method and surgeons preferring this technique recognize its limitations. Dividing the lower rectum, providing adequate traction low in the pelvis, and teaching trainees how to perform an appropriate total mesorectal excision are the current challenges. This procedure is less daunting for patients requiring an abdominal perineal resection. They are left without the morbidity of an abdominal wound as the specimen is routinely removed through the perineum.

Proponents of hand-assisted laparoscopy in the United States continuously have demonstrated equivalent outcomes for laparoscopic colon resection with reduced operative times. More recently results with hand-assisted methods for rectal cancer have also been reported with success^[23,24]. When the hand-assisted device is left in place and the pelvic dissection is performed laparoscopically these cases should be included with other minimally invasive approaches to rectal cancer. This approach may be favorable in patients with a bulky mesorectum or when additional tension is required to facilitate accurate transection of the low rectum.

Dividing the rectum laparoscopically is not always technically feasible. The limited angulation of the stapler and physical limitations of working in the bony confines of the pelvis are common deterrents^[25]. In this situation, having an assistant apply perineal pressure may elevate the pelvic floor enough to allow the first cartridge of the stapler to reach the anorectal junction. Furthermore, utilizing a suprapubic port or medicalizing the right lower quadrant port may help. Lastly, if these techniques are unsuccessful a limited lower midline or Pfannenstiel incision can be made and a 30 mm open stapler can be introduced. If an appropriate distal margin is not obtainable with these methods a mucosectomy with partial inter-sphincteric resection and hand-sewn coloanal anastomosis is performed.

In addition to the difficulty with transection, very low anteriorly based and bulky lesions are often challenging. Entering the appropriate plane anterior to Denonvillier's fascia laparoscopically, respecting the need for an adequate radial margin, and maintaining meticulous hemostasis is essential. In this location, tissue planes can be more ambiguous and any bleeding further obscures the appropriate anatomy. If there is considerable doubt that the correct tissue plane is being violated, immediate conversion is warranted. Ideally these tumors are approached by surgeons who are well past their learning curve for laparoscopic pelvic dissection.

The recognition of these technical limitations and the ongoing development of advanced technology led to the introduction of robotic applications for low pelvic dissection. Data for robotic approaches to rectal cancer have recently been published and presented in national and international forums. The advantage of operating with more degrees of freedom for low rectal cancer is apparent and is of particular benefit in a narrow male pelvis. However, concerns over significantly increased cost, operative times, and training have limited its widespread adoption. Furthermore, proponents seem to be employing this

approach *carte blanche* and looking for opportunities to expand its indications rather than using it as a tool. In the era of economic constraints and limited resident exposure to cases a costly technique with ill defined training methods should be used for select cases only.

CONCLUSION

Technical advances in the field of coloproctology have unquestionably improved patient outcomes. However, it is essential that we continue to strive to define the appropriate inclusion criteria for new approaches in regards to patient, disease, and surgeon experience. Historically, new technology, such as the PPH stapler, robotics, and laparoscopy, has become more than an optional approach or "tool". Surgeons inherently develop extraordinary comfort with the technology and tend to expand its indications, often illogically. Creativity and "pushing the envelope" should not be discouraged but when it becomes apparent that new approaches become simply a "means to an end" patients outcomes may be less than ideal.

The abundance of data for laparoscopic rectal resection for cancer supports its continued implantation and widespread usage by experienced surgeons for select patients. Until we become more adept at operating in the low narrow pelvis and transecting the rectum we must recognize that this approach is complementary to our open technique. To ensure the best outcomes we must continue to recognize the difference between the questions, "can you?" and "should you?" in regards to minimally invasive surgery.

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S- Editor Sun H L- Editor O'Neill M E- Editor Ma WH

Advances in diagnosis, treatment and palliation of pancreatic carcinoma: 1990-2010

Chakshu Sharma, Karim M Eltawil, Paul D Renfrew, Mark J Walsh, Michele Molinari

Chakshu Sharma, Karim M Eltawil, Paul D Renfrew, Mark J Walsh, Michele Molinari, Department of Surgery, Queen Elizabeth II Health Sciences Center, Dalhousie University, Halifax, Nova Scotia, B3H 2Y9, Canada

Author contributions: Sharma C, Eltawil KM and Molinari M contributed equally to this work; Sharma C performed the systematic literature review, contributed to the appraisal of the literature and wrote the paper; Eltawil KM performed the systematic literature review, contributed to the appraisal of the literature and wrote the paper; Renfrew PD and Walsh MJ contributed to the design framework of the systematic review; Molinari M performed the systematic literature review, contributed to the appraisal of the literature and wrote the paper.

Correspondence to: Michele Molinari, MD, Department of Surgery, Queen Elizabeth II Health Sciences Center, Dalhousie University, Rm 6-254, Victoria Building, 1276 South Park Street, Halifax, Nova Scotia, B3H 2Y9, Canada. michele.molinari@cdha.nshealth.ca

Telephone: +1-902-4737624 Fax: +1-902-4737639

Received: October 25, 2010 Revised: December 8, 2010

Accepted: December 15, 2010

Published online: February 21, 2011

Abstract

Several advances in genetics, diagnosis and palliation of pancreatic cancer (PC) have occurred in the last decades. A multidisciplinary approach to this disease is therefore recommended. PC is relatively common as it is the fourth leading cause of cancer related mortality. Most patients present with obstructive jaundice, epigastric or back pain, weight loss and anorexia. Despite improvements in diagnostic modalities, the majority of cases are still detected in advanced stages. The only curative treatment for PC remains surgical resection. No more than 20% of patients are candidates for surgery at the time of diagnosis and survival remains quite poor as adjuvant therapies are not very effective. A small percentage of patients with borderline non-resectable PC might benefit from neo-adjuvant chemoradiation therapy enabling them to undergo resection; however, randomized controlled studies are needed to prove the

benefits of this strategy. Patients with unresectable PC benefit from palliative interventions such as biliary decompression and celiac plexus block. Further clinical trials to evaluate new chemo and radiation protocols as well as identification of genetic markers for PC are needed to improve the overall survival of patients affected by PC, as the current overall 5-year survival rate of patients affected by PC is still less than 5%. The aim of this article is to review the most recent high quality literature on this topic.

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Key words: Diagnosis; Epidemiology; Palliation; Pancreatic cancer; Therapy

Peer reviewer: Hiroyuki Uehara, MD, PhD, Chief, Division of Pancreatology, Department of Gastroenterology, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari, Osaka 537-8511, Japan

Sharma C, Eltawil KM, Renfrew PD, Walsh MJ, Molinari M. Advances in diagnosis, treatment and palliation of pancreatic carcinoma: 1990-2010. *World J Gastroenterol* 2011; 17(7): 867-897 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/867.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.867>

INTRODUCTION

The vast majority (90%) of pancreatic cancers (PC) are malignant tumors originating from pancreatic ductal cells^[1]. Anatomically, 78% of PCs are located in the head, and the remaining 22% are equally distributed in the body and in the tail^[2]. The most common clinical presentations are progressive weight loss and anorexia, mid abdominal pain and jaundice^[3-5]. Over the past two decades many advances in the diagnosis, therapy and palliation of PC have taken place although the overall survival of affected patients has not improved significantly. The aim of this article is to review the most recent high quality literature on this topic.

SEARCH STRATEGY AND SELECTION CRITERIA

The literature search was targeted at studies that reported at least one of the following aspects of PC: epidemiology, diagnosis, therapy (e.g. surgery, radiotherapy, chemotherapy) and palliation. Randomized controlled trials (RCT) and prospective observational studies were given preference. Each of the topics was searched in MEDLINE, Ovid MEDLINE In-process, Cochrane Database of Systematic Reviews, Database of Systematic Reviews, Database of Abstracts of Review of Effects, EMBASE, PubMed, National Library of Medicine Gateway by established systematic review methods (Jadad Scale for RCT, as well as Downs and Black checklist for observational studies)^[6-8]. Articles from the authors' libraries and reference lists were further reviewed. We limited our search to English-language articles published from January 1990 to September 2010. We then developed a comprehensive and current database to catalog the medical literature on PC. To identify all potential papers, we searched the medical subject headings reported in Table 1. Three authors (Sharma C, Eltawil KM and Molinari M) independently performed the selection of the articles based on the content of titles and abstracts. When in doubt, each article was reviewed entirely. The decision to include articles in this review was reached by consensus. For conciseness, a full list of search strategies, search results, and quality assessment for each included study are available on request from the corresponding author.

EPIDEMIOLOGY

PC is the fourth leading cause of cancer related mortality in the United States with an estimated 42500 new cases and 35000 deaths from the disease each year^[9]. In industrialized countries, the incidence of PC (11 per 100000 individuals) ranks second after colorectal cancer among all gastrointestinal malignancies^[10]. While the mortality rate for males has decreased by 0.4% from 1990 to 2005, the mortality rate for females has increased by 4.4%^[9]. More than 80% of PCs are diagnosed in patients older than 60 and almost 50% have distant metastases at the time of presentation^[10-12]. Men are more frequently affected than women [relative risk (RR) = 1.3] and individuals of African American descent in comparison to Caucasians (RR = 1.5)^[10]. Analysis of overall survival shows that the prognosis of PC is still quite poor despite the fact that 1-year survival has increased from 15.2% (period between 1977-1981) to 21.6% (period between 1997-2001) and 5-year survival has increased from 3% (period between 1977-1986) to 5% (period between 1996-2004)^[10].

RISK FACTORS

Smoking

The risk of PC in smokers ranks second to lung cancer^[13] and it is proportionate to the frequency [≥ 30 cigarettes per day: odds ratio (OR) = 1.75], duration (≥ 50

years: OR = 2.13) and cumulative smoking dose (≥ 40 pack/years: OR = 1.78)^[14]. A meta-analysis of 82 studies from 4 continents has shown that cigarette smokers were diagnosed at significantly younger age and had a 75% increased risk of developing PC in comparison to the regular population^[15] and the risk persisted for 5 to 15 years after cessation^[16]. In a case-control study of 808 PC patients matched against 808 healthy controls, female smokers were at increased risk in comparison to males as they suffered from a synergistic interaction between cigarette smoking, diabetes mellitus (OR = 9.3) and family history of PC (OR = 12.8)^[17].

Diabetes

Nearly 80% of PC patients have either frank diabetes or impaired glucose tolerance^[18]. Diabetes is usually diagnosed either concomitantly or during the two years preceding the diagnosis^[19]. Several studies have assessed the role of diabetes in PC with conflicting results. A meta-analysis of 11 cohort studies found that the relative risk for diabetes was 2.1 [95% confidence interval (95% CI): 1.6-2.8]^[20]. These findings were supported by another cohort study of 100000 Danish diabetic patients which found a standardized incidence ratio of 2.1 (95% CI: 1.9-2.4) in a 4-year follow-up^[21]. A large prospective cohort study of 20475 men and 15183 women in the United States, has shown that the relative risk of PC mortality adjusted for age, race, cigarette smoking, and body mass index (BMI) was proportionate to the severity of abnormal glucose metabolism: RR was 1.65 for post load plasma glucose levels between 6.7 and 8.8 mmol/L; 1.60 for levels between 8.9 and 11.0 mmol/L, and 2.15 for levels equal or more than 11.1 mmol/L^[22]. Diabetes can be an early manifestation of PC as about 1% of new onset of diabetes in patients older than 50 is linked to PC^[23], but there is no evidence that screening for recent onset diabetes would reduce the mortality^[12] or lead to early diagnosis^[24].

The link between abnormal glucose and PC exists only for type II diabetes. A meta analysis of 36 studies has shown that the OR of PC for patients with type II diabetes for more than 5 years was 2.1^[25], while there are no reports on the association between PC and type I diabetes^[26].

Family history of diabetes does not appear to be a risk for PC. Compared to subjects with no family history, diabetics with a positive family history have an OR of 0.8 while non-diabetics with a positive family history have an OR of 1.0^[27].

A recent prospective study found that women with gestational diabetes have a relative risk of PC of 7.1 (95% CI: 2.8-18.0)^[28]. Gapstur and colleagues have proposed a mechanism to explain these findings^[22] by the fact that at high levels, insulin binds to the insulin-like growth factor I (IGF1) receptor^[24] and downregulates IGF binding protein 1^[25] causing an increase in cell growth in PC cell lines^[29,30].

Alcohol

The role of alcohol is controversial and several studies

Table 1 Summary of the terms used singly or in combination for evidence acquisition

Primary MeSH terms	Secondary MeSH terms (epidemiology, diagnosis)	Secondary MeSH terms (treatment, palliation)
Pancreatic neoplasm(s)	Epidemiology	Pancreaticoduodenectomy
Adenocarcinoma(s)	Classification	Resection
Carcinoma(s)	Diagnosis	Therapeutic(s)
Pancreatic diseases	Differential diagnosis	Treatment outcome(s)
Pancreas	Risk factor(s)	Surgery
Carcinoma, pancreatic ductal	Diagnostic imaging	Surgical procedures
Pancreatic duct(s)	Magnetic resonance imaging	Clinical trial(s)
Humans	Endosonography	Controlled clinical trial(s)
Adult	Ultrasonography	Randomized controlled trial(s)
	Emission computed tomography	Clinical trial (phase I)
	Radionuclide imaging	Clinical trial (phase II)
	Positron emission tomography	Clinical trial (phase III)
	Tomography	Clinical trial (phase IV)
	X-ray computed	Drug therapy
	Biopsy (fine needle)	Chemotherapy
	Biopsy (needle)	Neoadjuvant therapy
	Cytology	Adjuvant
	Cytodiagnosis	Antineoplastic combined chemotherapy protocols
	Tumor markers (biological) antigen(s)	Antineoplastic agent(s)
	Carcinoembryonic antigen	Antimetabolites, antineoplastic
	Ca 19-9 antigen	Combined modality therapeutic antineoplastic
	Ca 125 antigen	Combined chemotherapy protocols neoadjuvant
	Antigens, tumor-associated, carbohydrate	Therapy
	Endoscopic retrograde cholangiopancreatography	Radiotherapy
	Computed assisted image processing	Drainage
	Sensitivity and specificity	Cholestasis
	Endoscopy	Obstructive jaundice
		Celiac plexus
		Autonomic nerve block
		Nerve block
		Ethanol
		Injections, intralesional
		Cisplatin
		Deoxycytidine
		Epidermal growth factor
		Fluorouracil
		Endostatin
		Biological products
		Neoplasm proteins
		Immunotherapy
		Antibodies, monoclonal

have shown inconsistent findings. This might be attributed to multiple associations with confounding variables mainly smoking, socio-economic status^[31] and pancreatitis^[30]. A recent pooled analysis of 14 cohort studies with a sample of 862 664 individuals has shown a slight positive association between PC and alcohol intake only for consumption above 30 g/d (RR = 1.22; 95% CI: 1.03-1.45)^[32]. Contrasting findings were reported by a European epidemiological study with a smaller sample size ($n = 555$) that did not show any association between PC and alcohol consumption^[33].

Compared with light drinkers, men consuming large amounts of hard liquor suffered from a 62% increased risk of PC (95% CI: 1.24-2.10)^[16,34], but this was not observed for women or for beer and wine drinkers^[34].

Although moderate alcohol consumption is not a risk factor, African Americans were found to have a significantly higher OR when adjusted for their drinking habits, suggesting that racial differences might play a role in the development of PC^[35].

Pancreatitis

Several studies have shown a positive association between PC and history of pancreatitis, although the magnitude is still controversial^[36,37]. An international epidemiological study reported that both genders with chronic pancreatitis had an increased risk independently of the cause of pancreatitis^[37]. A large case-control study showed that chronic pancreatitis lasting more than 7 years was associated with a higher risk of PC (RR = 2.04; 95% CI: 1.53-2.72)^[38]. A large Italian study from 1983 to 1992 found similar results, as the risk increased after 5 or more years of chronic pancreatitis (RR in the first 4 years = 2.1, RR after 5 years = 6.9)^[34]. These findings have been challenged by an international study, as the risk was significantly increased only in the early years after diagnosis. This would suggest that pancreatitis might represent a manifestation of PC that becomes apparent only several years later, rather than a risk factor. The risk of PC in chronic pancreatitis has been shown to be especially true for patients affected by hereditary pancreatitis, who were found to have 53 times

the risk in comparison to normal individuals^[39]. This was confirmed by another study that estimated a 40% cumulative risk of PC in patients with hereditary pancreatitis by the age of 70. For patients with paternal inheritance, the cumulative risk of PC was even higher with risk up to 75%^[40]. Cytokines, reactive oxygen molecules and pro-inflammatory compounds seem to be responsible, as inflammation is a risk factor for many other solid tumors^[38].

Genetic predisposition for PC

Genetic predisposing factors have been a topic of intense research in the last decades. Case reports of families with multiple affected members suggest that PC might have a hereditary background^[41]. Yet, a large population study on twins identified hereditary factors for prostatic, breast and colorectal cancers, but not for PC^[42]. A Canadian study on patients with suspected hereditary cancer syndromes found that the standardized incidence rate of PC was 4.5 (CI 0.54-16.) when cancer affected one 1st degree relative, and increased to 6.4 (CI 1.8-16.4) and 32 (CI 10.4-74.7) when two and three 1st degree relatives were affected, respectively^[43]. This translates to an estimated incidence of PC of 41, 58 and 288 per 100 000 individuals, respectively, compared to 9 per 100 000 for the general population^[44].

Brentnall *et al.*^[45] and Meckler *et al.*^[46] described examples of autosomal dominant PC in individuals presenting at early age (median age 43 years) and with high genetic penetrance (more than 80%). A mutation causing a proline (hydrophobic) to serine (hydrophilic) amino acid change (P239S) within a highly conserved region of the gene encoding paladin (PALLD) was found in all affected family members and was absent in non-affected individuals of the same family (family X). Another study has shown that the P239S mutation was only specific for family X and was not a common finding in other individuals with suspected familial PC^[47]. Currently, genetic predisposition is thought to be responsible for 7% to 10% of all PC^[48]. Genetic factors including germline mutations in p16/CDKN2A^[49], BRCA2^[50-52] and STK 11^[53] genes increase the risk of PC. The combination of all these known genetic factors accounts for less than 20% of the familial aggregation of PC, suggesting the role of other additional genes.

A systematic review and meta analysis of studies that quantified familial risk of PC has shown that individuals with positive family history have an almost two-fold increased risk (RR = 1.80, CI 1.48-2.12)^[54]. Therefore, families with two or more cases may benefit from a comprehensive risk assessment involving collection of detailed family history information and data regarding other risk factors^[55]. A case-control study of PC in two Canadian provinces (Ontario and Quebec) assessed a total of 174 PC cases and 136 healthy controls that were compared for their family histories of cancer. Information regarding the ages and sites of cancer was obtained in 966 first degree relatives of the PC patients and for 903 first degree relatives of the control group. PC was the only malignancy in excess in relatives of patients with PC, compared to the control group (RR = 5, $P = 0.01$). The lifetime risk of PC was 4.7% for the first degree relatives and the risk was 7.2%

for relatives of patients diagnosed before the age of 60^[56].

Besides the isolated aggregation of PC in some families, several other hereditary disorders predispose to PC in known familial cancer conditions^[57]. These include hereditary pancreatitis, Puetz-Jeghers syndrome, familial atypical multiple mole melanoma, familial breast and ovarian cancer, Li-Fraumeni syndrome, Fanconi anaemia, Ataxia-telangiectasia, familial adenomatous polyposis, cystic fibrosis and possible hereditary non-polyposis colon cancer or Lynch syndrome^[11,55,58-60].

Familial PC registries

As the prognosis of PC is generally poor, there has been a strong interest in detecting genes or other markers that could help identify high risk patients at an early stage. Although a precise genetic marker for this scope is not currently available, geneticists and epidemiologists have been profiling traits of high risk families enrolled in registries established in North America and Europe^[61]. Even if there is no standardized definition for familial PC, most authors apply the term to families with at least two first degree relatives affected by PC in the absence of other predisposing familial conditions^[61]. The creation of familial PC registries has been used not only for identification of genetic mutations, but also for the screening of high risk individuals. In selected centers in North America and Europe, screening programs for high risk individuals have been implemented with the use of endoscopic ultrasound (EUS) and computed tomography (CT) scanning or magnetic resonance imaging (MRI). Such early diagnosis of PC within a comprehensive screening program is hoped to ultimately result in improved survival^[62]. The discovery of the genetic bases of inherited PC continues to be an active area of research, and in 2001 a multi-center linkage was formed to conduct studies aimed at the localization and identification of PC susceptibility genes (PAC-GENE)^[63]. The complex nature of pedigree data makes it difficult to accurately assess risk based upon the simple counting of the number of affected family members, as it does not adjust for family size, age of onset of PC, and the exact relationship between affected family members. Therefore, computer programs have been developed to integrate these complex risk factors and pedigree data. In April 2007, the 1st risk prediction tool for PC, PanaPro was released^[64]. This model provides accurate risk assessment for kindreds with familial PC as the receiver operating characteristic (ROC) curve was 0.75 which is considered good for predictive models.

Nutritional status

A number of studies have explored the relationship between BMI, lifestyle, diet and the risk of PC, but uncertainty regarding the strength of this relationship still exists. A recent case-control study of 841 patients and 754 healthy controls showed that individuals with a BMI of 25-29.9 had an OR of 1.67 (95% CI: 1.20-2.34) in comparison to obese patients (BMI of ≥ 30) who had an OR of 2.58 (95% CI: 1.70-3.90) independently of their diabetes status^[65]. The duration of being overweight was

Table 2 Known risk factors for pancreatic cancer

Age (more than 60 yr)
Smoking
Diabetes
Type II
Gestational diabetes
Impaired glucose tolerance
Alcohol
Pancreatitis
Acute
Chronic
Genetic predisposition
Family history
Hereditary disorders
Hereditary pancreatitis
Puetz-Jeghers syndrome
FAMMM
Familial breast and ovarian cancer
Li-Fraumeni syndrome
Fanconi anaemia
Ataxia-telangiectasia
Familial adenomatous polyposis
Cystic fibrosis
HNPCC
Lynch syndrome
Obesity

FAMMM: Familial atypical multiple mole melanoma; HNPCC: Hereditary non polyposis colon cancer.

significantly longer among patients with PC than controls. Being obese or overweight, particularly in early adulthood, resulted in earlier onset of PC (age at presentation of PC was 61 years for overweight patients and 59 years for obese) when compared to the median age of diagnosis (64 years) in the general population^[66]. A number of studies reported that central weight gain measured by waist circumference and/or waist-to-hip ratio had a statistically significant increased risk compared to those with peripheral weight gain (RR = 1.45, 95% CI: 1.02-2.07)^[67,68]. The known risk factors for PC are summarized in Table 2.

CLASSIFICATION

Anatomical classification

According to the location, PC can be divided in three groups: tumors of the head, body and tail. PCs of the head are at the right side of the superior mesenteric vessels, and tumors of the neck and body are located between the superior mesenteric vessels and the inferior mesenteric vein. PCs of the tail are located to the left of the inferior mesenteric vein.

A large epidemiological study^[2] of 100,313 patients in the United States has shown that 78% of PC presents in the head, 11% in the body and 11% in the tail (Figure 1).

Pathological classification

Recent advances in surgical pathology techniques integrated with molecular biology have allowed advances in the modern classification of PC. A summary of the clinico-pathological features of the different categories of PC is shown in Table 3.

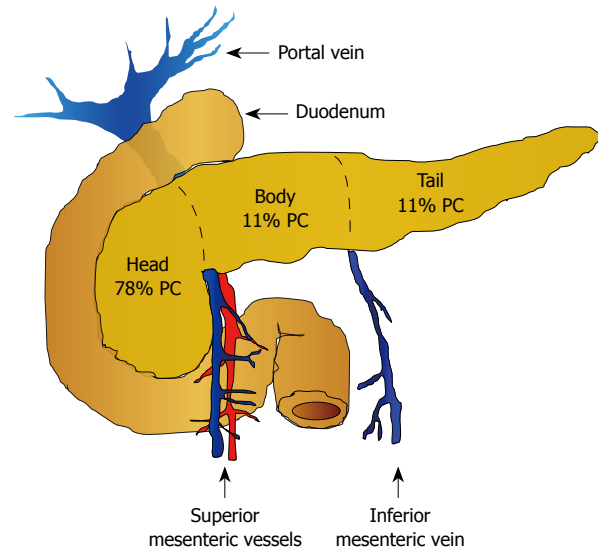


Figure 1 Graphical representation of the pancreas and frequency of pancreatic cancer in the three anatomical sections: head, body and tail. PC: Pancreatic cancer.

Ductal infiltrating adenocarcinoma

Ductal infiltrating adenocarcinoma (DIA) represents the most common type (85%-90%) of PC originating from ductal epithelial cells. Most DIAs appear as whitish masses, hard at palpation and poorly defined from surrounding tissues, predominantly solid although cystic degenerations can be seen in larger tumors^[89]. The microscopic appearance of DIA ranges from well-differentiated neoplasms difficult to distinguish from reactive gland, to poorly differentiated. The majority of DIAs are moderately to poorly differentiated and develop a dense desmoplastic stroma^[89]. Mutations in the KRAS2 or p16/CDKN2A genes are observed in 90% of patients, TP53 gene abnormalities in more than 75% and more than 55% of cases have changes in MADH4/DPC4 genes. Tumors showing loss of DPC4 expression have a worse outcome than those with intact DPC4^[90], and immunolabeling for DPC2 protein can help to classify metastatic carcinomas of unknown primary etiology^[91].

Solid pseudopapillary neoplasm

Solid pseudopapillary neoplasms (SPPN) represent a small proportion of PCs (3%) and present as solid, or solid and cystic masses. They are malignant epithelial neoplasms made of poorly cohesive cells that form pseudo-papillae around the blood vessels^[91]. The majority of SPNN are grossly well demarcated, but typically do not have a well formed capsule. The majority are solid, yellowish and soft^[92]. Larger tumors usually develop cystic degeneration filled with blood and necrotic debris. Cases that are almost completely cystic without a solid component have also been reported^[91].

Molecular analyses have shown that SPNN are different from ductal adenocarcinomas as they do not harbor mutations in the Kras 2, p16/CDKN2A, TP53, MADH4/DPC4 genes^[93]. In contrast, 90% of SPNN have a mutation on chromosome 3p (CTNNB1) respon-

Table 3 clinico-pathological features of the most frequent classes of pancreatic cancer

Classification	Frequency (%)	Author	yr	Survival (5-yr survival after surgical resection)
DIA (incidence per 100 000 patients at risk = 8.37) ^[69]	85-90 ^[1]	Conlon <i>et al</i> ^[70]	1996	10%
		Winter <i>et al</i> ^[71]	2006	18%
		Poultides <i>et al</i> ^[72]	2010	19%
SPPN (incidence per 100 000 patients at risk = NA) ^[69]	0.1-3 ^[73]	Papavramidis <i>et al</i> ^[74]	2005	95%
IPMN (incidence per 100 000 patients at risk = 0.03) ^[69]		Shin <i>et al</i> ^[76]	2010	Benign: 95%
				Malignant: 64%
IPMN with simultaneous DIA: (incidence per 100 000 patients at risk = NA) ^[69]	5 ^[75]	Poultides <i>et al</i> ^[72]	2010	42%
		Fan <i>et al</i> ^[77]	2010	57%
		Sohn <i>et al</i> ^[78]	2004	43%
Pancreatoblastoma (incidence per 100 000 patients at risk = NA) ^[69]	0.50 ^[79]	Dhebri <i>et al</i> ^[80]	2004	50%
		Saif <i>et al</i> ^[79]	2007	80%
Undifferentiated (incidence per 100 000 patients at risk = 0.03) ^[69]	2-7 ^[81]	Paal <i>et al</i> ^[82]	2001	3% (3-yr survival)
		Connolly <i>et al</i> ^[83]	1987	5 mo (average survival)
Medullary carcinoma (incidence per 100 000 patients at risk = NA) ^[69]	NA	Wilentz <i>et al</i> ^[84]	2000	11%
				14 mo (average survival)
Mucinous cystadenocarcinoma (incidence per 100 000 patients at risk = 0.43) ^[69]	1	Ridder <i>et al</i> ^[85]	1996	56%
Adenosquamous carcinoma (incidence per 100 000 patients at risk = 0.05) ^[69]	4	Madura <i>et al</i> ^[86]	1999	5-7 mo (median survival)
		Mulkeen <i>et al</i> ^[87]	2006	
Acinar cell carcinoma (incidence per 100 000 patients at risk = 0.02) ^[69]	2	Holen <i>et al</i> ^[88]	2002	38 mo after surgical resection (median survival)
				14 mo for unresectable disease (median survival)

DIA: Ductal infiltrating adenocarcinoma; SPPN: Solid pseudo-papillary neoplasm; IPMN: Intraductal papillary mucinous neoplasm; NA: Not applicable.

sible for the metabolism of β -catenin protein causing its accumulation in the cytoplasm and nucleus of neoplastic cells^[94]. As a result alteration in β -catenin protein expression disrupts E-cadherin which is a key regulator of cell junctions causing poor adhesion of neoplastic cells^[95]. Although there is some histological overlap between SPNN and other tumors of the pancreas, immunolabeling for β -catenin protein may help establish the diagnosis.

Intraductal papillary mucinous neoplasm

Intraductal papillary mucinous neoplasms (IPMNs) represent 5% of all PCs and are papillary epithelial mucin-producing neoplasms arising in the main pancreatic duct or in one of its branches. IPMNs are relatively common with increasing age of the population^[91] and the mean age at presentation is 65 years^[96]. IPMN is a potential premalignant condition and the risks of developing invasive adenocarcinoma increase with tumor size and when originating in the main pancreatic duct.

Adenocarcinoma is present in up to one-third of patients with IPMN and current guidelines recommend surgical resection when IPMNs are greater than 3 cm, in the presence of main pancreatic duct dilatation and when mural nodules are detected^[97].

Neoplastic cells of IPMN are columnar with gene profiles similar to infiltrating ductal carcinoma. About 25% of patients show loss of heterozygosity of the STK11/LKB1 gene^[98,99]. Other frequent gene mutations are TP53, KRAS2, and P16/CDKN2A^[100].

Pancreatic intraepithelial neoplasia

Pancreatic intraepithelial neoplasia (PanIN) represents a

neoplastic proliferation of mucin producing epithelial cells confined to the smaller pancreatic ducts and is considered a precursor to invasive ductal carcinoma^[101].

PanINs are usually characterized by lesions too small to be symptomatic or to be detected by current imaging technologies^[89]. Microscopically, PanINs are classified into three grades (PanIN-1, PanIN-2 and PanIN-3) based on the progressive degree of architecture abnormality and cellular atypia^[102]. PanIN-1 shows minimum cellular atypia, PanIN-2 moderate changes and PanIN-3 is equivalent to PC-*in-situ*. The discovery of specific molecular changes present in both PanIN and PC has helped to establish that these small lesions are the precursors to DIA^[103]. Early abnormalities of IPMNs are telomerase shortening and activating point mutations in the KRAS2 gene while intermediate mutation is the activation of the p16/CDKN2A gene and late events are alterations in the TP53, MADH4/DPC4, and BRCA2 genes^[102]. The understanding that many DIAs arise from PanIN lesions has prompted screening efforts on the detection of these small and potentially curable lesions^[104].

Pancreatoblastoma

Pancreatoblastoma is a rare malignant tumor (0.5% of PC) usually presenting in the pediatric age group. Generally, it appears as a soft and well demarcated mass with epithelial or acinar differentiation, but often it has cells with endocrine and mesenchymal characteristics^[79]. Most pancreato-blastomas affect children with a mean age of 5 years and are frequently associated with elevated levels of serum alpha fetoprotein. The median survival of patients with pancreato-blastomas is 48 mo and the 5-year

survival rate after successful resection is 50% (95% CI: 37%-62%)^[80,105].

The majority of pancreato-blastomas have loss of heterozygosity of chromosome 11p from the maternal side^[106]. These molecular findings unite pancreatoblastoma with other primitive neoplasms such as hepatoblastoma and nephroblastoma^[107]. Genetic alterations in the adenomatous polyposis coli (APC)/ β -catenin pathway have also been detected in most pancreato-blastomas including mutations in β -catenin (CTNNB1) and APC genes^[107].

Undifferentiated carcinoma

Undifferentiated PC (UPC) lacks differentiation direction^[91] and presents with symptoms similar to patients with DIA, but has a worse prognosis as it has a more aggressive behavior and tends to metastasize and infiltrate surrounding organs in early stages^[82]. The average time from diagnosis to death is about 5 mo and only 3% of patients are alive at 5 years after undergoing surgical resection. UPCs can form large locally aggressive masses and may present with severe hemorrhage and necrosis. The majority of UPCs have KRAS2 gene mutation suggesting that they arise from pre-existing ductal adenocarcinomas that transform into poorly differentiated tumors during their progression^[108].

Medullary carcinoma

Medullary carcinoma (MC) is a variant of PC characterized by poor differentiation and syncytial growth that has been described and recognized only in recent years^[84]. Patients with MC have a better prognosis and are more likely to have a family history of any kind of cancer^[109]. MC does not differ significantly from other classes of PC in its clinical presentation, age and gender. These tumors tend to form well demarcated soft masses and microscopically they are usually poorly differentiated with pushing rather than infiltrating features^[110]. Focal necrosis and intratumoral lymphocytic infiltration can be prominent similar to MC of the colon and other tumors with microsatellite instability^[89]. MCs have been shown to have loss of expression of one of the DNA mismatch repair proteins (M1h1 and Msh2) and mutation in the BRAF gene, which is a downstream effector of the k-ras pathway^[111]. Patients with MC and their families may benefit from genetic counseling and more frequent screening for early detection of other common cancers. The prognosis of MC is better than adenocarcinoma, although it is not responsive to adjuvant chemotherapy based on fluorouracil (5-FU), similar to colon cancer with microsatellite instability^[112].

Other rare classes of PCs

Mucinous cystadenocarcinoma: Malignant cystic neoplasms are rare entities that account for only 1% of all pancreatic tumors^[113]. Both serous and mucinous cystic neoplasms are tumors of the exocrine pancreas with different biological behaviors. Serous cystadenomas are considered benign tumors with almost no malignant potential often managed expectantly unless symptomatic. However, the preoperative differentiation between a benign serous

cystadenoma and malignant serous cystadenocarcinoma remains difficult^[114]. Histologically, cystadenocarcinomas appear identical to serous cystadenomas and are distinguished only by the presence of lymphovascular invasion or metastases^[115]. Mucinous cystadenocarcinomas resemble DIAs although some cell populations can present with undifferentiated features and other histological characteristics such as osteoclast-like giant cells, adenosquamous carcinoma, choriocarcinoma, or high-grade sarcoma^[116-119]. Mucinous cystic neoplasms of the pancreas are slowly growing and only about 20% show invasive features^[120,121].

The prognosis of cystadenocarcinoma is favorable compared to DIA with 5-year survival rates of 56% after radical resection^[85]. There is limited evidence on the role of chemotherapy for cystadenocarcinomas of the pancreas as they appear to be unresponsive to current chemotherapy agents and radiation therapy^[122,123].

Adenosquamous carcinoma: Adenosquamous carcinoma has previously been referred as adenoachantoma, mixed squamous and adenocarcinoma, and mucoepidermoid carcinoma. Histologically, they are characterized by mixed populations of adenomatous cells and cells with varying amount of keratinized squamous features. Usually this tumor affects patients in their seventh decade of life, with symptoms and pancreatic distribution similar to DIAs. Although it is reported that adenosquamous carcinomas represents 4% of all PCs (range 3%-11%), the literature on the natural history and survival is limited to case series only^[86]. The prognosis seems to be worse than DIAs, with a mean survival of 5-7 mo even after surgical resection^[86,87]. Lymphovascular and perineural invasion appear to be common and early features of adenosquamous carcinomas and the role of adjuvant chemo and radiation therapy is still not clear^[124].

Acinar cell carcinoma: Acinar cell carcinomas (ACCs) represent less than 2% of all pancreatic malignancies^[87,88]. ACCs are predominantly constituted by neoplastic cells with immunohistochemical staining characteristic for exocrine enzymes such as trypsin, chymotrypsin or lipase, and they present in older patients than DIAs and the prognosis is slightly better, although the literature is somewhat limited^[125,126]. Symptoms at presentation are aspecific and include abdominal pain and weight loss that are similar to all other PCs^[125]. Very rarely, patients with ACC can develop subcutaneous fat necrosis secondary to exceedingly high concentrations of serum lipase and contrary to DIAs, bile duct obstruction causing jaundice is not as common^[125]. Median survival for ACC confined to the pancreas treated by surgical resection is 38 mo, whereas it is 14 mo for individuals with unresectable disease^[88]. For the majority of patients, surgical management is not curative as distant recurrent disease is more frequent than in DIA, suggesting the presence of early micrometastases even when the tumors are in the early stages^[88]. Because ACCs are rare, there is a lack of studies on the role of chemotherapy, although radiation therapy seems to provide good responses in patients with regional unresectable disease^[88].

DIAGNOSIS

Clinical presentation

Early symptoms of PC are notoriously difficult to measure as educational and economic factors influence their perception and reporting^[127,128]. Cholestatic symptoms are more common in early PC of the head, while abdominal and back pain are more common in patients with distal PC and in patients with tumors infiltrating peripancreatic nerve tissue^[129]. The appearance of these symptoms usually indicates advanced disease (Table 4)^[129,130].

Early symptoms are usually vague such as anorexia, moderate weight loss, and early satiety^[131]. Diabetes might be a sign of PC particularly when presenting during or beyond the sixth decade of life in the absence of risk factors and family history^[20]. Diabetes is detected in 60%^[132] to 81%^[133] of PC patients within two years of their diagnosis. Early detection is possible if symptoms raise clinicians' suspicion, as 25% of patients report upper abdominal discomfort up to 6 mo prior to their diagnosis^[134,135].

In two European studies^[128,130], weight loss was present in 66%-84% of patients, jaundice (bilirubin level > 3 mg/dL) in 56%-61%, recent onset of diabetes in 97% and distended palpable gall bladder in 12%-94%, energy loss in 86%, abdominal pain in 78%, back pain in 48%, nausea in 50%, clay-coloured stools in 54%, dark urine in 58%, jaundice in 56% and pruritis in 32% of patients.

Serum tumor markers

Several serum tumor markers are associated with PC, however, to date, no single marker has been found to be optimal for screening.

Carbohydrate antigen 19-9: Carbohydrate antigens have been used as markers for several cancers^[136,137]. The production of these antigens seems to be caused by the up-regulation of glycosyl transferase genes^[138]. Among these carbohydrate antigen epitopes, Sialyl Lewis^a (sLe^a) detected by the 1116NS19-9 monoclonal antibody is commonly called carbohydrate antigen 19-9 (CA19-9)^[139]. The serum levels of CA19-9 at the time of diagnosis and during follow-up of PC provide useful diagnostic and prognostic information^[140,141]. Its sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are 70%-90%, 43%-91%, 72% and 81%, respectively^[142-145]. A worse survival was observed in patients with pre-operative CA19-9 levels above 370 U/mL (median survival 4.4 mo *vs* 9.5 mo if CA19-9 < 370 U/mL, *P* value < 0.01)^[146]. In another study, serum levels of CA19-9 > 200 U/mL were associated with a survival rate of 8 mo compared to 22 mo for patients with lower tumor antigen levels (*P* < 0.001)^[147]. In a prospective study of patients undergoing curative resection for PC, post-operative CA19-9 < 37 U/mL was associated with a longer median and disease-free survival compared to the control group^[148-150]. One of the limitations of CA19-9 is that high serum bilirubin can falsely increase its level and therefore the risk of false positive results in patients with jaundice. This is not observed for other markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 242 (CA 242)^[141].

Table 4 Presenting symptoms of advanced pancreatic cancer

Symptom	Percentage
Abdominal pain	78-82
Anorexia	64
Early satiety	62
Jaundice	56-80
Sleep disorders	54
Weight loss	66-84
Diabetes	97
Back pain	48
Nausea and weight loss	50-86

CEA: CEA is part of a subgroup of glycoproteins functioning as intracellular adhesion molecules. CEA was first detected in pancreatic secretions, and several studies have shown high levels of CEA in the pancreatic juice of patients with PC^[151-153]. A Japanese study found significantly higher CEA levels in the pancreatic juice of PC patients compared to those with benign pancreatic diseases. When the CEA cut off level in pancreatic juice was 50 ng/mL, the PPV, NPV, and the accuracy for diagnosis of carcinoma were 77%, 95% and 85%, respectively. CEA levels in pancreatic juice were higher in smaller tumors in comparison to advanced PC due to the incomplete obstruction of the pancreatic duct^[154]. A recent study examining single *vs* combined efficacy of tumor markers showed that CEA (> 5 ng/mL) alone had a sensitivity of 45% and a specificity of 75% in comparison to CA19-9 which had a sensitivity of 80% but lower specificity (43%) (*P* = 0.005)^[141,155]. The combination of CEA (> 5 ng/mL) and CA 19-9 (> 37 U/mL) decreased the sensitivity to 37%, but increased the specificity to 84%. Similarly, the combination of CEA (> 5 ng/mL) and CA242 (> 20 U/mL) decreased the sensitivity to 34% and increased the specificity to 92%. Yet, CEA and CA242 are currently not used as single tumor markers for PC, and the simultaneous use of CEA and CA19-9 provides the same information as CA19-9 alone^[156-158].

CA 242: CA 242, a sialylated carbohydrate was first defined by Lindholm *et al* in 1985 and has been used for diagnostic and prognostic purposes^[159,160]. For PC, its diagnostic sensitivity and specificity are 60% (*P* = 0.073) and 76% (*P* = 0.197), respectively, comparable to CEA. It also seems to be valuable in differentiating PC from benign pancreatic tumors as well as other hepatobiliary cancers and to predict outcomes as survival rates in CA 242 positive patients are lower than those with negative serum levels (*P* = 0.002)^[141].

In a study comparing CA 242 and CA19-9^[161], CA 242 appeared to be an independent prognostic factor for patients with resectable disease as serum levels of CA 242 < 25 U/mL were associated with a significantly better survival (*P* < 0.05). For patients with unresectable disease, poorer outcomes were observed when CA 242 levels were > 100 U/mL.

Similar results have been confirmed by Ni *et al*, who found that CA 242 is an independent prognostic factor

Table 5 Summary of the performance characteristics of serum tumor markers for the diagnosis of pancreatic cancer

Serum tumor marker	Author	Yr	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
CA19-9	Boeck <i>et al</i> ^[141]	2006	70-90	43-91	72	81	67
	Ni <i>et al</i> ^[142]	2005					
	Steinberg <i>et al</i> ^[143]	1990					
	Safi <i>et al</i> ^[144]	1997					
	Mu <i>et al</i> ^[162]	2003					
CEA in pancreatic juice	Ozkan <i>et al</i> ^[155]	2003	NA	NA	77	95	85
	Futakawa <i>et al</i> ^[154]	2000					
	Ni <i>et al</i> ^[142]	2005					
CEA in serum	Boeck <i>et al</i> ^[141]	2006	45	75	NA	NA	NA
CA19-9 + CEA	Ni <i>et al</i> ^[142]	2005	37	84	91	90	89
	Ozkan <i>et al</i> ^[155]	2003					
	Ma <i>et al</i> ^[163]	2009					
CA 242	Nilsson <i>et al</i> ^[160]	1992	60	76	63	61	71
	Röthlin <i>et al</i> ^[164]	1993					
	Carpelan-Holmström <i>et al</i> ^[165]	2002					
	Pålsson <i>et al</i> ^[166]	1993					
CEA + CA 242	Ni <i>et al</i> ^[142]	2005	34	92	67	90	87
	Ozkan <i>et al</i> ^[155]	2003					
	Hall <i>et al</i> ^[167]	1994					
CA19-9 + CA 242	Ni <i>et al</i> ^[142]	2005	59	77	65.3	87.8	65.1
	Röthlin <i>et al</i> ^[164]	1993					
	Jiang <i>et al</i> ^[158]	2004					
CA19-9 + CA 242 + CEA	Ni <i>et al</i> ^[142]	2005	29	96	NA	NA	NA

PPV: Positive predictive value; NPV: Negative predictive value; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CA 242: Carbohydrate antigen 242; NA: Not applicable.

in PC yielding more information than CA 19-9^[142,161]. In this study the use of combined tumor markers resulted in lower sensitivity, but higher specificity (Table 5). Despite these findings, CA 242 is not used in clinical practice as commonly as Ca 19-9 due to the limited number of laboratories equipped to run this test.

Other tumor markers

Recent studies have identified other serum molecules such as CA494^[168], CEACAM1^[169], PTHrP^[170], TuM2-PK^[171], CAM 17.1^[172] and serum beta HCG^[173] as potential markers for PC. Although preliminary results appear promising with sensitivity and specificity comparable and sometimes superior to CA19-9 and CEA, their clinical use has to be confirmed in larger studies and their role is currently confined to a limited number of medical centers and for research purposes.

Imaging modalities

Although PC may be detected with one particular diagnostic test, proper staging often requires the use of several imaging modalities^[174].

Abdominal ultrasound: Trans-abdominal ultrasound (US) is currently used as a screening test for patients with suspected PC^[175]. Its sensitivity ranges between 48%^[176] and 89%^[177], specificity between 40%^[178] and 91%^[179] and accuracy between 46%^[176] and 64%^[180]. PCs measuring less than 1 cm are detected by US in only 50% of cases, while the sensitivity increases to 95.8% for tumors larger than 3 cm^[177]. Other factors affecting the sensitivity of US are the operator's experience^[181] and the technical character-

istics of the machine. Newer US machines such as tissue harmonic imaging decrease artefacts and improve tissue contrast and therefore diagnostic accuracy^[182]. US has a relatively low performance profile for the staging of PC as its sensitivity for lymph node involvement only ranges between 8%^[159] and 57%^[177].

Color Doppler US has been used to assess the possible involvement of the portal vein and superior mesenteric vessels with a sensitivity ranging between 50%^[183] and 94%^[184], specificity between 80% and 100%^[183] and accuracy between 81% and 95%^[175].

The recent introduction of intravenous contrast has been shown to improve evaluation of the vascularity of pancreatic lesions allowing differentiation between PC and other conditions with 90% sensitivity, 100% specificity and 93% accuracy^[185]. Currently, US is considered a useful imaging modality for the initial screening of PC based on its ability to document unresectability (PPV = 94%)^[176]. However, the PPV for resectability is only 55%^[186], therefore, other imaging techniques are usually employed for better staging.

EUS: EUS provides high resolution images of the pancreas without interference by bowel gas^[187]. Despite the advancement of CT scans, EUS appears to have a higher sensitivity in detecting small PCs (98%) in comparison to CT (86%)^[188]. EUS has higher sensitivity compared to CT for local tumor staging (67% *vs* 41%), similar sensitivity for lymph node involvement (44% *vs* 47%) and potential tumor resectability (68% *vs* 64%)^[185]. EUS has a NPV of 100% for PC of the head^[186,189] and an accuracy of 90% for the assessment of portal and splenic vein inva-

sion^[178,190]. On the other hand, EUS does not appear to be accurate enough in assessing the invasion of SMA and superior mesenteric vein (SMV) with a NPV of 82% and sensitivity of only 50%^[191,192].

In order to improve EUS performance in PC staging, recent studies have assessed the benefits of using parenteral contrast agents. This technique has shown 92% sensitivity, 100% specificity, 100% PPV, 86% NPV and 95% accuracy^[193]. Although EUS is becoming a leading modality for staging and diagnosis of PC, drawbacks of this technique are the fact that it is invasive, highly operator dependent, costly and associated with a small risk of pancreatitis (0.85%)^[194], bleeding and duodenal perforation.

CT: On contrast CT, PC appears as an ill-defined, hypoattenuating focal mass with dilatation of the upstream pancreatic and/or biliary duct^[174]. Optimum visualization of the pancreas requires imaging acquisition obtained during both arterial and portal phases^[195]. Sensitivity and specificity of thin section triple phase helical CT is 77% and 100%, respectively, for lesions less than 2 cm^[196]. In a multicentric trial, the diagnostic accuracy of CT for resectability was 73% with a PPV for non resectability of 90%^[197].

With the advent of multi detector CT scanners (MDCT), the pancreas can be imaged at a very high spatial and temporal resolution^[198,199]. The dual phase pancreatic protocol MDCT using 1 to 3 mm slice collimation is one of the most sensitive techniques for metastatic disease to the liver and peritoneum^[186,200,201]. Recent studies have shown that MDCT has a NPV of 87% for tumor resectability compared to a NPV of 79% for conventional helical CT^[202] and with an accuracy between 85% and 95%^[203,204].

Images from MDCT can be used to visualize the biliary tree and normal vascular variants such as replaced hepatic arteries before surgical planning. Gangi *et al.*^[198] reported that pancreatic ductal dilatation in asymptomatic patients could be identified between 0 to 50 mo before PC diagnosis was confirmed. The sensitivity, specificity and accuracy of CT in the presence of hypo-attenuated pancreatic lesions, pancreatic ductal dilatation with cut-off, distal pancreatic atrophy, pancreatic contour abnormalities and common bile duct dilatation are reported in Table 6^[205].

Despite these improvements, interpretation of the CT scan is quite challenging in the setting of pancreatitis forming mass effects^[206] and in the presence of loco-regional lymph node involvement and small hepatic metastasis^[207].

Magnetic resonance imaging-magnetic resonance cholangiopancreatography: In most institutions, MRI is performed when other imaging modalities provide insufficient data for the clinical staging of the tumor, or when treatment planning can not be based on the images obtained by other techniques. Several studies have shown that MRI is superior to CT for the detection and staging of PC (100% *vs* 94%, respectively)^[208-211]. However, recent evidence has challenged this belief. The use of MRI-magnetic resonance cholangiopancreatography (MRCP) to better characterize PC is supported by a pro-

Table 6 Sensitivity, specificity and accuracy of computed tomography findings in pancreatic cancer patients

CT finding	Sensitivity (%)	Specificity (%)	Accuracy (%)
Hypoattenuation	75	84	81
Ductal dilatation	50	78	70
Ductal interruption	45	82	70
Distal pancreatic atrophy	45	96	81
Pancreatic contour anomalies	15	92	70
CBD dilatation	5	92	67

CT: Computed tomography; CBD: Common bile duct.

spective analysis that compared these two modalities in patients with periampullary cancers^[212]. MRI-MRCP was superior to CT in differentiating malignant from benign lesions (ROC = 0.96 *vs* 0.81, $P < 0.05$) and MRI-MRCP had better sensitivity (92% *vs* 76%), specificity (85% *vs* 69%), accuracy (90% *vs* 75%), PPV (95% *vs* 88%) and NPV (79% *vs* 50%) compared to CT. Another study confirmed the previous results with MRI-MRCP showing 97% sensitivity, 81% specificity and 89% accuracy^[213].

On the other hand, other studies comparing gadolinium-enhanced MRI with MDCT have shown that MRI and CT had equivalent sensitivity and specificity (83%-85% *vs* 83% and 63% *vs* 63%-75%, respectively). Both techniques had good to excellent agreement between radiologists, although MRI had a superior agreement for the evaluation of distant metastases (inter-observer agreement between MRI and CT scan; 0.78 *vs* 0.59 $P = 0.1$)^[214]. On the other hand, with the improvement in CT scan technology, recent studies have shown that MRI might have lower sensitivity in comparison to MDCT (82%-94% *vs* 100%)^[215]. This was confirmed by a recent meta-analysis comparing the accuracy of several imaging modalities which showed that helical CT had superior sensitivity compared to MRI (91% *vs* 84%) and transabdominal US (91% *vs* 76%)^[216]. Sensitivity for resectability of the tumor was equal for both MRI and helical CT (82% *vs* 81%, respectively)^[216].

Positron emission tomography: ¹⁸F-2fluoro-2-deoxy-D-glucose (FDG) accumulated by tumor cells provides positron emission tomography (PET) with the advantage of combining metabolic activity and imaging characteristics. Newly developed PET scanners can detect small PCs up to 7 mm in diameter and diagnose metastatic disease in about 40% of cases^[217,218]. A Japanese study found that the overall sensitivity of PET-CT was superior to contrast CT (92% *vs* 88%) and that PET was better at detecting bone metastases (100% *vs* 12%). However, CT scanning was superior for the evaluation of vascular invasion (100% *vs* 22%), involvement of para aortic regional lymph nodes (78% *vs* 57%), identification of peritoneal dissemination (57% *vs* 42%) and hepatic metastases (73% *vs* 52%)^[219]. Another Japanese study confirmed that PET had a sensitivity of 87%, a specificity of 67% and accuracy of 85%, and that tumors with metastatic

Table 7 Summary of the performance characteristics of imaging tests for the diagnosis of pancreatic cancer

Diagnostic modality	Author	Yr	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
US	Giovannini <i>et al</i> ^[176]	1994	48-95	40-91	92	100	46-64
	Böttger <i>et al</i> ^[177]	1998					
	Rösch <i>et al</i> ^[178]	1991					
	Niederer <i>et al</i> ^[179]	1992					
	Palazzo <i>et al</i> ^[180]	1993					
	Tanaka <i>et al</i> ^[231]	1996					
Doppler US	Candiani <i>et al</i> ^[232]	1998	50-94	80-100	79	88	81-95
	Casadei <i>et al</i> ^[184]	1998					
	Calculi <i>et al</i> ^[233]	2002					
EUS	Akahoshi <i>et al</i> ^[234]	1998	98	97	94	100	90
	Legmann <i>et al</i> ^[235]	1998					
Contrast enhanced US	Dietrich <i>et al</i> ^[185]	2008	90	100	100	86	93
CT	Bronstein <i>et al</i> ^[196]	2004	77	100	NA	NA	73
	Megibow <i>et al</i> ^[197]	1995					
MDCT	Park <i>et al</i> ^[214]	2009	83-91	63-75	80	87	85-95
	Vargas <i>et al</i> ^[202]	2004					
	Diehl <i>et al</i> ^[203]	1998					
	Schima <i>et al</i> ^[208]	2002					
MRI-MRCP	Andersson <i>et al</i> ^[212]	2005	83-92	63-85	95	79	89
PET	Maemura <i>et al</i> ^[217]	2006	87-100	67-77	94	100	85-95
	Delbeke <i>et al</i> ^[221]	1999					

PPV: Positive predictive value; NPV: Negative predictive value; US: Ultrasound; EUS: Endoscopic ultrasound; CT: Computed tomography; MDCT: Multi detector computed tomography; PET: Positron emission tomography; NA: Not applicable; MRI: Magnetic resonance imaging.

disease had significantly higher standardized uptake values [SUV = tissue concentration (millicuries/g)/injection dose (millicuries)/body weight (g)] than those without metastases^[220]. PET had superior sensitivity (100% *vs* 65%), specificity (77% *vs* 61%), NPV (100% *vs* 31%), PPV (94% *vs* 87%) and accuracy (95% *vs* 65%) in an American study comparing PET-CT with a SUV cut off of 2.0 *vs* contrast CT^[221]. A recent study enrolling 59 PC patients showed similar results, with 91% PPV and 64% NPV for PET-CT. One of the most interesting results was that the clinical management of patients undergoing PET was changed in 16% of cases deemed resectable after routine staging ($P = 0.031$) preventing unnecessary surgery because of distant metastases^[222].

Diffuse uptake of FDG is frequent in pancreatitis in comparison to PC (53% *vs* 3%, $P < 0.001$), and therefore PET is extremely useful in distinguishing these two conditions in controversial cases^[218,223]. Animal studies have shown that ¹¹C-acetate-PET appears to be superior to FDG PET for the detection of early PC and might be useful in differentiating inflammatory processes from malignancies as ¹¹C-acetate-PET is less affected by the presence of inflammation in human tissues^[224].

Another very important characteristic of PET-CT is its ability to provide useful information on tumor viability, and this technique also allows monitoring of tumor response to treatment^[217] and the metabolic features of PET help predict the prognosis as a SUV less than 3 appears to be a positive predictive factor^[222,225-229].

Similar results were found by Zimny *et al*^[230] who showed that better survival trends were noted in patients with PC and a SUV less than 6.0 in comparison to those with a higher SUV. Sensitivity and specificity of imaging modalities are summarized in Table 7.

STAGING

Pathological staging

In the 7th edition of the American Joint Committee on Cancer the different categories of PC are classified according to only one TNM staging system, even if neuroendocrine tumors have a different biology and a better prognosis than ductal carcinomas. Yet, the TNM system provides a reasonable discrimination and prognostic validity for these patients^[236].

The TNM system classifies PC into 3 clinically important categories: (1) patients with Tis-T2 PC have localized cancer within the pancreas; (2) patients with T3 cancer have locally invasive disease; and (3) patients with T4 tumors have unresectable PC^[237] (Table 8).

Prognostic features of PC include perineural and lymphovascular invasion, elevated serum CA19-9 levels and incomplete tumor resection. Therefore, gross and microscopic assessment of the resection margins is of major importance even if it is not included in the TNM staging system. Patients undergoing resections with grossly or microscopically positive margins have no survival benefits compared to individuals undergoing palliative chemo-radiation therapy alone.

Clinical staging

Surgery is the only chance of cure and the presence of negative resection margins of the primary tumor represent the strongest prognostic factor. Preoperative staging modalities include the combination of several imaging techniques such as CT scan, MRI, EUS, staging laparoscopy and laparoscopic ultrasound which aim to identify patients with resectable disease. There is consensus that patients with distant metastases (liver, lungs, peritoneum)

Table 8 American Joint Committee on Cancer staging of pancreatic cancer

AJCC 6th edition TNM staging system for pancreatic cancer		
TX	Primary tumor cannot be assessed	
T0	No evidence of primary tumor	
Tis	Carcinoma <i>in situ</i>	
T1	Tumor limited to the pancreas, 2 cm or less in greatest diameter	
T2	Tumor limited to the pancreas, greater than 2 cm at greatest diameter	
T3	Tumor extends beyond pancreas but no involvement of celiac axis or superior mesenteric artery	
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable)	
NX	Regional nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
MX	Distant metastasis cannot be assessed	
M0	No distant metastasis	
M1	Distant metastasis	
Stage grouping		
Stage 0	Tis N0 M0	Localized within pancreas
Stage I A	T1 N0 M0	Localized within pancreas
Stage I B	T2 N0 M0	Localized within pancreas
Stage II A	T3 N0 M0	Locally invasive, resectable
Stage II B	T1, 2, or 3 N1 M0	Locally invasive, resectable
Stage III	T4 Any N M0	Locally advanced, unresectable
Stage IV	Any T Any N M1	Distant metastases

AJCC: American Joint Committee on Cancer.

or local invasion of the surrounding organs (stomach, colon, small bowel) are usually not surgical candidates.

The criteria for unresectability of PC include tumor encroachment (defined as tumor surrounding the vessel more than 180 degrees) of arteries such as the celiac artery, hepatic artery, superior mesenteric artery (SMA) or massive venous invasion with thrombosis. Portal or superior mesenteric venous invasion without thrombosis or obliteration of vessels can still be classified as resectable PC^[204,238]. A recent study comparing the roles of EUS, CT, MRI and angiography in the assessment of PC staging and resectability has shown that CT scanning was the most accurate in assessing the stage of the tumor (73%), loco-regional invasion (74%), vascular involvement (83%), distant metastases (88%), final TNM stage (46%) and overall tumor resectability (83%)^[239]. EUS appeared to be superior in detecting smaller tumors not visualized by CT. A decision analysis demonstrated that the best strategy to assess tumor resectability was based on CT as an initial test and the use of EUS to confirm the results of resectability by CT^[221].

Laparoscopic staging

Diagnostic laparoscopy for PC was first introduced as a staging procedure in the late 1980s by Cuschieri *et al.*^[240] and Warshaw^[241,242]. Staging laparoscopy is considered a simple, minimally invasive technique to identify radiographically occult distant metastatic disease and to prevent non-therapeutic laparotomies. Laparoscopic examination allows direct visualization of intra-abdominal contents and has been reported to identify hepatic and peritoneal metastases not shown by other modalities^[243] as reported in some studies where 20%-48% of patients considered resectable by CT were found to be unresectable during surgery^[244-246].

Diagnostic laparoscopy involves a general exploration

of the abdominal surfaces including palpation of the liver with two instruments when necessary. The hilum of the liver is visualized, the foramen of Winslow is examined and periportal lymph nodes are biopsied when enlarged. The transverse colon and omentum are reflected cephalad and the base of the transverse mesocolon is examined with particular attention to the mesocolic vessels. The gastrotocolic ligament/omentum is incised and the lesser sac is examined^[247].

Laparoscopic ultrasonography (LUS) has been introduced as an additional procedure to increase the detection of intrahepatic metastases, identify enlarged and suspicious lymph nodes and to evaluate local growth in the vascular structures^[248]. Some studies have demonstrated that LUS has improved the accuracy of predicting resectability up to 98%^[249-251].

Despite these results, the routine use of staging laparoscopy and LUS in patients with radiographically resectable PC remains controversial as imaging modalities have significantly improved, thus reducing the risk of discovering non-resectable disease at the time of surgery. In addition, staging laparoscopy adds costs and it can be time consuming. Sustainers of staging laparoscopy are supported by a study by Kwon *et al.*^[250], which revealed that staging laparoscopy was able to detect unsuspected metastases and changed the surgical approach in 37% of patients even when using CT, MRI, ERCP and angiography for preoperative staging. Another study by Conlon *et al.*^[247], supported the use of staging laparoscopy as only 67 out of 115 patients (58%) with PC had resectable disease after completion of the laparoscopic examination. On the other hand, a more recent study from the same group at the Memorial Sloan-Kettering Cancer Center has shown that the yield of staging laparoscopy was only 8.4% when good imaging modalities were obtained at the referral center^[252].

Based on the fact that minimally invasive approaches for the diagnosis of PC as well as radiological imaging techniques will continue to advance, the selective use of staging laparoscopy and LUS would play a role in cases where detection of unresectable disease is more likely. Factors which suggest a higher yield with diagnostic laparoscopy include a large primary tumor (diameter larger than 4 cm), a tumor in the body or tail of the pancreas, equivocal findings after imaging tests, severe weight loss, abdominal or back pain, hypoalbuminemia and significantly elevated tumor markers^[240].

TREATMENT

Patients with suspected or confirmed diagnosis of PC should be assessed by a multidisciplinary team and stratified as resectable (stage I or II), borderline resectable (stage IIa or IIb), locally advanced unresectable (stage III) or metastatic disease (stage IV). Treatment should be planned according to local expertise and established guidelines, as resectable and borderline patients should be referred to surgeons, unresectable and metastatic patients should be referred to medical and radiation oncologists and palliative care teams. A multidisciplinary approach to PC is necessary to improve the overall outcome of these patients, especially for borderline resectable or unresectable disease as neo-adjuvant chemo-radiation therapy may play a role in downstaging and the conversion to potentially curable disease^[253,254].

SURGICAL THERAPY

Surgical treatment is the only potential cure for PC^[255]. Although pancreatic surgery is considered challenging and technically demanding, improvements in surgical techniques and advances in perioperative supportive care have reduced the mortality rates to less than 5% in high-volume centers^[256-258]. According to the United States Surveillance and Epidemiology End Results registries, the 5-year relative survival for the period between 1999 and 2006 was 22.5% for localized and 1.9% for metastasizing PC (Table 9)^[259].

Because only 20% of patients with PC are candidates for radical resection at the time of diagnosis^[260], accurate staging is important in identifying surgical candidates and sparing the risk and cost of surgery for patients who are affected by advanced disease^[261]. Unresectable PC is commonly defined when there is tumor invasion of the SMA, inferior vena cava, aorta or celiac arteries; encasement or occlusion of the SMV-portal venous system or by distant metastasis (e.g. hepatic, extra-abdominal, peritoneum, omentum, lymph nodes outside the resection zone)^[262]. An Italian study has recently demonstrated that the duration of symptoms (mainly jaundice and celiac pain) of more than 40 d, CA 19-9 levels above 200 U/mL and G3-G4 histological grade of the tumor are poor prognostic parameters, even if the disease is resectable by preoperative staging^[263].

Table 9 Stage distribution of pancreatic cancer and 5-year relative survival by stage at diagnosis for 1999-2006, all races and both sexes (SEER registries)

Stage at diagnosis	Stage distribution (%)	5-yr relative survival (%)
Localized (confirmed to primary site)	8	22.5
Regional (spread to regional LNs)	26	8.8
Distant (cancer had metastasized)	53	1.9
Unknown (unstaged)	14	5

SEER: Surveillance Epidemiology and End Results.

Tumor of the head of pancreas

Preoperative biliary decompression vs immediate surgical resection: Obstructive jaundice is a common presentation for tumors located in the periampullary area or in the head of the pancreas. To reduce perioperative complications and mortality in patients with obstructive jaundice undergoing pancreaticoduodenectomy (PD), preoperative biliary drainage appears to have a positive impact supported by the findings of several observational studies^[264-266]. On the other hand, several other non-randomized studies failed to show any advantage of preoperative biliary decompression in these patients, as they developed a higher incidence of bacteriobilia and fungal colonization causing more wound infections, postoperative sepsis and longer hospital stay^[267-270]. Two meta-analyses of randomized controlled trials and a systematic review of descriptive series have shown that the outcome of patients undergoing biliary decompression prior to PD was inferior to early surgery as they had higher rates of infectious complications and perioperative mortality^[271,272]. These findings were confirmed by a recent multicenter randomized controlled study from the Netherlands which showed that the rates of serious complications were 39% for patients who underwent early surgical resection in comparison to 74% in the group that underwent pre-operative biliary decompression ($P < 0.001$)^[264]. Similarly, surgical complications occurred in 37% of patients undergoing early resection in comparison to 47% for individuals who had preoperative biliary decompression. Although the difference did not reach statistical significance ($P = 0.14$), the overall mortality and hospital stay were comparable between the two groups^[273].

During the last decade, there has been an increasing interest in treating patients with neo-adjuvant chemoradiotherapy to improve disease-free and overall survival in patients undergoing surgery. Although there are still no phase III randomized controlled studies to support the use of this strategy, several phase II randomized trials have shown that neo-adjuvant chemo and chemo-radiation therapy are relatively well tolerated, do not reduce the resectability rate and seem to increase the percentage of patients who undergo R0 resections^[274-283]. For jaundiced PC patients, candidates for neo-adjuvant therapy must undergo biliary decompression to prevent liver decompensation and stent patency is required for several months. Currently, the only study assessing the outcome

of patients undergoing chemo-radiation therapy prior to PD has shown that plastic stents do not provide patency of the biliary system for long enough to complete the preoperative protocols. In fact, 55% of cases required unplanned repeat ERCP with stent exchange for recurrence of jaundice or ascending cholangitis^[284]. For these patients, self expanding metallic stents should be used as the direct costs associated with repeating ERCP and hospital admissions for recurrent biliary obstruction and ascending cholangitis appear to be superior to the initial higher cost of using metallic stents^[285].

Standard vs pylorus preserving PD: Walter Kausch first described PD in 1912^[286], and Allan Whipple later popularized the procedure that bears his name^[287]. The classic Whipple (CW) operation consists of an *en-bloc* removal of the pancreatic head, the duodenum, the common bile duct, the gall bladder and the distal portion of the stomach together with the adjacent lymph nodes^[288]. This operation can lead to specific long-term complications such as early and late dumping syndrome, post-operative weight loss^[289] and post-operative acid and bile reflux^[290].

Pylorus preserving PD (PPPD) was first introduced by Watson in 1942^[291], and the procedure was popularized by Traverso and Longmire in 1978^[292]. Although it was originally described for the treatment of periampullary tumors, many surgeons nowadays perform PPPD for PC in the head of the pancreas. In order to retain a functioning pylorus, the stomach and the first 2 cm of the duodenum are preserved along with their neurovascular supply. The rationale behind preservation of the stomach is to improve long-term gastrointestinal function^[293]. There is still some controversy as to which is the best surgical treatment for PC of the head of the pancreas. In comparison to CW, PPPD has the advantages of reduced operative time^[294], less blood loss, better access to the biliary anastomosis for post-operative endoscopy in patients with recurrent biliary obstruction, improvement of post-operative weight gain and quality of life^[295]. On the other hand, some series have reported that PPPD has a higher incidence of delayed gastric emptying^[296,297]. Moreover, it has not been unequivocally shown that PPPD is oncologically equivalent to CW^[298]. A number of RCTs and meta-analyses have demonstrated that both perioperative morbidity and long-term outcome are equal in CW and PPPD^[263,299,300].

Pancreatic reconstruction: The most significant cause of morbidity and mortality after PD is the development of complications caused by leakage of pancreatic secretions and pancreatic fistulae observed in up to 20% in specialized centers^[301,302]. The meticulous reconstruction of pancreatico-enteric continuity is the key to preventing pancreatic fistulae^[303]. Pancreatico-jejunostomy and pancreatico-gastrostomy (PG) are the most commonly employed techniques for pancreatocenteric reconstruction. PG was believed to be an easier technique and less prone to ischemia as a result of the close proximity between the stomach and the pancreatic stump and the presence

of a better vascular supply in the stomach in comparison to the jejunum. However, RCTs have not demonstrated superiority of one technique over the other in terms of post-operative complication rates or incidence of pancreatic fistulae^[304,305].

Tumor of the body/tail of pancreas

Distal pancreatectomy: Distal pancreatectomy is the surgical procedure of choice for PC of the body and tail of the pancreas. It entails resection of the portion of the pancreas extending to the left of the superior mesenteric vessels and not including the duodenum and the distal bile duct^[306]. The spleen is conventionally removed in an *en-bloc* fashion^[307]. However, splenic preservation could be accomplished without an increased rate of complications, operative time or the duration of post-operative hospital stay^[295,308]. Several closure techniques have been introduced for the pancreatic remnant in an attempt to reduce pancreatic fistulae. They include hand-sewn suture techniques, staple closure techniques or a combination of both^[309-312], ultrasonic dissection devices^[313], pancreatico-enteric anastomosis^[314], application of meshes, seromuscular^[315] and gastric serosal patches^[316], or sealing the pancreatic stump with fibrin glue^[199].

Cancers of the body and tail of pancreas usually present at a later stage of the disease in comparison to PC of the head due to lack of early symptoms^[317]. There are no survival differences between resections for equal TNM stage tumors of the head vs tumors of the body and tail as shown by a retrospective study that reported a 5-year survival of 17% after resection of the pancreatic head vs 15% for left-sided tumors in stage I cancers^[318].

Laparoscopic pancreatic resection: Laparoscopic pancreatic surgery represents one of the most challenging abdominal operations^[319,320]. Gagner and Pomp were the first to describe a laparoscopic duodeno-pancreatectomy in 1994^[321]. Since then, the total number of laparoscopic duodeno-pancreatectomies has remained small due to technical difficulties associated with this operation^[322]. A recent study from the Mayo clinic with 65 patients who underwent total laparoscopic PD (TLPD) outlined that TLPD is safe, feasible and its results appear to be comparable to the open approach^[323] (Table 10).

Nevertheless, larger prospective studies are required in order to better assess the advantages of TLPD.

Laparoscopic distal pancreatic resection is currently the most frequently performed laparoscopic pancreatic surgery^[327]. Most of the studies on distal laparoscopic pancreatectomy are case series with a relatively small number of patients^[328]. Although recent studies have shown that laparoscopic distal pancreatectomy is feasible and safe^[329-331], the morbidity, mortality and hospital stay are similar to those after open surgery^[332]. This is probably due to the fact that morbidity after pancreatic surgery results from retroperitoneal dissection, length of the operation and pancreatic fistulae rather than the incision. In addition, a recent prospective observational study comparing 85 open vs 27 laparoscopic distal pancreatectomies has shown

Table 10 Published results on laparoscopic pancreaticoduodenectomies

Author	Yr	Patient No.	Morbidity (%)	Pancreatic fistula (%)	Mean hospital stay	Mortality (%)
Kendrick <i>et al</i> ^[323]	2010	62	42	18	7	1.6
Palanivelu <i>et al</i> ^[324]	2007	42	28.6	7.1	10.2	2.4
Dulucq <i>et al</i> ^[325]	2006	25	31.8	4.5	16.2	0
Pugliese <i>et al</i> ^[326]	2008	19	31.6	15.8	18	0

that the number of lymph nodes removed during the minimally invasive procedure was significantly inferior (mean number: 5.2) in comparison to the open approach (mean number: 9.4)^[333]. These findings suggest that at this time there is a lack of evidence to support oncological equipoise between laparoscopic and open resections for PC.

Total pancreatectomy: Total pancreatectomy has been employed in selected patients with chronic pancreatitis^[334], multifocal islet cell tumors or diffuse IPMN^[335]. Total pancreatectomy for PC was initially proposed to avoid the risk of pancreato-enteric leaks and to remove potential undetectable synchronous disease in other parts of the gland^[336]. However, the indication of total pancreatectomy to avoid the risks of pancreatic fistulae is still controversial^[337]. Improvement in operative techniques, advances in nutritional support, critical care and interventional radiology have significantly decreased the incidence of life-threatening sequels of pancreatoenteric leaks^[338]. In addition, the permanent endocrine insufficiency associated with total pancreatectomy impacts enormously on the quality of life and long-term outcome of these patients^[339]. Some studies have demonstrated a significant increased risk of perioperative morbidity and mortality associated with total pancreatectomy compared with PD^[318]. A recent study by Reddy *et al*^[335] showed that long-term survival rates were equivalent after total pancreatectomy and PD (19.9% *vs* 18.5%), supporting the fact that there is no oncological benefit of total pancreatectomy *vs* a more limited resection in PC. Currently, total pancreatectomy should be performed in patients with PC if it is the only oncologically sound treatment option^[335].

Vascular resections and extended lymphadenectomy:

With the advancement in operative techniques and perioperative management of patients with PC, more radical surgical procedures with vascular resection and extended lymphadenectomy have been proposed for selected cases^[340]. The results of extended vascular and lymphatic resections remain controversial.

The principal use of venous resection and reconstruction is to allow complete tumor clearance when precluded by tumor involvement of the superior mesenteric or portal vein, and when the surgeon expects to achieve a negative resection margin^[341]. Post-operative morbidity and mortality rates following portal or superior mesenteric vein resections seem to be similar to those of patients with standard PD (42%-48.4% *vs* 47.1%, 3.2%-5.9% *vs* 2.5%, respectively)^[342,343]. Another study showed that patients undergoing pancreatic resection with venous recon-

struction (VR) had a median survival of 22 mo compared to 20 mo for those who had classic PD ($P = 0.25$)^[344]. In another study, a slight survival benefit was noted in patients who did not require VR (33.5%) compared to those with VR (20%, $P = 0.18$), although this did not reach statistical significance^[345].

Pancreatectomies with major arterial resections (common hepatic artery/celiac axis and superior mesenteric artery) have been reported in recent years with acceptable outcomes. Nevertheless, arterial reconstruction during pancreatectomies remains a challenging procedure with increased risk of complications compared to classic PD and PD with VR. In addition, most PCs with arterial invasion are for the majority, advanced tumors with distant lymph node involvement and metastases, and therefore indicated only in a very select group of patients^[346]. Recent data on pancreatectomies requiring arterial resections at high volume tertiary centers have shown operative mortality rates of 4.3%^[346], peri-operative mortality rates (60 d) of 17%^[347], morbidity rates of 48%^[348] and 3-year survival rates of 17%-23.1%, which are much higher than for classic PD^[346,347].

It has been noted that lymph node involvement outside the standard PD specimens occurs in more than 30% of cases^[349]. This has led to the evaluation of the need for a more extended lymph node dissection (ELND) in the surgical management of PC. To date, the definitions of a standard lymphadenectomy as well as ELND are still not very clear^[341]. A number of Japanese studies have shown an increased survival rate in patients who have undergone ELND compared to conventional PD^[350-352]. However, these studies were not randomized and their data were not validated by other centers^[353].

The first RCT comparing standard PD and ELND was reported by Pedrazzoli *et al*^[354] in 1998. In this study, standard lymph node dissection was defined as the removal of lymph nodes from the anterior and posterior pancreatoduodenal region, pyloric region, biliary duct, superior and inferior pancreatic head and body. In addition to the above, ELND included removal of lymph nodes from the hepatic hilum and along the aorta from the diaphragmatic hiatus to the inferior mesenteric artery and laterally to both renal hila, with circumferential clearance of the origin of the celiac trunk and SMA. This study showed no difference in morbidity, mortality or 4-year survival rates between the two groups.

Recently, a meta-analysis on standard PD and PD + ELND for PC patients showed comparable morbidity and mortality rates with a trend towards higher rates of delayed gastric emptying in the ELND group. The weighted

Table 11 Survival data after resection of pancreatic cancer

Author	Yr	Resection (n)	RO resection (n)	Overall 5-yr survival (%)	RO 5-yr survival (%)	Median survival (mo)
Fatima <i>et al.</i> ^[371]	2010	617	468	17.4	20	18
Kato <i>et al.</i> ^[376]	2009	138	115	9.9	13.2	12.3
Raut <i>et al.</i> ^[373]	2007	360	300	NA	NA	24.9
Cameron <i>et al.</i> ^[258]	2006	1000	NA	18	23	33
Shimada <i>et al.</i> ^[372]	2006	88	66	19	26	22
Howard <i>et al.</i> ^[375]	2006	126	158	4	67	18
Moon <i>et al.</i> ^[374]	2003	81	20	10.8	67.8	11.8

NA: Not applicable.

mean log hazard ratio for overall survival was 0.93 (CI: 0.77-1.13), revealing no significant outcome differences between the standard and extended procedure ($P = 0.480$) suggesting that ELND does not benefit overall survival and has a trend towards increased morbidity^[355].

CLINICAL VOLUME AND OUTCOMES

During the last two decades, several large observational studies in the U.S., Canada and the Netherlands have shown that the institutional volume of pancreatic resections affects patients' outcomes. Higher perioperative morbidity, mortality and decreased use of multimodality therapy have been observed more frequently in low volume centers^[356-363]. In 1993, Edge and colleagues reported that case load did not correlate with mortality after pancreatic resection^[364]. However, surgeons who performed fewer than 4 resections per year had more complications. Recent studies have shown significant improvements in perioperative morbidity and mortality in patients undergoing pancreatic resections in high volume centers. For example, investigators at Memorial Sloan Kettering Cancer Center found that in a cohort of 1972 patients, high-volume centers defined as performing more than 40 cases per year in New York State had significantly less mortality (4% *vs* 12.3%) than low volume centers^[356].

The definition of high and low volume varied among all these studies, but the findings were consistent and were confirmed by Birkmeyer *et al.*^[365] who showed that very low volume centers (0-1 procedure per year), low volume hospitals (1-2 procedures per year) and higher volume hospitals (more than 5 procedures per year) had significantly different mortality rates (16% and 12% *vs* 4% respectively; $P < 0.001$). The largest difference in operative mortality between very low volume (17.6%) and high volume (3.8%) centers is even more significant for PD when compared to other major surgeries as shown in a retrospective analysis of data from the national Medicare claims database and the Nationwide Inpatient Sample^[257].

A recent study involving 301 033 patients with PC included in the National Cancer Database evaluated the treatment patterns of 1667 hospitals over a 19-year period^[366]. During that time the pancreatectomy rate as well as the use of multimodality adjuvant therapy for patients with stage I and II disease increased significantly (pancreatectomy rate increased from 39.6% to 49.3%; $P < 0.001$, and

the use of multimodality therapy increased from 26.8% to 38.7%; $P < 0.001$). Furthermore, patients were more likely to receive multimodality therapy at academic institutions, particularly those considered to be high volume hospitals. Despite these important advances, it appears that there is still a high percentage (71.4%) of patients with potentially resectable disease who are still not referred for surgical resection as reported by Bilimoria *et al.*^[367]. These findings would suggest that a persistent nihilism of clinicians towards PC and pancreatectomy may be the most significant correctable factor that contributes to the current poor long-term outcomes of PC.

ADJUVANT CHEMORADIATION THERAPY

Several single agent chemotherapeutic agents have been tried in the treatment of PC. 5-FU has been used in PC for more than 25 years with response rates of 8%-15%^[368]. The addition of Leucovorin to 5-FU doubled the response rate to 26%, however, it showed no benefit in terms of survival^[369]. The only chemotherapeutic agent that demonstrated prolonged survival in comparison to 5-FU and Leucovorin was Gemcitabine^[370].

After pancreatic resection, the 5 year survival rate is only 20% or less as PC has a high loco-regional recurrence rate and a tendency towards early liver metastasis (Table 11)^[258,371-376].

Based on these observations it appears necessary to employ adjuvant therapy in combination with surgical resection in order to improve survival. Only a few years ago there was no valid data on adjuvant chemoradiation therapy after curative surgical resection^[377].

The first RCT that showed benefit from adjuvant chemoradiation therapy in comparison to surgery alone was the Gastrointestinal Tumor Study Group (GITSG) trial, where patients receiving 40 cGy followed by 5-FU showed a mean survival of 18 mo in comparison to 11 mo for those who received surgery alone ($P = 0.05$). The two- and five-year survival rates of the two groups were 43% *vs* 18% and 19% *vs* 0%, respectively^[378].

The EORTC (European Organization for Research and Treatment of Cancer) study showed that patients undergoing chemoradiation therapy (5-FU protocol) had a median survival of 17.1 mo compared to 12.6 mo for the

controls ($P = 0.099$). The two- and five-year overall survival rates were 37% and 20% for the experimental arm and 23% and 10% for the control arm ($P = \text{NS}$)^[379].

The European Study Group for PC 1 trial (ESPAC-1) compared four groups of patients who underwent pancreatic resection; (1) surgery alone; (2) 5-FU and Leucovorin adjuvant chemotherapy; (3) combination of adjuvant radiation therapy and 5-FU chemotherapy; and (4) adjuvant chemoradiation followed by chemotherapy^[380]. In this study, the five-year survival rate for patients who received adjuvant chemotherapy was 21% compared to 8% for patients who did not ($P = 0.009$). Patients who underwent chemoradiation therapy had an inferior five-year survival rate (10% *vs* 20%) in comparison to patients who did not receive radiation ($P = 0.05$).

In 2006, the Radiation Therapy Oncology Group trial compared patients receiving adjuvant chemoradiation (5040 cGy in combination with continuous 5-FU) followed by 5-FU *vs* similar chemoradiation therapy followed by Gemcitabine. For patients affected by PC of the head, the arm treated with Gemcitabine had a superior median (18.8 mo *vs* 16.7 mo) and overall survival at 3 years [31% *vs* 21% ($P = 0.047$)], but with a higher incidence of toxicity (80% *vs* 60%)^[381].

In 2007, a RCT conducted in Germany and Austria (CONKO-1 [Charite Onkologie Clinical Studies in GI Cancer 001]) compared patients undergoing R0 or R1 pancreatic resection alone *vs* resection followed by Gemcitabine-based chemotherapy. The median disease-free survival for patients treated with Gemcitabine was 13.9 mo *vs* 6.9 mo in the observation arm ($P < 0.001$), although there was no difference in the overall survival between the two groups (22 mo *vs* 20 mo)^[382]. From the results of these studies, adjuvant chemotherapy has become the standard of care for patients who can tolerate the treatment after surgical resection.

NEOADJUVANT THERAPY

Neoadjuvant therapy is defined as the preoperative intervention aiming to convert unresectable PCs to resectable tumors or to increase the probability of complete microscopic tumor resection^[383]. One of the limitations of the role of neoadjuvant therapy for PC is the fact that there is no standardized definition for tumor resectability and there is no data from randomized phase three trials on the benefit of neoadjuvant therapy. In addition, data from prospective and retrospective studies have several biases due to heterogeneity of inclusion and exclusion criteria, preoperative quality of imaging tests, and surgical pathology reports on lymph node involvement and resection margin status.

A recent systematic review^[383] evaluating retrospective and prospective studies on neoadjuvant chemo and radiation therapy from 1966 to 2009 included a total of 111 studies and 4,394 patients. The results of this meta-analysis showed that the majority of patients were treated with Gemcitabine, 5-FU or oral analogue Mitomycin-c, and Platinum compounds. Patients undergoing neoadjuvant treatment received radiotherapy in the range of 24-63 Gy.

The analysis showed that neoadjuvant treatment in patients with unresectable tumor was able to convert 33.2% of patients to resectable candidates, providing a median survival of 20.5 mo which was equivalent to patients undergoing resection followed by adjuvant therapy who had median survival of 20.1 to 23.6 mo. On the other hand, neoadjuvant therapy for patients with resectable cancer did not seem to improve overall outcome.

RADIATION THERAPY

Persistent loco-regional disease after pancreatic surgery is a major determinant of recurrence^[384]. Although there is supportive evidence for the use of adjuvant chemotherapy^[380,385], the role of adjuvant radiation remains unresolved. Generally it is believed that external-beam radiotherapy (EBRT) alone is a suboptimal treatment for locally advanced PC as most patients will die of systemic disease^[386].

In the Mayo clinic clinical trial and the GITSG trial, patients who were randomized to receive EBRT only had a median survival of 5.3-6.3 mo which was inferior to EBRT plus 5-FU^[387,388].

Among 210 patients who underwent surgical resection for PC [PD (73%), total and/or distal pancreatectomy (25%), Appleby procedure (2%)] followed by intraoperative electron beam radiotherapy (IOERT), some patients received a single fraction of IOERT alone (25 Gy), whereas others (30%) received additional EBRT and 54% received various forms of adjuvant chemotherapy. The study demonstrated excellent local control with the addition of IOERT (75%). Despite the benefit in local control, the overall median survival was similar to other studies with adjuvant chemotherapy or chemoradiation (19 mo)^[389]. A combined study of extended resection and intraoperative radiation therapy (IORT) concluded that IORT contributed to local control; however, it provided no overall survival benefits (14.6% 5-year survival)^[390].

In the United States, chemoradiation with concurrent 5-FU followed by Gemcitabine continues to represent the standard for adjuvant therapy of tumor of the pancreatic head. A direct comparison of chemo-radiation therapy and chemotherapy alone seems to be difficult to achieve and additive chemotherapy before or after chemo-radiation-therapy will have to be tested in randomized studies in order to determine the optimal sequencing^[391].

PALLIATIVE MEASURES

Palliative treatment of patients with PC plays a very important role as 80% to 90% of newly diagnosed tumors are not resectable due to local invasion or presence of distal metastatic disease^[392]. Median survival for patients with unresectable PC located in the head and body of the gland is approximately 7 mo, while for PC located in the tail median survival is significantly less [3 mo ($P = 0.0002$)], as they are usually diagnosed in more advanced stages^[393]. For these patients, relief of symptoms secondary to gastric outlet obstruction, jaundice and pain are essential to

improve their quality of life and overall survival. In the past, surgical palliation was more common as the diagnosis of unresectable disease was frequently done in the operating room and patients underwent one or more of the following procedures: gastric bypass, hepatico-enteric decompression and celiac plexus neurolysis for pain relief during the same surgery. With the improvement in diagnostic imaging tests, the role of surgical staging has decreased as the vast majority of patients can be currently classified as suffering from unresectable disease by non-invasive modalities such as CT and MRI or by endoscopic US. Nevertheless, there are still controversies on the best palliative strategies for these patients as there is a lack of randomized controlled trials and abundant contrasting data from observational studies.

Gastro-duodenal decompression

There is still some controversy on the use of routine gastro-intestinal bypass for PC diagnosed as unresectable at the time of exploratory laparoscopy or laparotomy.

In a large observational study of 155 patients with unresectable PC staged by extended laparoscopy at the Memorial Sloan Kettering Cancer Center, only 4% of patients required surgical intervention for gastric outlet obstruction before their death: 2 patients required open gastro-jejunal anastomosis alone and 1 patient underwent a combined gastro and hepatico-jejunostomy a few days after laparoscopy^[393]. In addition, 1 patient required a percutaneous endoscopic gastrostomy for palliation of gastric outlet obstruction a few weeks before demise. The authors concluded that the routine use of gastric bypass in patients with unresectable PC is not indicated. On the other hand, several other retrospective studies^[394,395] have suggested that up to 25% of patients with unresectable PC would develop gastric outlet obstruction requiring surgical intervention.

A recent prospective randomized trial compared 44 patients who were found unresectable at the time of surgery and who underwent a retrocolic gastro-jejunostomy to 43 patients who did not^[396]. The two groups had similar morbidity (32% *vs* 33%), mortality (0%) and hospital stay. On the other hand, patients who had gastric bypass did not develop any gastric outlet obstruction, while 19% of patients in the control group did ($P < 0.01$). Although this study would suggest that gastric bypass should be performed in all patients found unresectable at the time of surgery, the introduction of metallic self-expanding intestinal stents has changed the options for palliation.

A prospective multicenter cohort study of 51 patients with malignant gastric outlet obstruction treated with self-expandable metallic stents showed that in 98% of cases the stent was successfully deployed and that the median duration of patency was 10 mo. Only 14% of patients had stent dysfunction, and migration was observed in only 2% of cases^[397]. Similar results were reported by another study from South Korea which showed a median stent patency of 385 d, and only 1% serious complications (gastrointestinal bleeding or perforation)^[398]. Other observational studies have shown that compared with palliative surgery,

stent placement provides a shorter hospital stay, earlier resumption of oral intake, fewer complications and lower hospital costs^[399,400]. The only randomized controlled study that compared duodenal stent and laparoscopic gastrojejunostomy favored endoscopic therapy as it was associated with less discomfort, shorter hospital stay and improved physical health scores at 1 mo^[401]. In this small study, only a third of patients were alive at 1 year and no cases of stent occlusion were observed. The two groups had similar overall survival supporting equipoise between endoscopic and surgical palliation. Nevertheless, surgical palliation can still play an important role when patients have a long life-expectancy, need biliary and gastric bypass in combination with celiac neurolysis for pain control.

Biliary decompression

The majority of PCs occur in the head of the pancreas and obstructive jaundice is one of the early symptoms for 50%-80% of patients^[396]. In the past, staging laparotomy and biliary bypass were frequently performed for unresectable PC of the head^[402,403]. During the last decades, the development of interventional radiology and endoscopy has allowed palliation of obstructive jaundice by the insertion of percutaneous or endoluminal stents with minimal morbidity and mortality. Currently, endoscopic biliary stenting is the treatment of choice for unresectable PC with obstructive jaundice. Percutaneous transhepatic stenting is reserved only for patients in whom endoscopic stenting has failed as it is associated with a higher complication rate than endoscopic palliation (61% *vs* 35%)^[404,405]. High risk surgical patients are best managed by biliary stenting, however, it is still unclear whether palliative surgical biliary decompression is superior to other interventions for patients who are fit for surgery or who have a longer life expectancy. A European randomized controlled study comparing surgical biliary decompression *vs* endoscopic plastic stenting showed that both interventions were equally successful in palliating jaundice (95% *vs* 94%, respectively) and provided equal overall survival. Nevertheless, major complications (29% *vs* 11%) and procedure-related mortality (14% *vs* 3%) were significantly higher for surgical patients^[406]. In addition, surgical decompression was more expensive than stenting, although recurrent biliary obstructions and late gastric bypasses were more common in patients undergoing endoscopic treatment even if that did not reach statistical significance. Similar results were reported in a more recent Brazilian study which found that endoscopic therapy with self-expandable metallic stents was more cost-effective than surgical decompression (US\$2832 *vs* US\$3821, $P = 0.031$) and provided better quality of life at 30 ($P = 0.04$) and 60 d ($P = 0.05$)^[407]. The only available meta-analysis of randomized controlled studies comparing surgery with endoscopic stenting included only 3 studies where none tested the use of metallic self-expanding stents^[408]. Although the reintervention rate was 3% (0%-16%) in surgically treated patients compared with 36% (28%-43%) in stented patients, because of the limited number of studies with a relatively small group of patients and heterogeneous quality, the authors

concluded that they could not identify which treatment was preferable.

The patency of biliary stents has greatly improved with the introduction of expandable metallic stents (EMS) as they offer a larger diameter for drainage and are associated with a lower occlusion rate than plastic stents^[409,410]. The concurrent use of chemotherapeutic agents in patients palliated with SEMS was thought to increase the risk for ascending cholangitis. However, a Japanese retrospective study has demonstrated that the combination of SEMS and palliative chemotherapy for unresectable PC did not change the incidence of biliary infectious complications^[411]. In patients with combined biliary and duodenal obstructions, concomitant biliary and duodenal stenting is now feasible and justified as the need to repeat endoscopic therapies is rarely required even in long-term survival patients^[412].

Currently, surgical biliary bypass is advocated only for patients with obstructive jaundice who fail endoscopic or percutaneous stent placement.

Pain control

About 70% of patients with unresectable PC develop clinically important pain during their lives^[413]. Pain is the main cause of the significant drop in quality and quantity of life of these patients and good palliation is necessary as pain incidence and severity increases with disease progression^[414].

For the majority of patients, pain from PC can be managed with opioid analgesics. However, approximately one third of patients experience inadequate control of pain with oral analgesics alone^[415]. For these patients, radiation therapy, chemotherapy and celiac plexus neurolysis have been used. Percutaneous neurolytic celiac plexus block with injection of 50%-100% ethyl alcohol under radiological guidance has become the most commonly recognized method of splanchnicectomy with a 70%-96% success rate^[416]. The celiac plexus block has several advantages as it has been proven to ease pain without the side effects of opioids and can be administered intraoperatively, percutaneously, or by endoscopic ultrasonography. Recent studies have shown that endoscopic ultrasonography-guided neurolysis is effective and has minimal risk of the potentially serious complications associated with surgical or percutaneous approaches^[417,418].

A recent double-blind randomized controlled study comparing patients treated with celiac plexus block *vs* systemic analgesic therapy showed that splanchnic neurolysis provided superior pain relief and quality of life scores, but overall opioid consumption, frequency of opioid adverse effects and overall survival did not reach statistical significance between the two groups^[419]. For the majority of PC patients, pain is still controlled pharmacologically even if other modalities such as surgical thoracoscopic splanchnicectomy, epidural anesthesia, subcutaneous injection with octreotide, hypofractionated-accelerated radiotherapy and more recently photodynamic therapy have shown some temporary success^[414,420-423].

Nutritional supportive care

The median survival of patients with unresectable PC is 33 wk and for advanced metastatic disease is only 10 wk^[424]. About 90% of patients with PC have significant weight loss at the time of diagnosis and all of them develop progressive cachexia due to neoplastic metabolic derangements. Secondary events such as pancreatic exocrine insufficiency due to pancreatic duct obstruction, fat malabsorption due to biliary obstruction and poor oral caloric intake caused by nausea or gastric outlet obstruction are also responsible for the progressive weight loss. Even if weight loss has been found to have a prognostic effect on survival, most of the palliative care interventions for PC are directed at correcting biliary obstruction, gastric outlet obstruction and pain, and relatively little attention has been paid to interventions that can prevent or reduce the progressive weight loss of these patients^[425]. Recently, a placebo-controlled trial comparing patients receiving enteric coated pancreatic enzyme supplements *vs* placebo showed that after 2 mo, patients receiving pancreatin had gained 1.2% of their body weight in comparison to controls who lost 3.7% ($P = 0.02$), and that they had higher daily total energy intake (8.4 MJ *vs* 6.6 MJ, $P = 0.04$)^[424]. Although the Karnofsky performance status between the two groups was not different and survival analysis was not performed to determine if body weight gain translates into better prognosis, this study was the first to show an effective palliative strategy able to increase the intestinal absorptive function of patients who suffer from steatorrhea.

CONCLUSION

In recent decades, diagnostic modalities, and the surgical and palliative treatments of PC have clearly progressed although the overall prognosis has barely changed. The management of patients affected by PC is complex and requires expertise in many fields. Multidisciplinary teams are necessary to optimize the overall care, and palliative techniques have to be mastered as the majority of PCs are diagnosed in advanced stages. Better outcomes are reached if PC patients are appropriately referred to tertiary centers for assessment by surgical, medical and radiation oncologists, gastroenterologists, palliative care specialists and other dedicated health care providers. Despite recent progress, there is still a very limited ability to detect PC at an early stage, and there is a need for more studies to better understand genetic predisposing factors and to discover new markers that could assist physicians in this task. Randomized controlled studies are necessary to explore the role of neo-adjuvant therapies and new protocols for adjuvant strategies in patients undergoing pancreatic resection.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP

Neuroprotective action of *Ginkgo biloba* on the enteric nervous system of diabetic rats

Glasiella Gonzalez Perez da Silva, Jacqueline Nelisis Zanoni, Nilza Cristina Buttow

Glasiella Gonzalez Perez da Silva, Jacqueline Nelisis Zanoni, Nilza Cristina Buttow, Department of Morphological Sciences, Universidade Estadual de Maringá, Av. Colombo, 5790 Bloco H-79, CEP 87020-900, Maringá, PR, Brazil

Author contributions: da Silva GGP performed data collection and wrote the paper; Buttow NC designed the study, contributed analytical tools and corrected the paper; Zanoni JN analyzed the data.

Correspondence to: Nilza Cristina Buttow, PhD, Department of Morphological Sciences, Universidade Estadual de Maringá, Av. Colombo, 5790 Bloco H-79, CEP 87020-900, Maringá, PR, Brazil. ncbuttow@uem.br

Telephone: +55-44-30111370 Fax: +55-44-30114340

Received: February 18, 2010 Revised: March 24, 2010

Accepted: March 31, 2010

Published online: February 21, 2011

Abstract

AIM: To investigate the effect of *Ginkgo biloba* extract on the enteric neurons in the small intestine of diabetic rats.

METHODS: Fifteen Wistar rats were divided into three groups: control group (C), diabetic group (D) and diabetic-treated (DT) daily with EGb 761 extract (50 mg/kg body weight) for 120 d. The enteric neurons were identified by the myosin-V immunohistochemical technique. The neuronal density and the cell body area were also analyzed.

RESULTS: There was a significant decrease in the neuronal population (myenteric plexus $P = 0.0351$; submucous plexus $P = 0.0217$) in both plexuses of the jejunum in group D when compared to group C. With regard to the ileum, there was a significant decrease ($P = 0.0117$) only in the myenteric plexus. The DT group showed preservation of the neuronal population in the jejunum submucous plexus and in the myenteric plexus in the ileum. The cell body area in group D increased significantly ($P = 0.0001$) in the myenteric plexus of

both segments studied as well as in the ileum submucosal plexus, when compared to C. The treatment reduced ($P = 0.0001$) the cell body area of the submucosal neurons of both segments and the jejunum myenteric neurons.

CONCLUSION: The purified *Ginkgo biloba* extract has a neuroprotective effect on the jejunum submucous plexus and the myenteric plexus of the ileum of diabetic rats.

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Key words: Diabetes mellitus; *Ginkgo biloba*; Myenteric plexus; Submucous plexus; Neuroprotection

Peer reviewer: Claudio Daniel Gonzalez, MD, Professor of Pharmacology, Department of Pharmacology, CEMIC University Hospital, Buenos Aires, Argentina

da Silva GGP, Zanoni JN, Buttow NC. Neuroprotective action of *Ginkgo biloba* on the enteric nervous system of diabetic rats. *World J Gastroenterol* 2011; 17(7): 898-905 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/898.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.898>

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by high levels of glucose due to the lack of insulin and/or the inability of insulin to properly exercise its effects^[1]. Long-term hyperglycemia induces morbid states in patients, resulting in macroangiopathy^[2] complications, microangiopathy (retinopathy and nephropathy)^[3] and neuropathies^[4].

Neuropathy is the most common late complication in diabetic patients^[5,6]. It compromises the sympathetic, parasympathetic and enteric nerves, causing a variety of abnormalities such as ulcerations of the lower limbs, sud-

den death by cardiac arrhythmia, gangrene, amputations, sexual dysfunction and gastrointestinal alterations^[6,7].

The gastrointestinal tract is seriously affected by DM. Nearly 75% of diabetic patients may suffer with disorders such as late gastric emptying, vomiting, nausea, diarrhea, abdominal pain, swelling and constipation^[8]. These disorders are usually correlated with enteric neuron lesions^[9-12].

Oxidative stress plays an important role in the development and progression of diabetic neuropathy^[13,14]. Hyperglycemia has been identified as the main cause in the development of oxidative stress by the production of reactive oxygen species (ROS) and reduction of endogenous antioxidants^[15] due to auto-oxidation of blood glucose, excessive formation of AGEs (advanced glycation end products) and activation of the polyol pathway^[13]. The excessive activation of the polyol pathway reduces the cytosolic NADPH, thus decreasing reduced glutathione (GSH), an important endogenous antioxidant. At the same time, this pathway produces an accumulation of sorbitol which causes cellular osmotic stress, also leading to oxidative stress^[16].

ROS or free radicals such as superoxide anion (O_2^-), hydroxyl radical (OH) or intermediate species such as hydrogen peroxide (H_2O_2), damage all classes of cell macromolecular components and organelles (e.g. mitochondria, endoplasmic reticulum, proteins, *etc.*), which can lead to cell death. These free radicals also degrade the cell membrane phospholipids through a process called lipid peroxidation^[17].

The use of antioxidants has beneficial effects in the treatment of diabetic complications^[17-19]. *Ginkgo biloba* extract, obtained from *Ginkgo biloba* leaves, has medicinal properties and is one of the most sold natural supplements in the world. This extract has antioxidant activity and neuroprotective effect, inhibiting cell death^[20,21]. Husstedt *et al.*^[22] noticed that treatment with *Ginkgo biloba* reduced symmetrical polyneuropathy when they analyzed clinical and neurophysiological parameters and the hemorheologic changes in patients with diabetes.

The immunohistochemical technique to identify protein myosin-V has been used to estimate the total neuronal population in different regions of the gastrointestinal tract^[11,12]. This technique confers specificity in the identification of enteric neurons, because this protein is located in neuronal cytoplasm, allowing visualization of cell bodies and their projections^[12].

Our aim was to analyze the effects of standardized extract of *Ginkgo biloba* (EGb 761) on neurons of the myenteric and submucous plexuses in the jejunum and ileum of streptozotocin-diabetic rats. To do so, a morphometric and quantitative study of enteric neurons after 120 d of treatment was carried out.

MATERIALS AND METHODS

Animals

Fifteen male *Wistar* rats (*Rattus norvegicus*) were used, obtained from the Central Vivarium of the Universidade

Estadual de Maringá (UEM). The animal procedures described in this work were conducted in accordance with the ethical principles of the Brazilian Academy in Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Experimentation of UEM.

The weight of animals at the beginning of the experiment was 400 g, corresponding to an approximate age of 150 d. The animals were kept for 120 d in groups of five per box in a room with a light cycle of 12/12 h (7:00 to 19:00) and at constant room temperature of 21-22°C. They were fed with Nuvilab standard diet and water *ad libitum*.

Experimental design

The animals were divided into 3 experimental groups, each group comprised of 5 animals: control group (C) (normoglycemic); diabetic group (D); diabetic-treated with *Ginkgo biloba* extract (EGb 761) group (DT).

To induce diabetes the rats in groups D and DT were weighed and fasted for 16 h. Then, they were injected intravenously with streptozotocin (Sigma, St. Louis, MO, USA) at a dose of 35 mg/kg of body weight.

Blood glucose levels were determined after 7 d by the glucose oxidase method to confirm the disease onset. Only animals with blood glucose higher than 200 mg/dL were kept in groups D and DT.

Besides their normal diet, the DT group animals were treated daily by gavage with the *Ginkgo biloba* (EGb 761) extract (Tebonin, Altana Pharma, Jaguariúna, São Paulo, Brazil) at a dose of 50 mg/kg of body weight throughout the experiment.

Collection and processing of material

At the end of the 120-d trial period, all animals were anesthetized intraperitoneally with thiopental (40 mg/kg body weight) (Abbott Laboratories, Chicago, IL, USA). Blood was collected through cardiac puncture to assess the glycemia. After a laparotomy, the jejunum and ileum segments were collected. These segments were washed with 0.9% saline solution, the ends tied up and inflated with a fixative solution [periodate-lysine-paraformaldehyde (10 mmol/L sodium periodate, 75 mmol/L lysine, and 1% paraformaldehyde in 37 mmol/L phosphate buffer, pH 7.4)]. They were kept in vials containing the same solution for one and half hours. Thirty minutes later, two small holes were made near each end, and the fixative content was drained.

In order to improve the antibody tissue permeability, fragments of the jejunum and ileum were dehydrated in increasing series of alcohols (50%, 70%, 80%, 90%, 95%, 100% I, 100% II), cleared in xylol and rehydrated in decreasing series of alcohol up to 70%.

The dissection procedures were performed by cutting transversely the cylindrical segments of the jejunum and ileum, which were then opened longitudinally at the mesenteric insertion in order to obtain rectangular pieces. The procedure was carried out under a stereoscopy microscope and samples handled with watchmaker tweezers to obtain myenteric plexus membrane whole mounts. The

mucosa and submucosal tunica were removed from the myenteric plexus, while the external muscular layer was kept. The mucosa was removed from the submucosal plexus with the aid of a wooden spatula.

Immunohistochemistry of the myenteric and submucosal plexuses

The myenteric and submucous plexuses were stained by the anti-myosin-V immunohistochemical technique as described by Buttow *et al.*^[23]. The final concentration of antibody was 0.89 mg/mL. The dilution used was 1:1000 (v/v). The membranes were first immersed in a blocking solution of 0.1 mol/L PBS containing 2% bovine serum albumin (BSA) and 0.5% Triton X-100 and normal goat serum at a ratio of 1:50 (v/v) for 3 h. The material was incubated with primary antibody for 48 h at room temperature (RT); this was performed in a solution of 0.1 mol/L PBS containing 1% BSA and 0.1% Triton X-100 and normal goat serum in the proportion of 1:50 (v/v). After the incubation, the material was washed twice for 15 min with PBS solution 0.1 mol/L and Triton X-100 0.1% and then also washed twice in PBS 0.1 mol/L and Tween 20 at a concentration of 0.05% for 15 min. The whole-mounts were then incubated with anti-rabbit secondary antibody produced in goat, peroxidase-conjugated [ImmunoPure® Goat Anti-Rabbit IgG, (Fc), Peroxidase Conjugated, brand Pierce] in a blocking solution containing 0.1 mol/LPBS, 1% BSA and 0.05% Tween 20 for 24 h at RT. Normal goat serum at 1:50 (v/v) was also added to this blocking solution. The material was washed 4 times for 15 min in a solution of 0.1 mol/L PBS containing 0.05% Tween 20. The membranes were developed with the use of a diaminobenzidine solution (Sigma, St. Louis, MO, USA) for approximately 10 min at a concentration of 0.14 mg/mL. After developing, the material was mounted on histological slides with glycerol-gel (containing 50% glycerol, 0.07 g/mL gelatin in PBS, and 2 μ L/mL phenol). The slides were then placed in refrigerator (4°C), in order to slowly dry the whole-mounts.

Density analysis of myosin-V immunoreactive neurons

Enteric neurons were counted on a BX 40 Olympus microscope under a 40 \times lens. Forty microscopy fields, randomly selected, were counted for each preparation. The area of each field was 0.229 mm². The results were expressed in number of neurons per cm².

Morphometric analysis of myosin-V immunoreactive neurons

Images of the ganglia were taken and then measured with the aid of the image analysis software Image Pro-Plus 3.0.1 (Media Cybernetics, Silver Spring, MD, USA) to study the area of neurons in different groups. The area (μ m²) of 100 cell bodies per animal was measured, for a total of 500 neurons (5 animals per group). Neurons were classified into the class interval of 10 μ m², and the percentage of each group was calculated for each interval.

Table 1 Final weight and glycemia in groups: control, diabetic and EGb 76-treated diabetic (mean \pm SE)

Group	Final weight (g)	Blood glucose (mg/dL)
C	445.6 \pm 63.04	78.97 \pm 5.12
D	264.6 \pm 22.88	253 \pm 64.97
DT	308 \pm 19.27	322 \pm 20.42

n = 5/groups. C: Control; D: Diabetic; DT: EGb 76-treated diabetic.

Statistical analysis

To compare the parameters of the studied groups we used analysis of variance (ANOVA). When there was a significant difference we used Tukey's test. For this study we used the Prism software version 3.0. Results were considered significant when *P* < 0.05. The results were shown as mean \pm SE, *n* indicating the number of samples in each group.

RESULTS

Streptozotocin caused diabetic syndrome onset in animal groups D and DT, as evidenced by the significant increase in blood glucose, as well as a significant reduction in body weight, when compared to group C (Table 1). Other typical symptoms of the disease (polyuria, polydipsia and polyphagia) were observed during the experimental period.

Neuronal density

There was a significant reduction (*P* < 0.05) in the neuronal density of myenteric neurons in the jejunum in group D when compared to C (Table 2). There was no significant difference in the DT group when compared to groups C and D. The neuronal density of submucosal neurons decreased significantly (*P* < 0.05) in group D when compared to C. No significant difference in the neuronal density was observed when group DT was compared to C (Table 2).

The neuronal density of myenteric neurons in the ileum decreased significantly (*P* < 0.05) in group D when compared to C (Table 3). No significant difference was seen when comparing group DT to C. There was no significant reduction in the neuronal density in the ileum submucous plexus when the three groups were compared (Table 3).

Areas of neuronal cell bodies

The results obtained with the measurements of 500 neurons per studied group were distributed according to the relative frequency of areas of neuronal cell bodies at intervals of 10 μ m² (Figures 1 and 2). The cell body area in the jejunum ranged between 81.33 and 538.9 μ m² for animals in group C; between 119.9 and 588.9 μ m² in group D; and between 101.0 and 609.2 μ m² in group DT. There were no significant differences in the mean areas of the jejunum myenteric neurons when comparing groups C and D. However, there was a significant reduction in the mean area (*P* < 0.05) of the DT group when compared to the other two groups (Table 2). The cell body area in the

Table 2 Neuronal density and mean area of cell bodies of myenteric and submucosal neurons in the jejunum of rat groups: control, diabetic and EGb 761-treated diabetic (mean \pm SE)

Group	Myenteric plexus		Submucous plexus	
	Neuronal density (cm ²)	Mean area of cell body (μ m ²)	Neuronal density (cm ²)	Mean area of cell body (μ m ²)
C	15 884 \pm 712.0	234.2 \pm 88.10	12 602 \pm 233.8	230.6 \pm 62.89
D	13 483 \pm 617.9	245.6 \pm 77.19	11 383 \pm 159.6	235.4 \pm 67.99
DT	14 426 \pm 301.2	218.2 \pm 72.10	12 682 \pm 353.4	216.2 \pm 62.03

$n = 5$ /myenteric plexus group; $n = 3$ /submucous plexus group. C: Control; D: Diabetic; DT: EGb 76-treated diabetic.

Table 3 Neuronal density and mean area of cell bodies of myenteric and submucosal neurons in the ileum of rat groups: control, diabetic and EGb 761-treated diabetic (mean \pm SE)

Group	Myenteric plexus		Submucous plexus	
	Neuronal density (cm ²)	Mean area of cell body (μ m ²)	Neuronal density (cm ²)	Mean area of cell body (μ m ²)
C	16 522 \pm 625.5	232.7 \pm 82.97	11 657 \pm 403.9	210.0 \pm 59.18
D	14 568 \pm 424.7	251.4 \pm 98.23	11 275 \pm 281.9	231.3 \pm 74.37
DT	16 884 \pm 366.1	239.3 \pm 81.19	11 943 \pm 299.3	204.5 \pm 57.36

$n = 5$ /myenteric plexus group; $n = 3$ /submucous plexus group. C: Control; D: Diabetic; DT: EGb 76-treated diabetic.

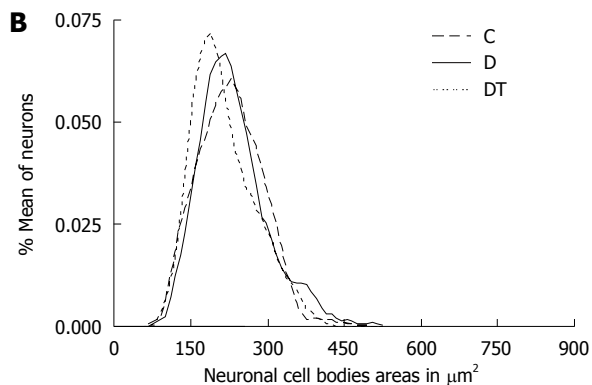
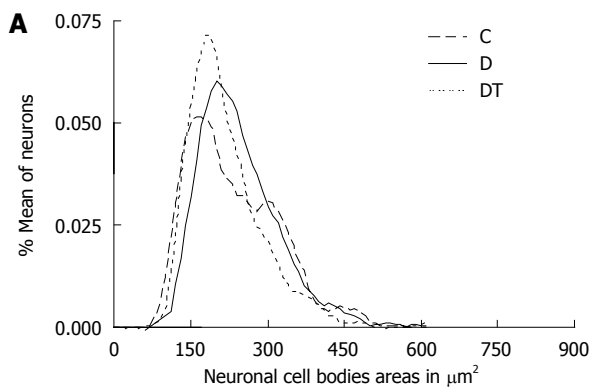


Figure 1 Neuronal behavior: area of cell body of myenteric (A) and submucosal (B) neurons, myosin-V immunoreactive in the jejunum, of control (C), diabetic (D) and diabetic-treated with EGb 761 (DT).

submucosal neurons in the jejunum ranged between 106.1 and 474.4 μ m² in group C, between 102.3 to 523.4 μ m² in group D and between 91.73 to 401.1 μ m² in group DT. There were no significant differences between the mean cell body areas in groups C and D ($P > 0.05$). However, there was a significant reduction ($P < 0.05$) in group DT when compared to groups C and D (Table 2).

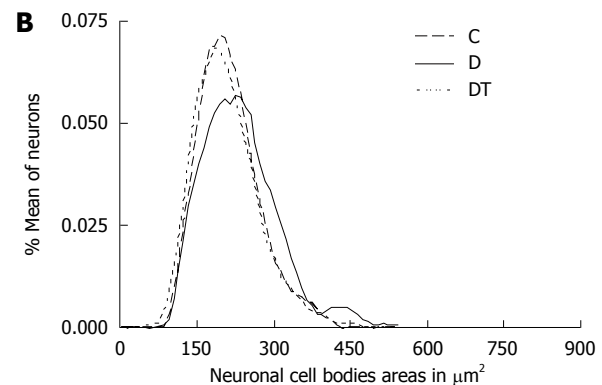
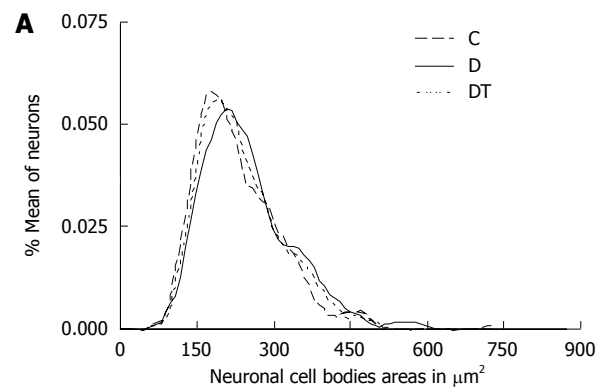


Figure 2 Neuronal behavior: area of cell body of myenteric (A) and submucosal (B) neurons, myosin-V immunoreactive in the ileum, of control (C), diabetic (D) and diabetic-treated with EGb 761 (DT).

The cell body area of myenteric neurons in the ileum ranged between 97.70 and 725.7 μ m² in group C, between 101.5 and 595.5 μ m² in group D, and between 96.32 and 512.9 μ m² in group DT. There was a significant increase ($P < 0.05$) in group D when compared to C. No significant difference was observed when comparing group DT to groups C or D (Table 3). As for the ileum submucous

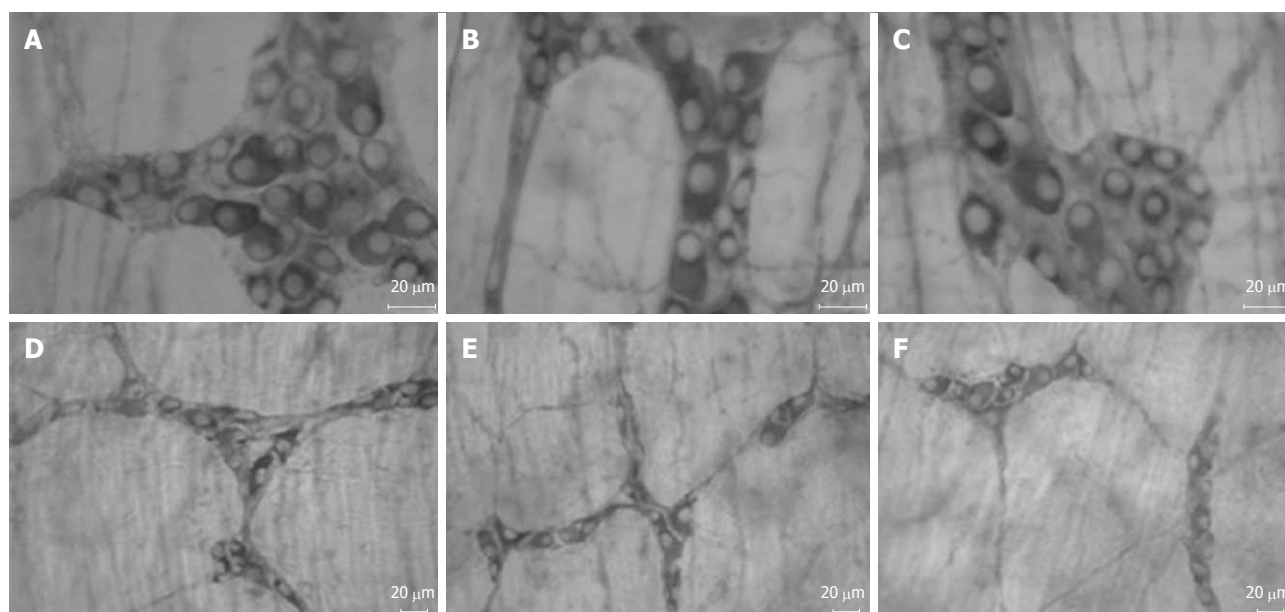


Figure 3 Myosin-V immunoreactive myenteric neurons in the jejunum (A-C) and myosin-V immunoreactive submucosal neurons in the jejunum (D-F). There is a significant reduction in the neuronal density in the myenteric (B) and submucous (E) plexus in group diabetic. The neuronal density in the submucous plexus (F) was preserved in group EGb 76-treated (DT) (F). There was a significant reduction in the neuronal cell body area in group DT of both plexuses (C and F).

plexus, the area ranged between 89.54 and 426.2 μm^2 , between 99.52 and 534.0 μm^2 in group D, and between 72.77 and 435.0 μm^2 in group DT. There was a significant increase in the mean cell body area in group D ($P < 0.05$) when compared to C. The DT group showed no significant difference in mean cell body area when compared to group C (Table 3). In the submucous plexus, reduction in neuronal profile area was greater than in the myenteric plexus; the values in the submucous plexus just below those of the control group.

The distribution of the relative frequency of areas of cell bodies in the jejunum showed a displacement curve to the right in the myenteric plexus; thus showing a higher relative frequency of neurons at about 160 μm^2 in both plexuses (Figure 1). There was a similarity in the curves of groups C and DT in both plexuses in the ileum (Figure 2). Group D showed a displacement to the right in both plexuses.

DISCUSSION

Streptozotocin (STZ) is widely used in experimental animal models to induce DM. Its cellular action includes irreversible changes in genetic material causing lethal alterations in the metabolism of β cells^[24]. There is a reduction in overall myenteric plexus neuron population in animal models with chronic STZ-diabetes^[11,12,25,26]. There are no studies of changes caused by diabetes in the overall neuronal population of the submucous plexus. Our study showed that the 120-d treatment with purified *Ginkgo biloba* extract (EGb 761) has a neuroprotective effect on the ileum myenteric plexus and on the jejunum submucous plexus of STZ-diabetic rats.

Characteristic diabetic symptoms (polydipsia, polyuria

and polyphagia) were observed in animals of D and DT groups. These data support the experimental model of streptozotocin-induced diabetes^[27-29]. The immunohistochemical technique, anti-myosin-V (Figures 3 and 4), was used to assess the effect of *Ginkgo biloba* extract (EGb 761) on the enteric neuronal population. The protein myosin-V is present in cell bodies and projections of enteric neurons^[30] and is being used as a pan-neuronal marker.

The reduction of the myenteric neuron density in the jejunum was 15.12% in group D when compared to C ($P < 0.05$). The submucosal neuron density was 9.61% lower in group D when compared to C ($P < 0.05$). A reduction of 11.83% in myenteric neuron density was observed in the ileum in group D when compared to C ($P < 0.05$). The submucosal neuron density in the ileum was similar among the three groups. Several authors report the reduction of myenteric neuron density in rats with STZ-diabetes in different regions of the gastrointestinal tract, including the cecum^[31], ileum^[11,26], jejunum^[25] and proximal colon^[12]. There are no studies in the submucosal plexus of the total neuronal population in STZ-diabetes models. Pereira *et al.*^[26] reported a 24% reduction in the number of myosin-V myenteric neurons in the ileum (after 120 d) of diabetic rats when compared to non-diabetic ones. De Freitas *et al.*^[25] observed a 37.9% neuronal loss of myosin-V myenteric neurons in the jejunum of diabetic rats when compared to non-diabetic animals, also after 120 d. These studies used 90-d-old animals at the beginning of the experiment and our study was carried out with 150-d-old rats, which may have contributed to the neuronal loss variation due to age.

The degenerative changes that affect the enteric nervous system seen in DM are due to metabolic disorders. High oxidative stress, resulting from the imbalance be-

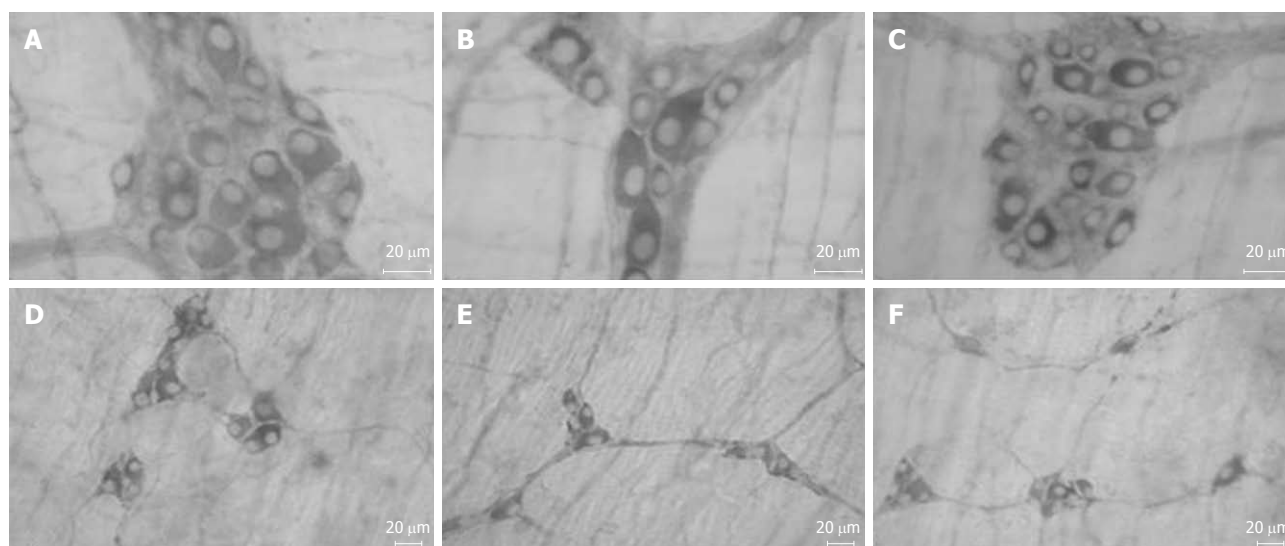


Figure 4 Myosin-V immunoreactive myenteric neurons in the ileum (A-C) and myosin-V immunoreactive submucosal neurons in the jejunum (D-F). There is a significant reduction in the neuronal density in the myenteric plexus (B), but the neuronal density was preserved in this plexus in group EGb 76-treated (DT) (C). There is a significant increase in the neuronal cell body area in group diabetic in the myenteric (B) and submucous (E) plexuses. There was a significant reduction in the neuronal cell body area in group DT in the submucous plexus (F).

tween ROS production and neutralization, is a well established mechanism of diabetic neuropathy pathogenesis and other complications^[32,33]. The levels of endogenous and exogenous antioxidants are reduced in this condition. New studies have confirmed the destruction of endogenous antioxidants in peripheral nerves and the increased production of free radicals in the vasa nervorum^[4].

Ginkgo biloba extract is widely used for its neuroprotective and antioxidant activity in several cardiovascular and neurologic disorders^[34,35]. The *Ginkgo biloba* extract (EGb 761) was given at a daily dose of 50 mg/kg body weight for 120 d in this experiment. This standardized extract contains 24% flavonoid glycosides (quercetin, kaempferol, isorhamnetin) and 6% terpene lactones (ginkgolides, bilobalides). The EGb 761 extract components eliminate free radicals such as the hydroxyl radical and the superoxide anion^[36]. Quercetin is a powerful antioxidant within the flavonoid family due to its molecular configuration which is capable of eliminating free radicals^[37].

The myenteric neuronal density in the jejunum in the DT group was 9.17% lower when compared to C, though this reduction is not significant. On the other hand, the submucosal neuronal density in DT had very similar values to those of group C. The treatment with EGb 671 resulted in the preservation of the neuronal population in the ileum, represented by very similar values to those of the control group (Table 2), thus demonstrating a neuroprotective effect on this complex. The submucosal neuronal density in this segment was similar in all three groups. The *Ginkgo biloba* extract reduces the oxidative stress in diabetic rats by increasing the activity of antioxidant enzymes^[38]. Wu *et al.*^[39] reported that this extract may be vital to postpone diabetic cataract, since their studies showed that, besides inhibiting aldose reductase activity, *Ginkgo biloba* also inhibits apoptosis induced by high glu-

cose levels by reducing the Bax/Bcl2 ratio. This high ratio harms the mitochondria which release apoptosis-inducing proteins, such as the apoptosis-inducing factor, leading to the activation of caspase-3 *via* caspase 9. The myenteric plexus neuroprotection, seen only in the ileum, is similar to results in aging models^[40] where 120-d treatment of rats with the same dose of *Ginkgo biloba* extract was more efficient in the ileum myenteric plexus than in the jejunum.

Few studies have been carried out in the submucous plexus due to the difficulty of dissection. Some authors have reported changes in neuronal subpopulations through the neurotransmitter immunoreactivity. Belai *et al.*^[41] observed an increase in VIP and neuropeptide Y immunoreactivity when analyzing the submucous plexus in the ileum of STZ-diabetic rats aged 8 and 16 wk. They also observed a reduction in calcitonin gene-related peptide (CGRP) immunoreactivity. However, no change in substance P immunoreactivity or dopamine beta hydroxylase was seen. VIP-ergic neurons of diabetic rats show increased immunoreactivity in the jejunum^[42] and ileum^[43] submucous plexus.

The mean cell body areas of myenteric neurons in the jejunum were similar in groups C and D. These results are similar to those observed by De Freitas *et al.*^[25], who did not observe an increase in the mean area of the cell body of immunoreactive myosin-V neurons in the jejunum of diabetic rats when compared to non-diabetic rats. The mean areas of cell bodies of submucosal neurons in the jejunum were similar in groups C and D. Studies on morphometric changes in the submucosal plexus caused by diabetic syndrome report an increase in the mean area of the cell body of neuronal subpopulations. Defani *et al.*^[42] observed an increase in the mean area of the cell body of submucous VIP-ergic neurons in the jejunum. The technique used to stain the total population showed no change

in the mean area of submucosal neurons in the jejunum. The mean area of the cell body of myenteric neurons in the ileum was 7.44% ($P < 0.05$) higher in group D than in group C in our study. This increase was also observed by Zanoni *et al.*^[11] and Pereira *et al.*^[26] in Wistar rats after a 120-d experimental period. The mean area of the body cell of submucosal neurons in the ileum showed a statistically significant increase of 9.2% ($P < 0.05$) in group D when compared to C. Zanoni *et al.*^[43] reported an increase in the mean area of the body cell of submucous VIP-ergic neurons in the ileum.

The increase in the neuronal cell body area in rats with chronic diabetes may be the result of neuronal edema^[11]. The aldose reductase hyperactivity observed in diabetes is associated with increased levels of sorbitol^[44] which increases the intracellular osmolarity, resulting in edema and neuronal lesions^[43].

The EGb 761 treatment induced a reduction of 6.8% in the mean area of the cell body in the jejunum myenteric neurons in DT when compared to C ($P < 0.05$). The mean area of the cell body of submucosal neurons decreased 6.2% in group DT when compared to C ($P < 0.05$). The mean area of the cell body of myenteric and submucosal neurons in the ileum in DT was reduced to values similar to group C. Schneider *et al.*^[40] observed that the EGb 761 treatment reduced the mean area of myenteric neuronal cell bodies in the jejunum and ileum of aging rats. However, studies by Perez *et al.*^[45] in the large intestine treated with EGb 761 at a dose of 50 mg/kg of body weight observed that the EGb 761 extract promotes an increase in the mean area of myenteric neurons in rats in the aging process. These results show that the response to the use of antioxidants such as the *Ginkgo biloba* extract may be different according to the segment evaluated.

This study showed that treatment with *Ginkgo biloba* extract reduced the area of the cell body of myenteric and submucosal neurons in the jejunum and ileum of diabetic-treated rats (group DT) when compared to non-treated diabetic rats (group D). However, the reduction in the mean area of the cell body of myenteric neurons in the ileum was not significant. The inhibitory action of *Ginkgo biloba* on aldose reductase^[19] enzyme activity may be responsible for the reduction in the mean area of neuronal cell bodies observed in rats treated with EGb 761 (DT group).

In conclusion, our results show that the 50 mg/kg of body weight dose of standardized *Ginkgo biloba* extract (EGb761) has a neuroprotective effect on the ileum myenteric plexus and on the jejunum submucous plexus of STZ-diabetic rats.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Enilza Maria Espreafico (USP-Ribeirão Preto, Brazil) for her invaluable assistance and support in the production of the anti-myosin-V antibody, Maria Eurides do Carmo Cancino, Maria dos Anjos Fortunato, Valdir Trombeli and José Antônio de Souza (UEM, Brazil) for their excellent technical support, and

Ali Suleiman Mahmoud for translating the text. We gratefully acknowledge Altana Pharma for supplying the *Ginkgo biloba* extract (EGb 761).

COMMENTS

Background

Ginkgo biloba extract possesses various biological activities and has been shown to be useful in diabetes treatment. Oxidative stress has been known to play an important role in the development and progression of diabetes mellitus (DM), and reactive oxygen species (ROS) production is a direct consequence of hyperglycemia. Chronic hyperglycemia in diabetes is involved in direct neuronal damage caused by intracellular glucose which leads to altered neurotransmitter functions and reduced motor activity. Oxygen free radicals are also thought to play an important role in the diabetic and hypoxic condition of cells. Success of *Ginkgo biloba* application is determined by its main active substances, flavonoids (flavone glycosides, primarily composed of quercetin) and terpenoids (ginkgolides and bilobalides). *Ginkgo biloba* can improve hemodynamics, scavenge ROS, suppress platelet-activating factor (PAF) and relax vascular smooth muscle.

Research frontiers

Gastrointestinal (GI) afflictions are not normally life threatening but do profoundly affect quality of life. Diabetic patients experience a wide range of GI discomforts including nausea, vomiting, heartburn, diarrhea, constipation, abdominal pain and fecal incontinence. The high morbidity, high socioeconomic costs and lack of specific treatments are key factors that define the relevance of DM for human health and the importance of research on neuronal protective agents. Some studies provide a strong case for the application of *Ginkgo biloba* in diabetic nephropathy therapy.

Innovations and breakthroughs

Ginkgo biloba has been ascertained to be protective against DM. However, there has been little in the literature reporting on the protective effects of *Ginkgo biloba* on the enteric nervous system of the small intestine of streptozotocin-induced diabetic rats *in vivo*.

Applications

This study indicated that standardized extract of *Ginkgo biloba* (EGb 761) could improve antioxidant ability and protect the enteric nervous system of the small intestine of streptozotocin-induced diabetic rats *in vivo*. These biological activities have considerable potential in diabetes mellitus treatment.

Peer review

The authors investigated the effect of *Ginkgo biloba* extract on the enteric neurons on the small intestine of diabetic rats. They found purified *Ginkgo biloba* extract has a neuroprotective effect on the jejunum submucous plexus and the myenteric plexus of the ileum of diabetic rats. This is a well written paper.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM

Outcome of non surgical hepatic decompression procedures in Egyptian patients with Budd-Chiari

Ahmed Eldorrry, Eman Barakat, Heba Abdella, Sara Abdelhakam, Mohamed Shaker, Amr Hamed, Mohammad Sakr

Ahmed Eldorrry, Mohamed Shaker, Department of Radiodiagnosis and Interventional Radiology, Faculty of Medicine, Ain Shams University, Cairo 11341, Egypt

Eman Barakat, Heba Abdella, Sara Abdelhakam, Amr Hamed, Mohammad Sakr, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo 11341, Egypt

Author contributions: Eldorrry A and Sakr M contributed equally to this work; Sakr M, Eldorrry A, Barakat E, Abdella H, Abdelhakam S and Hamed A designed the research; Abdelhakam S, Hamed A and Shaker M performed the research; Eldorrry A, Sakr M, Barakat E, Abdella H, Abdelhakam S and Shaker M contributed analytic tools; Sakr M, Eldorrry A, Barakat E, Abdella H, Abdelhakam S and Hamed A analyzed the data; Barakat E, Abdella H and Abdelhakam S wrote the paper.

Correspondence to: Sara Abdelhakam, MD, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo 11341, Egypt. saratropical@yahoo.com

Telephone: +20-101601548 Fax: +20-2-22598751

Received: August 4, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: February 21, 2011

Abstract

AIM: To evaluate outcome of patients with Budd-Chiari syndrome after balloon angioplasty ± stenting or transjugular intrahepatic portosystemic shunt (TIPS).

METHODS: Twenty five patients with Budd-Chiari syndrome admitted to Ain Shams University Hospitals, Tropical Medicine Department were included. Twelve patients (48%) with short segment occlusion were candidates for angioplasty; with stenting in ten cases and without stenting in two. Thirteen patients (52%) had Transjugular Intrahepatic Portosystemic Shunt. Patients were followed up for 12-32 mo.

RESULTS: Patency rate in patients who underwent angioplasty ± stenting was 83.3% at one year and at end of follow up. The need of revision was 41.6% with one year survival of 100%, dropped to 91.6% at end

of follow up. In patients who had Transjugular Intrahepatic Portosystemic Shunt, patency rate was 92.3% at one year, dropped to 84.6% at end of follow up. The need of revision was 38.4% with one year and end of follow up survival of 100%. Patients with patent shunts showed marked improvement compared to those with occluded shunts.

CONCLUSION: Morbidity and mortality following angioplasty ± stenting and TIPS are low with satisfactory outcome. Proper patient selection and management of shunt dysfunction are crucial in improvement.

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Key words: Angioplasty; Stenting; Transjugular Intrahepatic portosystemic shunt; Patency rate

Peer reviewer: Bijan Eghtesad, Dr, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, United States

Eldorrry A, Barakat E, Abdella H, Abdelhakam S, Shaker M, Hamed A, Sakr M. Outcome of non surgical hepatic decompression procedures in Egyptian patients with Budd-Chiari. *World J Gastroenterol* 2011; 17(7): 906-913 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/906.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.906>

INTRODUCTION

Budd-Chiari syndrome (BCS) results from hepatic venous outflow obstruction at any level, from hepatic venules to the right atrium^[1]. If obstruction is due to endoluminal venous lesion like thrombosis, primary BCS is considered. In secondary BCS, the cause originates from neighboring structures like extrinsic compression or tumor invasion^[2].

Imaging studies combined with clinical information

are often essential for reaching a definitive diagnosis^[3].

The goals of treatment are to prevent extension of thrombosis in hepatic veins (HVs) and to alleviate venous obstruction in order to decrease hepatic congestion. Few patients respond to medical treatment (anticoagulation \pm thrombolytic therapy, diuretics). However, most patients need intervention to restore the hepatic blood flow^[4].

If there is a possibility of restoring hepatic venous outflow in one of the major HVs by balloon dilatation, recanalization, or stent insertion, then this is the procedure of choice as it is the most physiological method. However, in cases where blood flow cannot be restored or when the approach fails, transjugular intrahepatic portosystemic shunt (TIPS) is used as a decompressing non-surgical procedure^[5].

MATERIALS AND METHODS

Study Design & Sampling: This prospective follow-up study was conducted on twenty five patients with confirmed diagnosis of primary BCS and eligible criteria for radiological intervention, who were presented to the Budd-Chiari Study Group and admitted to the Tropical Medicine Department, Ain Shams University Hospitals.

Patients were subjected to: (1) Complete Clinical Evaluation; and (2) Radiological Assessment, with special stress on the patency of HVs, portal vein and inferior vena cava (IVC) by abdominal Duplex/US. Abdominal MRI, MR venography or multislice CT scan were done to confirm diagnosis and to delineate vascular anatomy before intervention.

They were divided into two groups: (1) Patients with short segment occlusion of any of HVs who were candidates for angioplasty \pm stenting; and (2) Patients with complete occlusion of all HVs who were candidates for TIPS.

Exclusion criteria: (1) Secondary BCS; (2) Retro or suprahepatic IVC obstruction; (3) Complete portal vein thrombosis; (4) Presence of comorbid etiology for liver disease in addition to BCS (e.g.: viral hepatitis); (5) Hepatocellular carcinoma; (6) Cardiac contraindications to TIPS (congestive heart failure and severe pulmonary hypertension); (7) Marked coagulopathy (INR > 5) and Thrombocytopenia (platelets $< 20\,000$)^[6]; (8) Biliary obstruction; and (9) Uncontrolled sepsis.

Details of the study and interventions were explained to recruited patients who signed a written consent form.

Pre-intervention assessment and preparation

Routine laboratory investigations and thrombophilia workup were done aiming at identification of etiology of BCS, in addition to assessment of liver disease severity.

Patients' general health was assessed according to WHO performance status scale^[7]: 0: patient is fully active, able to carry on all pre-disease performance without restriction; 1: patient is restricted in physically strenuous

activity but ambulatory and able to carry out work of a light or sedentary nature, e.g. light house work; 2: patient is ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours; 3: patient is capable of only limited self care, confined to bed or chair more than 50% of waking hours; 4: patient cannot carry on any self care and totally confined to bed or chair.

Patients were classified as follows: According to Rotterdam prognostic classification^[8] into 3 classes with scores according to the equation: $1.27 \times \text{encephalopathy} + 1.04 \times \text{ascites} + 0.72 \times \text{prothrombin time} + 0.004 \times \text{bilirubin}$ [Ascites and hepatic encephalopathy were scored as present (1) or absent (0) and prothrombin time as higher (1) or lower (0) than 2.3 INR. Bilirubin was included as a continuous variable]. Where Class I (0-1.1): good prognosis; Class II (1.1-1.5): intermediate prognosis and Class III (> 1.5): poor prognosis.

According to Child-Pugh score into 3 classes (A, B and C)^[9].

All patients started anticoagulation therapy when diagnosis of BCS was evident; in the form of low molecular weight heparin (LMWH) or unfractionated heparin. Then oral warfarin was added till INR reached its target (2-3), then continued on oral therapy alone after withdrawal of LMWH or unfractionated heparin.

Five days before procedure, oral anticoagulation therapy was stopped with administration of LMWH or unfractionated heparin only; to be stopped (6-12 h) before intervention in case of unfractionated heparin and (12-24 h) in case of LMWH to avoid intra or postoperative bleeding^[10].

Antibiotic prophylaxis was administered for all patients (1-2 h) before intervention in the form of combination of ampicillin- sulbactam 1.5 gm IV and cefotaxime 1 gm IV^[11].

Technical considerations

All procedures were performed in an angiographic interventional room with high resolution C-arm fluoroscopy, and digital subtraction angiography.

Interventions were done under general anesthesia.

All cases of TIPS or angioplasty with stenting had self expandable non covered metallic stents.

Post intervention management

Patients were admitted to hospital for 1 wk after procedure for early detection and management of any procedure-related complications and adjustment of anticoagulation.

Antibiotics regimen taken before procedure was continued for 5 d after.

Oral warfarin was introduced together with parental anticoagulation (LMWH after 24 h or unfractionated Heparin after 6 h) till INR reaches (2-3) then oral therapy was continued alone for life^[12].

Duplex U/S was performed to detect shunt patency at days 1, 3, and 7 after the procedure.

Follow up

Patients were followed up clinically, by laboratory investigations (mainly liver profile and PT and PTT for monitoring of anticoagulation) and radiologically by duplex U/S.

Follow up after intervention was every three mo or when indicated (e.g.: clinical manifestations suggestive of angioplasty or TIPS dysfunction). Follow up was intended to be at least one year (Minimum: 12 mo, Maximum: 32 mo).

Aims of follow up were

(1) Assessment of patients' survival and shunt survival (i.e.; shunt patency and function) (at one year interval and at the end of follow up); (2) Description of procedures related complications and their management; and (3) Assessment of patients' improvement after intervention by comparison of clinical, laboratory and performance status criteria before intervention and one year after.

Statistical analysis

Descriptive statistics: (1) Quantitative data: mean, standard deviation (\pm SD); and (2) Qualitative data: frequency and percentage.

Analytical statistics: (1) Quantitative data: Wilcoxon Signed Ranks Test; and (2) Qualitative data: McNemar Test.

Levels of significance: (1) $P > 0.05$ = non significant (NS); (2) $P < 0.05$ = significant (S); (3) $P < 0.01$ = highly significant (HS); and (4) $P < 0.001$ = very highly significant (VHS).

Survival: (1) Patient Survival was *defined* as the duration between diagnosis of BCS, and patient death or loss to follow up. Survival rates were Kaplan-Meier estimates; (2) Shunt Survival was *defined* as the duration between shunt application, and shunt occlusion or loss to follow up. Survival rates were Kaplan-Meier estimates.

RESULTS

Descriptive data

This study was conducted on twenty five patients with BCS who underwent non surgical hepatic decompression procedures in the form of either angioplasty \pm stenting or TIPS. They were 16 females (64%) and 9 males (36%) with a mean age of 28.28 ± 8.93 years (range 14-57 years). BCS was chronic form in 21 patients (84%), acute in three patients (12%), and fulminant in 1 patient (4%). When tested for underlying thrombophilia, 8 were negative (idiopathic), 4 primary antiphospholipid antibody syndrome (APS), 4 protein C deficiency, 3 Antithrombin III deficiency, 1 myeloproliferative disorder, 1 combined protein C, S deficiency, 1 combined protein C, S, Antithrombin III deficiency, 1 combined Antithrombin III deficiency + factor V Leiden mutation (FVLM), 1 combined protein S deficiency + FVLM and 1 was primary APS + FVLM.

According to Child Classification, 5 patients (20%)

Table 1 Clinical manifestations and radiological criteria in studied patients

Findings	Patients, <i>n</i> (%)
Clinical manifestations	
Abdominal pain	23 (92)
Jaundice	9 (36)
Lower limb edema	10 (40)
Dilated veins over abdomen and trunk	5 (20)
Tender hepatomegaly	16 (64)
Ascites	24 (96)
Radiological criteria	
Hepatomegaly	24 (96)
Splenomegaly	22 (88)
Ascites	
Absent	1 (4)
Present	24 (96)
Liver mottling appearance	17 (68)
Intra hepatic collaterals	16 (64)
Caudate lobe hypertrophy	12 (48)
Hepatic Veins:Short segment occlusion	
RHV	2 (8)
MHV	7 (28)
LHV	5 (20)
Total occlusion	
RHV	23 (92)
MHV	18 (72)
LHV	20 (80)

Radiological criteria were obtained using duplex ultrasound, magnetic resonance venography and/or multislice computed tomography scan. RHV: Right hepatic vein; MHV: Middle hepatic vein; LHV: Left hepatic vein.

were Child A, 16 (64%) were Child B and 4 (16%) were Child C. According to Rotterdam Classification, 7 patients (28%) were Class I, 15 (60%) were Class II and 3 (12%) were Class III. The Performance status score was "0" in none of the patients, "1" in 4 patients (16%), "2" in 5 patients (20%), "3" in 11 patients (44%) and "4" in 5 patients (20%).

Pre-intervention clinical and investigational data

Clinical manifestations and baseline radiological criteria of studied patients using duplex U/S, MRV and/or Multislice CT scan are shown in Table 1.

Intervention details: The main indications for intervention in the studied patients were ascites associated with large esophageal varices; uncontrollable ascites only; large esophageal varices only and fulminant hepatic failure in 56%; 36%; 4% and 4% of patients respectively.

Twelve patients (48%) were candidates for angioplasty; of those; 10 patients (40%) had stenting (5; 20% in MHV, 4; 16% in LHV and 1; 4% in RHV) and 2 patients (8%) had angioplasty without stenting (1 patient in both LHV and MHV and the other patient in both RHV and MHV, where they shared a common short stenotic segment at their entrance into IVC).

Thirteen patients (52%) were candidates for TIPS.

The need of revision was 41.6% (5 out of 12 patients) in cases of angioplasty \pm stenting and 38.4% (5 out of 13 patients) in cases of TIPS as shown in Table 2.

Table 2 Details of patients who needed revisions and their follow up (*n* = 10)

Patient	Intervention	Time of dysfunction	Action taken	No of revisions	1 yr patency	End of FUP patency
23 yr F	Angioplasty without stenting	Day 7 and Day 10	TIPS was done, occluded at day 10; then re-angioplasty was done ¹	2	Patent	Patent at 20th mo
27 yr M	Angioplasty and stenting	Day 7 and 2nd yr	Angioplasty was done-then angioplasty + thrombectomy	2	Patent	Patent at 24th mo
28 yr F	Angioplasty and stenting	4th mo	Angioplasty + local thrombolytic therapy	1	Patent	Patent at 12th mo
30 yr F	Angioplasty and stenting	1st, 4th, 6th and 9th mo	TIPS was done-then angioplasty (3 times)	4	Occluded at 9th mo	Occluded at 24th mo
28 yr M	Angioplasty and stenting	3rd mo and 14th mo	Angioplasty + stent was done-then mesoatrial shunt	1	Occluded at 1 yr	Dead ² at 17th mo
27 yr F	TIPS	Day 1	(stent occlusion and migration to portal vein) - Re (TIPS)	1	Patent	Patent at 20th m
33 yr F	TIPS	Day 3	Angioplasty + thrombectomy + systemic thrombolytic therapy	1	Patent	Patent at 32nd mo
37 yr F	TIPS	Day 7 and 1st mo	Angioplasty (2 times)	2	Patent	Patent at 12th mo
27 yr M	TIPS	Day 7, 3rd and 8th mo	Angioplasty (3 times)	3	Patent	Occluded at 20th mo
17 yr M	TIPS	1st mo	Patient refused intervention	0	Occluded	Occluded at 12th mo

¹Patient had angioplasty dysfunction at Day 7, so transjugular intrahepatic portosystemic shunt (TIPS) was done but was occluded at Day 10, so angioplasty of TIPS stent was done; ²Cause of death: Intraperitoneal bleeding. Follow up period: Minimum (12 mo), Maximum (32 mo). F: Female; M: Male; yr: Years old; FUP: Follow up.

Table 3 Patient survival *n* (%)

	Angioplasty	TIPS	Total
One year			
Alive	12 (100)	13 (100)	25 (100)
Dead	0 (0)	0 (0)	0 (0)
End of follow up			
Alive	11 (91.6)	13 (100)	24 (96)
Dead	1 (8.4)	0 (0)	1 (4)

Because of death of one patient only out of 25; Kaplan-Meier curve couldn't be drawn for patient survival. TIPS: Transjugular intrahepatic portosystemic shunt.

Figure 1 shows frequency of all complications in total procedures done [Twenty six angioplasty ± stenting procedures (12 as primary intervention and 14 as a trial for maintenance of previously occluded angioplasty or TIPS) and 16 TIPS procedures (13 as primary intervention and 3 in patients with occluded stents following angioplasty in whom redilatation was not possible)].

In total procedures done (whether primary or revision procedures), the frequency of angioplasty dysfunction was 53.85% (14 out of 26 procedures) and the frequency of TIPS dysfunction was 43.75% (7 out of 16 procedures).

Statistical analysis

The mean duration of follow up was 20.04 ± 7.817 mo (ranging from 12-32 mo). One year survival rate was 100% for all patients and at the end of follow up survival rate was 96% due to death of one patient at the 17th mo of follow up as shown in Table 3.

Figure 2A shows patency rate in patients who underwent angioplasty ± stenting procedures; it was 11/12 (91.7%) at 9 mo (due to persistent shunt occlusion in one

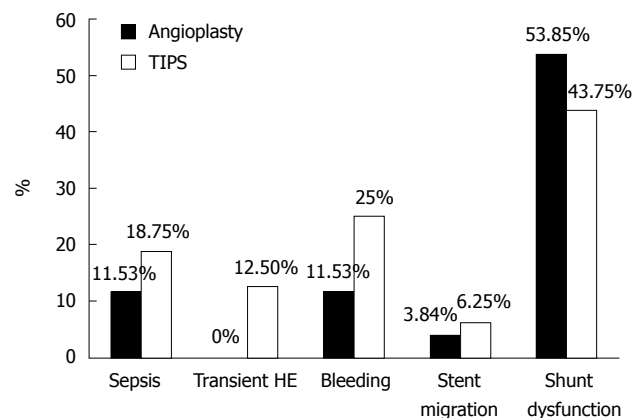


Figure 1 Procedure complications. Transient hepatic encephalopathy (HE): HE lasting 2-3 d after procedure with rapid response to treatment. Bleeding was either intra-peritoneal or hemobilia. TIPS: Transjugular intrahepatic portosystemic shunt.

patient). Patency rate dropped to 10/12 (83.3%) at one year and continued till the end of follow up at 32 mo. (There was persistent shunt occlusion in 2 patients in spite of repeated revisions and optimal anticoagulation therapy).

Figure 2B shows patency rate in patients who had TIPS procedures; it was 12/13 (92.3%) at one year (due to persistent shunt occlusion in one patient despite repeated revisions). Patency rate dropped to 11/13 (84.6%) at 20 mo and this continued till the end of follow up at 32 mo (due to persistent shunt occlusion in another patient).

At one year of follow up, only three patients of 25 (12%) had occluded shunts. Patients with occluded shunts showed no improvement regarding their clinical manifestations, laboratory profile and performance status. On the contrary, patients with patent shunts (22 of 25; 88%) showed marked improvement as shown in Tables 4 and 5.

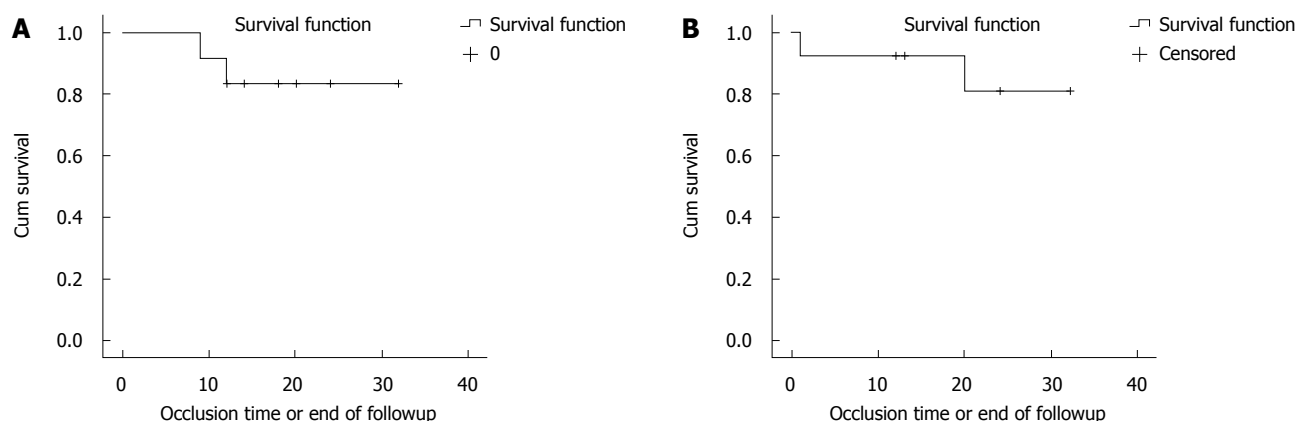


Figure 2 Patency rate in patients who underwent angioplasty \pm stenting (A) and transjugular intrahepatic portosystemic shunt (B). A: Type of intervention: angioplasty \pm stenting of hepatic veins, it was 91.7% at 9 mo and dropped to 83.3% at one year till the end of follow up at 32 mo; B: Type of intervention: transjugular intrahepatic portosystemic shunt, it was 92.3% at one year and dropped to 84.6% at 20 mo till the end of follow up at 32 mo.

Table 4 Clinical data of patients before and after intervention

	Before intervention		One year after intervention		P value	Sig
	+VE	-VE	+VE	-VE		
Patients with occluded shunts (n = 3)						
Abdominal pain	3	0	2	1	> 0.05	NS
Jaundice	1	2	0	3	> 0.05	NS
Lower limb edema	2	1	1	2	> 0.05	NS
Dilated veins	1	2	0	3	> 0.05	NS
Ascites	3	0	3	0	> 0.05	NS
Patients with patent shunts (n = 22)						
Abdominal pain	20	2	1	21	< 0.001	VHS
Jaundice	8	14	0	22	< 0.01	HS
Lower limb edema	8	14	1	21	< 0.05	S
Dilated veins	4	18	0	22	> 0.05	NS
Ascites	21	1	1	21	< 0.001	VHS

Sig: Significance; NS: Non significant; S: Significant; HS: Highly significant; VHS: Very highly significant; -VE: Negative; +VE: Positive.

DISCUSSION

This is the first study that addresses the short term outcome of interventional radiology procedures in management of Egyptian patients with BCS. In this study, 12 patients (48%) had short segment occlusion that enabled us to perform angioplasty with stenting in ten cases and without stenting in two cases. Thirteen patients (52%) were not suited for angioplasty and had TIPS.

According to Xu *et al.*^[13], short-term results of balloon angioplasty alone without stenting were excellent but the sustained patency rate was only 50% at two years after the procedure. In this study, one of the cases that had angioplasty alone was still having patent shunt at 24 mo after the procedure without any need for shunt revision; the other one had occluded shunt on the seventh day that necessitated re-intervention in the form of TIPS which was still patent at 20 mo after procedure.

Patency rate in patients who underwent angioplasty \pm stenting procedures was 10/12 (83.3%) at one year and at the end of follow up due to persistent shunt occlusion in 2 patients in spite of repeated revisions and optimal

anticoagulation therapy. This is a more or less satisfactory outcome; however it might have been influenced by the relatively short follow up period (ranging from 12 to 32 mo) as well as most of the patients having good or intermediate prognosis according to Rotterdam score. The need of revision in cases with angioplasty \pm stenting was 41.6% (5 out of 12 cases). One year survival was 100% and at the end of follow up, survival dropped to 91.6% due to death of one patient who had occluded shunt after one year and was also referred for mesoatrial shunt due to occlusion of IVC.

Although angioplasty is considered a simple procedure; some complications were reported in the current study. Twenty six angioplasty \pm stenting procedures have been done (12 procedures as primary intervention and 14 procedures as a trial for maintenance of previously occluded angioplasty or TIPS); of these procedures, angioplasty dysfunction was reported in 53.85%. This is consistent with Senzolo *et al.*^[14] who stated that although long-term patency rates can reach 80%-90% in angioplasty \pm stenting procedures; angioplasty may later be required in 50% of these cases to overcome angioplasty dysfunction.

Table 5 Lab data and performance status of patients before and after intervention

	Before intervention		One year after intervention		P value	Sig
	mean	SD	mean	SD		
Patients with occluded shunts (n = 3)						
ALT (N = 7-40 IU/L)	70.33	75.070	29.66	24.66	> 0.05	NS
AST (N = 7-37 IU/L)	42	24.240	42.33	32.51	> 0.05	NS
Total bilirubin (N = 0.2-1.2 mg/ dL)	2.9	2.940	1.26	0.832	> 0.05	NS
Direct bilirubin (N = 0-0.3 mg/ dL)	1.53	1.560	0.53	0.577	> 0.05	NS
Albumin (N = 3.5-5.3 g/ dL)	3.7	0.800	3.56	0.901	> 0.05	NS
Performance status	3.33	0.577	2.00	1.730	> 0.05	NS
Patients with patent shunts (n = 22)						
ALT (N = 7-40 IU/L)	66.95	117.265	26.45	8.528	< 0.05	S
AST (N = 7-37 IU/L)	53.95	33.832	32.22	9.586	< 0.01	HS
Total bilirubin (N = 0.2-1.2 mg/ dL)	2.818	3.198	1.21	0.414	< 0.01	HS
Direct bilirubin (N = 0-0.3 mg/ dL)	1.29	2.022	0.51	0.296	< 0.01	HS
Albumin (N = 3.5-5.3 g/ dL)	3.5	0.475	3.93	0.576	< 0.01	HS
Performance status	2.59	1.007	0.18	0.664	< 0.001	VHS

N: Normal range; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Sig: Significance; NS: Non significant; S: Significant; HS: Highly significant; VHS: Very highly significant.

Table 6 Comparison of different transjugular intrahepatic portosystemic shunt studies in Budd-Chiari syndrome with the current study

Points of comparison	Mancuso <i>et al</i> ^[18]	Perelló <i>et al</i> ^[19]	Rössle <i>et al</i> ^[20]	Hernández-Guerra <i>et al</i> ^[21]	Current study
No. of patients	15	13	35	25 (9 covered stents)	13
Mean age in years (range)	40 (20-73)	36 (17-67)	43 (12-74)	40 (17-54)	29 (14-57)
Median child score	11	9	9	9	8
Acute, fulminant/chronic presentation	8/6	4/6	11/13	ND	2/11
Mean follow-up (mo)	24	48	37	20	18
Stent stenosis (%)	36	72	47	67 (19% covered stents)	38.4
Anticoagulation (%)	100	95	100	ND	100
Patients with acute presentation who died	4	ND	2	ND	0
Patients with chronic presentation who died	0	ND	1	ND	0
Death total (%)	30	10	9	0	0
Liver transplantation	0	1	2	0	0
Surgical portocaval shunt	0	2	0	0	0

ND: Not determined; Anticoagulation: Percent of patients who were adherent to anticoagulation therapy.

Stent migration, which is very rare, occurred in one angioplasty procedure (3.84%) where stent migrated to the heart just after insertion. However, no serious complications occurred and stent was embedded in the wall of right atrium and the patient was quite well.

Post procedure (angioplasty \pm stenting) bleeding was encountered in 3 procedures (11.53%), 2 of which were intraperitoneal and one of which was hemobilia. All 3 cases were managed conservatively by temporary stoppage of anticoagulation and blood transfusion when indicated. This complication could be attributed to the application of a transhepatic approach in these procedures. Beckett and Olliff^[5] stated that this approach has the merit of simplicity over a transjugular or transfemoral approach, as well as feasibility with major superior vena caval obstruction but with a potentially greater risk of bleeding.

Post procedure sepsis occurred in 3 procedures (11.53%) in spite of antibiotic prophylaxis with cefotaxime in combination with ampicillin-sulbactam. This could be due to infection from resistant organisms. According to McDermott *et al*^[15], pathogens that precipitated infection after angio-

plasty and stent were *Staphylococcus aureus* and *S. epidermidis*, which were sensitive to cefazolin.

In this study, the results of angioplasty \pm stenting agreed with Fisher *et al*^[16] who stated that, with appropriate case selection, many patients with BCS caused by short length HV stenosis or occlusion may be managed successfully by angioplasty \pm stenting with a good outcome following the procedure, provided that anticoagulation is maintained. According to the authors' comparative study between percutaneous angioplasty and operative shunt surgery; both groups had the same re-occlusion rate and both were related to suboptimal dose of anticoagulation.

In the current study, 13 patients (52%) were not candidates for angioplasty and underwent TIPS. The need for revision was 38.4% (compared to 41.6% in angioplasty \pm stenting). One year and end of follow up survival rates following TIPS were 100%. This could be attributed to the relatively short follow up duration (ranging from 12 to 32 mo) and good selection of cases, as most of our patients had good or intermediate predictable prognosis according to Rotterdam score.

Patency rate in patients who had TIPS procedures was 12/13 (92.3%) at one year due to persistent shunt occlusion in one patient despite repeated revisions. At the end of follow up; patency rate dropped to 11/13 (84.6%) due to persistent shunt occlusion in another patient.

The results of the current study are much better than what had been reported by Valla^[17], namely that secondary thrombosis or shunt dysfunction requiring revision occurs in about 70% of cases by 6 mo. However, the results of this study are more or less comparable to those reported by Senzolo *et al*^[14] who stated that 36%-72% of patients needed reintervention after TIPS. The authors also reported a long-term patency rate of about 50% despite of routine anticoagulation therapy.

Comparison between the results of the current study, regarding TIPS, with other studies is shown in Table 6.

Sixteen TIPS procedures have been done throughout the current study (13 as primary intervention and 3 in patients with occluded stents following angioplasty in which predilatation was not possible).

Post TIPS sepsis occurred in 3 procedures (18.75%), in spite of prophylactic antibiotics. According to Dravid *et al*^[22]; an infection rate of 13% following TIPS was reported.

According to Ryan *et al*^[11], acute infection related to TIPS placement appears to be uncommon. Whether or not prophylactic antibiotics are of value remains undetermined. Options for prophylactic antibiotics for TIPS are: (1) no prophylaxis; (2) 1 g ceftriaxone single dose intravenously before procedure; and (3) 1.5-3 g ampicillin/sulbactam single dose intravenously before procedure. We adopted the third strategy successfully in combination with cefotaxime 1 gm IV and completed the course of antibiotics for five days after intervention.

Hepatic encephalopathy after TIPS occurred in 2 patients (12.5%) and was transient, lasting only for 2-3 d and responded well to anti hepatic encephalopathy measures.

Post procedure bleeding was encountered in 4 procedures (25%), 2 intraperitoneal and 2 hemobilia; all were managed conservatively with temporary stoppage of anticoagulation and blood transfusion if indicated.

In the current study, the overall 1 year shunt patency of all procedures (angioplasty \pm stenting and TIPS) was 22/25 (88%) as 3 patients had occluded shunts in spite of repeated trials of dilatation and adherence to anticoagulation therapy. We compared clinical and laboratory characteristics before and after intervention in patients with patent shunts (22 patients) and in those with occluded shunts (3 patients) irrespective of the type of procedure performed. We observed that patients with occluded shunts showed no improvement compared to those with patent shunts even after multiple revisions in terms of clinical manifestations, laboratory profile and performance status.

These observations are consistent with Bachet *et al*^[23] who concluded that, in patients with BCS treated with portosystemic shunting, shunt dysfunction has a major impact on morbidity and mortality and maintenance of shunt patency is of major importance for better long-term outcome.

In conclusion; Budd Chiari syndrome is a potentially life-threatening disorder that requires a multidisciplinary approach with hepatologist, hematologist, interventional radiologist and vascular surgeon. Morbidity and mortality following both angioplasty \pm stenting and TIPS are low with satisfactory stent and patient survival. Proper selection of procedure candidates and maintenance of shunt patency by strict adherence to anticoagulation and early management of shunt dysfunction are crucial in clinical, laboratory and radiological improvement of BCS patients.

COMMENTS

Background

Budd-Chiari syndrome (BCS) results from hepatic venous outflow obstruction at any level from hepatic venules to the right atrium. Few patients respond to medical treatment (anticoagulation \pm thrombolytic therapy, diuretics). However, most patients need intervention to restore the hepatic blood flow. Restoring outflow in one of the major hepatic veins by balloon dilatation \pm stenting is the management of choice. When not possible or failed, Transjugular Intrahepatic Portosystemic Shunt is used.

Research frontiers

Follow up of patients after radiological intervention is crucial in order to assess patient improvement, shunt patency and function and to manage any procedure related complications. In this study, the authors demonstrate that morbidity and mortality following angioplasty \pm stenting and transjugular intrahepatic portosystemic shunt (TIPS) are low with satisfactory outcome.

Innovations and breakthroughs

This is the first Egyptian study that addresses the short term outcome of interventional radiology procedures in management of BCS.

Applications

This study may represent a future strategy for good selection of procedure candidates, maintenance of shunt patency by strict adherence to anticoagulation and early management of shunt dysfunction which are all crucial in clinical, laboratory and radiological improvement of BCS patients.

Terminology

Angioplasty means balloon dilatation of hepatic vein; it may be with or without stent insertion. This procedure is performed in BCS patients with short segment stenosis or occlusion of the hepatic veins with significant patent segments. This approach will re-establish hepatic venous outflow via the physiological route. In cases where blood flow cannot be restored or where the approach fails (usually because the remaining patent veins are too small or have insufficient flow), Transjugular Intrahepatic Portosystemic Shunt is used; in which the shunt connects the hepatic vein to the portal vein to bypass the obstruction.

Peer review

The authors evaluated the outcome of patients with BCS after non surgical hepatic decompression procedures (either balloon angioplasty \pm stenting or TIPS). It revealed that morbidity and mortality following both procedures are low with satisfactory stent and patient survival. Thus, proper selection of procedure candidates and maintenance of shunt patency by strict adherence to anticoagulation and early management of shunt dysfunction are crucial in clinical, laboratory and radiological improvement of those patients. Their results are excellent on managing a very challenging group of patients and their program should be commended for this outcome.

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S- Editor Cheng JX L- Editor Rutheford A E- Editor Ma WH

Body mass index is associated with age-at-onset of HCV-infected hepatocellular carcinoma patients

Takumi Akiyama, Toshihiko Mizuta, Seiji Kawazoe, Yuichiro Eguchi, Yasunori Kawaguchi, Hirokazu Takahashi, Iwata Ozaki, Kazuma Fujimoto

Takumi Akiyama, Toshihiko Mizuta, Yuichiro Eguchi, Yasunori Kawaguchi, Hirokazu Takahashi, Iwata Ozaki, Kazuma Fujimoto, Department of Internal Medicine, Saga Medical School, Saga 8498501, Japan

Takumi Akiyama, Seiji Kawazoe, Department of Hepatology, Saga Prefectural Hospital, Saga 8408571, Japan

Author contributions: Akiyama T and Mizuta T designed research and analyzed data; Kawazoe S, Eguchi Y, Kawaguchi Y, Takahashi H and Ozaki I provided the patient data; Fujimoto K reviewed the manuscript; Akiyama T wrote the paper; Mizuta T reviewed and edited the manuscript.

Correspondence to: Toshihiko Mizuta, MD, PhD, Department of Internal Medicine, Saga Medical School, 5-1-1 Nabeshima, Saga 8498501, Japan. mizutat@med.saga-u.ac.jp
 Telephone: +81-952-342362 Fax: +81-952-342017

Received: August 10, 2010 Revised: September 29, 2010

Accepted: October 6, 2010

Published online: February 21, 2011

Abstract

AIM: To identify factors associated with the age at onset of hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: Five hundred and fifty-six consecutive patients positive for HCV antibody and treatment-naïve HCC diagnosed between 1995 and 2004 were analyzed. Patients were classified into three groups according to age at HCC onset: < 60 years ($n = 79$), 60-79 years ($n = 439$), or ≥ 80 years ($n = 38$). Differences among groups in terms of sex, body mass index (BMI), lifestyle characteristics, and liver function were assessed. Factors associated with HCC onset in patients < 60 or ≥ 80 years were analyzed by logistic regression analysis.

RESULTS: Significant differences emerged for sex, BMI, degree of smoking and alcohol consumption, mean bilirubin, alanine aminotransferase (ALT), and γ -glutamyl transpeptidase (GGT) levels, prothrombin activity, and

platelet counts. The mean BMI values of male patients > 60 years old were lower and mean BMI values of female patients < 60 years old were higher than those of the general Japanese population. BMI > 25 kg/m² [hazard ratio (HR), 1.8, $P = 0.045$], excessive alcohol consumption (HR, 2.5, $P = 0.024$), male sex (HR, 3.6, $P = 0.002$), and GGT levels > 50 IU/L (HR, 2.4, $P = 0.014$) were independently associated with HCC onset in patients < 60 years. Low ALT level was the only factor associated with HCC onset in patients aged ≥ 80 years.

CONCLUSION: Increased BMI is associated with increased risk for early HCC development in HCV-infected patients. Achieving recommended BMI and reducing alcohol intake could help prevent hepatic carcinogenesis.

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Key words: Age-at-onset; Hepatocellular carcinoma; Hepatitis C virus; Body mass index; Alcohol consumption; Sex difference

Peer reviewers: Heitor Rosa, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania - GO 74093-080, Brazil; Jian Wu, Associate Professor of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817, United States

Akiyama T, Mizuta T, Kawazoe S, Eguchi Y, Kawaguchi Y, Takahashi H, Ozaki I, Fujimoto K. Body mass index is associated with age-at-onset of HCV-infected hepatocellular carcinoma patients. *World J Gastroenterol* 2011; 17(7): 914-921 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/914.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.914>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer in men and the eighth most common cancer in women worldwide. The incidence and mortality associated with HCC have been reported to be increasing in countries in North America, Europe and Asia. Infection with hepatitis C virus (HCV) infection is likely to play an important role in the pathogenesis of HCC^[1-3]. In Japan, over 70% of cases of HCC diagnosed in the last 20 years are related to HCV infection^[3].

One report estimates that 3%-35% of patients progress to cirrhosis 25 years after infection with HCV and 1%-3% progress to HCC 30 years after infection^[4]. However, the factors that influence the development of HCC in patients infected with HCV remain largely unknown. Previous studies have suggested that host factors, such as sex, alcohol consumption, smoking, diabetes mellitus, and obesity, are important risk factors for HCC^[5-11]. In addition, recent studies have suggested that HCV infection causes insulin resistance and leads to oxidative stress, potentiating fibrosis and hepatic carcinogenesis^[12-14].

Therefore, we hypothesized that obesity influences the time to onset of HCC related to HCV infection, which is reflected in the patient's age at onset. To test this hypothesis, we investigated the relationship between body mass index (BMI) and lifestyle factors and age at onset of HCC in HCV-infected patients.

MATERIALS AND METHODS

Study participants

The study was conducted in accordance with the Helsinki Declaration. Written informed consent on the use of clinical records for research purposes was obtained from all subjects.

From January 1995 to December 2004, 656 consecutive patients positive for HCV antibodies and diagnosed with HCC for the first time at Saga Medical School Hospital and Saga Prefectural Hospital, without prior HCC treatment, were recruited for this study. Patients were excluded from the study if they were positive for hepatitis B surface antigen ($n = 8$), were previously treated with interferon ($n = 23$), had uncontrolled ascites ($n = 27$), or had an advanced tumor stage accompanied by tumor thrombus in portal tract or extrahepatic metastasis ($n = 42$). The remaining 556 patients (351 men, 205 women), with a median age at HCC onset of 67.8 years (range, 41-92 years) were enrolled in this study.

Diagnosis and staging of HCC

Diagnosis of HCC was confirmed by combined ultrasonography and dynamic computed tomography (CT), dynamic magnetic resonance imaging, or CT during angiography, demonstrating a hypervascular contrast pattern of the nodule in the arterial phase and a hypovascular pattern in the portal phase. If the nodule contrast patterns were not consistent with those typical for HCC, a needle biopsy of the tumor was taken for pathological diagnosis.

Tumor stage was classified according to the 5th Edition of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer, 2008, published by

the Liver Cancer Study Group of Japan^[15]. This classification system assumes three conditions: (1) tumor diameter of ≤ 2 cm; (2) a single tumor is present; and (3) no vascular invasion of the tumor. If all three conditions are met, the tumor is classified as stage I; if two conditions are met, it is classified as stage II; if only one condition is met, it is classified as stage III; and if none of the conditions are met, it is classified as stage IV.

Exposure and laboratory data

At the time of HCC diagnosis, blood tests were performed and BMI was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). Prothrombin activity and serum albumin and total bilirubin levels were measured and used to determine the Child-Pugh status. Blood samples were also used to measure alanine aminotransferase (ALT) and γ -glutamyl transpeptidase (GGT) levels and other liver function tests.

Patients were classified according to the World Health Organization (WHO) BMI criteria: underweight, BMI < 18.5 kg/m^2 ; normal weight, BMI 18.5-25 kg/m^2 ; overweight, BMI 25-30 kg/m^2 ; and obese, BMI ≥ 30 kg/m^2 ^[16]. Diagnosis of diabetes mellitus was made either by reviewing medical history or by assessing glucose levels with fasting plasma glucose level of ≥ 7.0 mmol/L or a 2-h plasma glucose level of ≥ 11.1 mmol/L^[17]. Patients were questioned by nurses about their smoking and drinking habits during the last 10 years. We defined heavy drinking as > 60 g of alcohol consumed per day and habitual smoking as > 20 pack years.

Statistical analysis

To identify factors associated with age at onset of HCC in patients with chronic hepatitis C, we compared clinical factors in two groups of patients; those aged < 60 years at HCC onset and those aged ≥ 80 years. We then analyzed risk factors affecting earlier (onset age < 60 years) and later (onset age ≥ 80 years) development of HCC in patients with chronic HCV.

We used the Kruskal-Wallis test or the χ^2 test to compare clinicopathological variables between three groups of patients. The differences in age at onset of HCC between the two groups stratified by BMI were analyzed by the Tukey-Kramer method. Univariate and multivariate logistic regression analyses were performed to identify factors associated with earlier or later onset of HCC.

Data processing and analysis were performed by using the SAS (SAS Institute Inc.). Two-tailed P values of < 0.05 were considered significant.

RESULTS

Patient characteristics

A histogram showing age at onset of HCC in 556 HCV-infected patients is depicted in Figure 1. The median age of patients was 67.8 years, with a nearly normal age distribution for the study population.

The clinical characteristics were categorized into three groups according to age at onset of HCC; < 60 years

Table 1 Clinical characteristics of patients classified with hepatocellular carcinoma occurrence age

Factors	Occurrence age of HCC (years old)			P
	< 60 (n = 79)	60-80 (n = 439)	≥ 80 (n = 38)	
Sex				
Male/Female, n	70/9	264/175	17/21	< 0.0001 ^a
BMI (kg/m ²)	23.8 ± 3.4	22.9 ± 3.4	21.8 ± 3.3	0.02 ^b
< 25/≥ 25, n	50/29	325/114	32/6	0.039 ^a
Diabetes mellitus				
With/without, n	16/63	86/353	2/36	0.088 ^a
Smoking (pack years)				
< 20/≥ 20, n	43/36	137/302	9/29	0.0001 ^a
Alcohol consumption (g/d)				
< 60/≥ 60, n	65/14	416/23	36/2	< 0.0001 ^a
Tumor stage				
I / II / III, n	21/33/25	116/196/127	11/16/11	0.981 ^a
Child-Pugh class				
A/B/C, n	55/23/1	349/87/3	34/4/0	0.145 ^a
Albumin (g/dL)	3.57 ± 0.53	3.64 ± 0.50	3.63 ± 0.41	0.586 ^b
< 3.5/≥ 3.5, n	30/49	151/288	14/24	0.433 ^a
Total bilirubin (mg/dL)	1.23 ± 0.62	1.05 ± 0.55	0.80 ± 0.30	0.0002 ^b
< 2.0/≥ 2.0, n	70/9	413/26	38/0	0.047 ^a
Prothrombin activity (%)	76.1 ± 16.6	79.9 ± 15.1	89.1 ± 12.2	0.0002 ^b
< 70/≥ 70, n	26/53	98/341	3/35	0.003 ^a
Platelet count (× 10 ⁴ /μL)	10.4 ± 7.8	11.0 ± 5.5	13.1 ± 5.8	0.005 ^b
< 10/≥ 10, n	45/34	223/216	14/24	0.125 ^a
ALT (IU/L)	78.3 ± 39.3	71.8 ± 44.1	41.7 ± 19.3	< 0.0001 ^b
< 80/≥ 80, n	47/32	288/151	36/2	0.0004 ^a
GGT (IU/L)	123.7 ± 102.5	86.0 ± 86.6	54.6 ± 36.1	< 0.0001 ^b
< 50/≥ 50, n	15/64	173/266	20/18	0.0002 ^a

Continuous variables are expressed as mean ± standard deviation. Statistical analysis was done using a: the χ^2 test or b: the Turkey-Kramer test. HCC: Hepatocellular carcinoma; BMI: Body mass index; ALT: Alanine aminotransferase; GGT: γ -glutamyl transpeptidase.

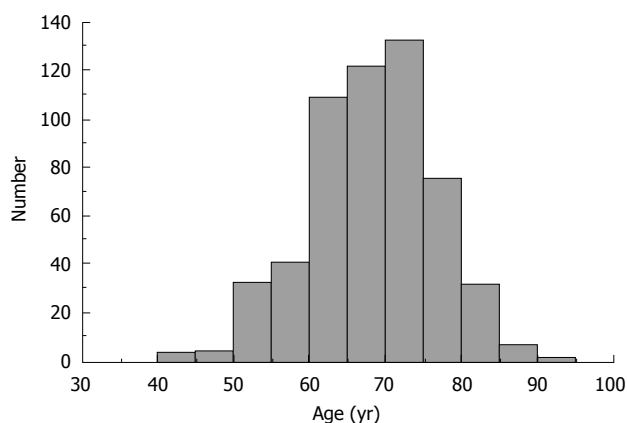


Figure 1 Histogram showing age at onset of hepatocellular carcinoma in hepatitis C virus-infected patients (n = 556). Median age, 67.8 years; range, 41-92 years.

(n = 79), 60-79 years (n = 439), and ≥ 80 years (n = 38) (Table 1). Of those aged < 60 years, 88.6% were men, a much higher percentage than in those aged 60-79 years (60.1%) and those aged ≥ 80 years (44.7%). In terms of BMI, the mean value increased, and the percentage of patients with BMI < 25 kg/m² decreased while that of patients with BMI > 25 increased with decreasing age at onset of HCC. However, this is a normal phenomenon in the general population. Therefore, we compared the mean BMI values according to the age at onset of HCC for

patients in this study with BMI values of the general Japanese population in 2005 and 2006, which were published by the Ministry of Health, Labour and Welfare, Japan (<http://www.mhlw.go.jp/>). The mean BMI of male HCC patients aged > 60 years was lower whereas that of female HCC patients aged < 60 years was higher than those of the general population (Figure 2). This indicates that the association between BMI and age at onset of HCC observed in this study was affected by factors independent of natural aging. We found that there were significantly more heavy drinkers ($P < 0.0001$) and habitual smokers ($P = 0.0001$) among patients aged < 60 years, compared with the other two age groups. Although the three groups did not differ in terms of Child-Pugh status, total bilirubin, ALT, and GGT levels were higher, and prothrombin activity and platelet counts were lower in patients aged < 60 years at HCC onset. No differences emerged in terms of the prevalence of diabetes mellitus or the distribution of tumor stage among the three groups.

Factors associated with the development of HCC at < 60 years of age

We investigated risk factors associated with the development of HCC at a younger age (i.e. < 60 years of age) (Table 2). In univariate analysis, the following were found to be significant risk factors for earlier age at onset of HCC: male sex [hazard ratio (HR), 5.4; 95% CI, 2.65-11.12; $P < 0.0001$], BMI > 25 kg/m² (HR, 1.7; 95% CI, 1.04-2.85;

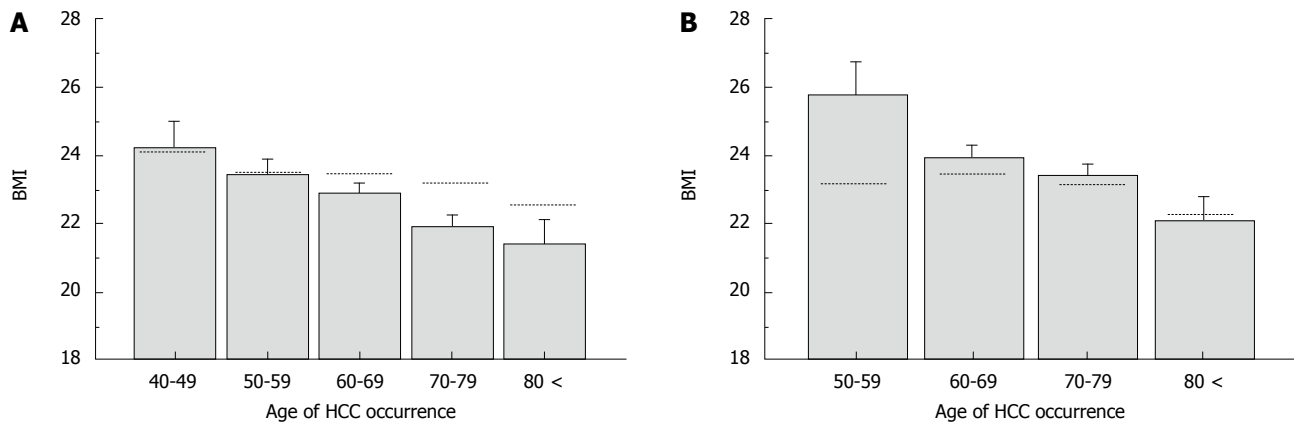


Figure 2 Mean body mass index in each age group at onset of hepatocellular carcinoma (A: Men; B: Women). The bars show the mean body mass index (BMI) \pm SD in patients with hepatocellular carcinoma (HCC). The dashed lines show the mean BMI for the general Japanese population in 2005 and 2006, which was surveyed by the Ministry of Health, Labour and Welfare, Japan.

Table 2 Analysis of factors affecting development of hepatocellular carcinoma at younger age (under 60 yr old)

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Sex						
Female	1			1		
Male	5.43	2.647-11.120	< 0.0001	3.58	1.580-8.133	0.002
BMI						
< 25	1			1		
≥ 25	1.73	1.044-2.851	0.033	1.82	1.015-3.270	0.045
Diabetes mellitus						
Without	1			1		
With	1.12	0.619-2.037	0.703	1.00	0.516-1.952	0.991
Smoking (packs year)						
< 20	1			1		
≥ 20	2.71	1.669-4.393	< 0.0001	1.64	1.904-2.991	0.104
Alcohol (g/d)						
< 60	1			1		
≥ 60	3.89	1.926-7.874	0.0002	2.51	1.130-5.563	0.024
Total bilirubin (mg/dL)						
< 2.0	1			1		
≥ 2.0	2.23	1.003-4.958	0.049	2.33	0.898-6.033	0.082
Prothrombin activity (%)						
≥ 70	1			1		
< 70	1.91	1.111-3.262	0.019	1.60	0.859-2.987	0.139
Platelet ($\times 10^4/\mu\text{L}$)						
≥ 10	1			1		
< 10	1.34	0.829-2.166	0.232	1.60	0.877-2.886	0.118
ALT (IU/L)						
< 80	1			1		
≥ 80	1.44	0.884-2.350	0.142	1.17	0.656-2.090	0.542
GGT (IU/L)						
< 50	1			1		
≥ 50	3.24	1.731-6.053	0.0002	2.38	1.194-4.727	0.014

HR: Hazard ratio; BMI: Body mass index; ALT: Alanine aminotransferase; GGT: γ -glutamyl transpeptidase.

$P = 0.033$), habitual smoking (HR, 2.7; 95% CI, 1.67-4.39; $P < 0.0001$), heavy drinking (HR, 3.9; 95% CI, 1.93-7.87; $P = 0.0002$), total bilirubin > 2.0 mg/dL (HR, 2.2; 95% CI, 1.00-4.96; $P = 0.049$), prothrombin activity $> 70\%$ (HR, 1.9; 95% CI, 1.11-3.26; $P = 0.019$), and GGT level > 50 IU/L (HR, 3.2; 95% CI, 1.73-6.05; $P = 0.0002$). In multivariate analysis, independent risk factors for earlier age at onset of HCC were male sex (HR, 3.6; 95% CI,

1.58-8.13; $P = 0.002$), BMI > 25 kg/m² (HR, 1.8; 95% CI, 1.015-3.270; $P = 0.045$), heavy drinking (HR, 2.5; 95% CI, 1.13-5.56; $P = 0.024$), and GGT > 50 IU/L (HR, 2.4; 95% CI, 1.19-4.73; $P = 0.014$).

Factors associated with the development of HCC at ≥ 80 years of age

We also investigated factors associated with the develop-

Table 3 Analysis of factors affecting development of hepatocellular carcinoma at older age (over 80 yr old)

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Sex						
Female	1			1		
Male	0.45	0.229-0.867	0.017	0.47	0.200-1.119	0.089
BMI						
<25	1			1		
≥ 25	0.49	0.201-1.201	0.119	0.48	0.174-1.321	0.155
Diabetes mellitus						
Without	1			1		
With	0.23	0.054-0.957	0.043	0.32	0.074-1.412	0.133
Smoking (packs year)						
< 20	1			1		
≥ 20	0.58	0.270-1.258	0.169	0.81	0.306-2.164	0.680
Alcohol (g/d)						
< 60	1			1		
≥ 60	0.72	0.167-3.118	0.663	0.45	0.056-3.606	0.451
Total bilirubin (mg/dL)						
< 2.0	1			1		
≥ 2.0	1.00	-	0.97	1.00	-	0.98
Prothrombin activity (%)						
≥ 70	1			1		
< 70	0.10	0.014-0.755	0.025	0.15	0.020-1.166	0.07
Platelet ($\times 10^4/\mu\text{L}$)						
≥ 10	1			1		
< 10	0.54	0.275-1.076	0.080	0.62	0.287-1.360	0.236
ALT (IU/L)						
< 80	1			1		
≥ 80	0.10	0.024-0.427	0.002	0.13	0.030-0.569	0.007
GGT (IU/L)						
< 50	1			1		
≥ 50	0.51	0.262-0.984	0.045	1.01	0.479-2.146	0.971

HR: Hazard ratio; BMI: Body mass index; ALT: Alanine aminotransferase; GGT: γ -glutamyl transpeptidase.

ment of HCC at an older age (i.e. ≥ 80 years of age) (Table 3). In univariate analysis, the following were significantly and negatively associated with age at onset of HCC ≥ 80 years: male sex (HR, 0.45; 95% CI, 0.23-0.87; $P = 0.017$), diabetes mellitus (HR, 0.23; 95% CI, 0.05-0.96; $P = 0.043$), prothrombin activity $< 70\%$ (HR, 0.1; 95% CI, 0.01-0.76; $P = 0.025$), ALT > 80 IU/L (HR, 0.1; 95% CI, 0.02-0.43; $P = 0.002$), and GGT > 50 IU/L (HR, 0.51; 95% CI, 0.26-0.98; $P = 0.045$). In multivariate analysis, ALT > 80 IU/L was the only independent factor associated with age at onset of HCC ≥ 80 years (HR, 0.13; 95% CI, 0.03-0.57; $P = 0.007$).

Age at onset of HCC stratified by BMI in relation to sex or alcohol consumption

Differences in age at onset of HCC stratified by BMI were assessed in relation to sex or alcohol consumption. In men, age at onset decreased significantly with increasing BMI (mean age \pm SD; underweight, 71.1 ± 7.4 years; normal weight, 67.0 ± 8.5 years; overweight, 63.6 ± 8.1 years; obese, 57.0 ± 7.0 years) (Figure 3A). Although a similar trend was noted in women, this was not significant (underweight, 73.6 ± 7.8 years; normal weight, 70.4 ± 7.0 years; overweight, 68.9 ± 6.4 years; obese, 67.0 ± 7.5 years) (Figure 3B).

Although an association between BMI and age at onset of HCC was found among non-heavy drinkers (Figure 4A), no association was found among heavy drinkers (Figure 4B).

DISCUSSION

The results of this study revealed that higher BMI, heavy alcohol consumption, male sex, and high GGT levels are independent risk factors for younger age at onset of HCC in patients with chronic HCV infection. This study confirms the previously reported risk factors for HCC and is the first to investigate the relationship between age and HCC development.

It seems plausible that the duration of HCV infection plays a role in the age at which cirrhosis progresses to HCC. However, Hamada *et al.*^[18] reported a significant negative correlation between the time from HCV infection to onset of HCC and the patient's age at the time of infection, and as a result, the onset of HCC was considered to occur in patients during their 60 s regardless of their age at time of infection. This indicates that factors other than duration of HCV infection may be associated with the age at onset of HCC in HCV-infected patients.

Recent studies have shown that HCV proteins, such

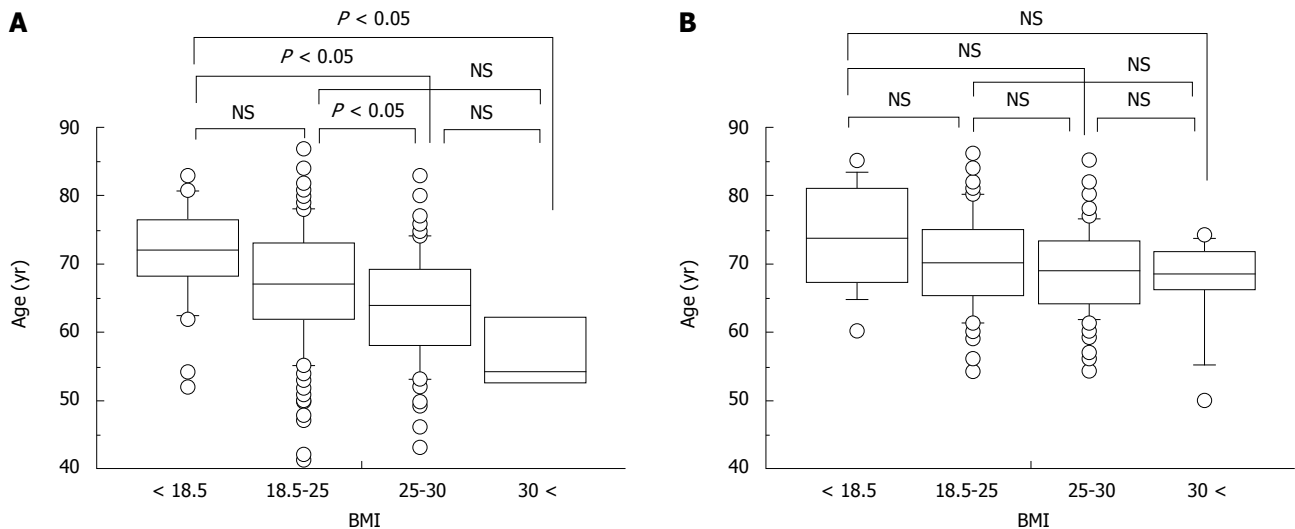


Figure 3 Differences in age at onset of hepatocellular carcinoma stratified by body mass index according to sex (A: Men; B: Women). Statistical analysis was performed using the Tukey-Kramer method. NS: Not significant; BMI: Body mass index.

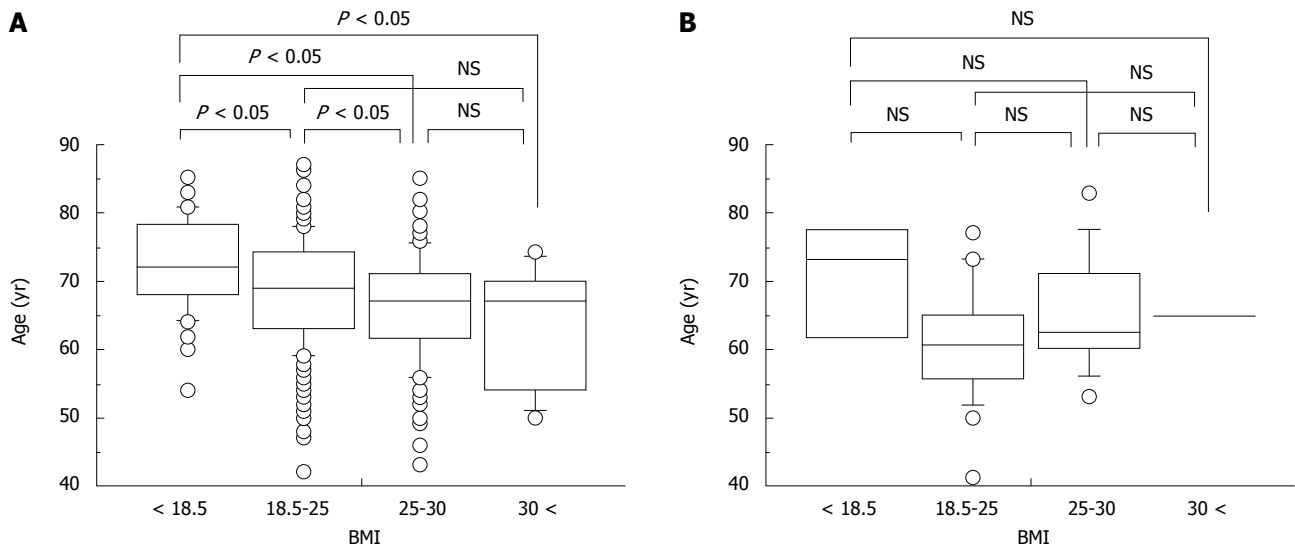


Figure 4 Differences in age at onset of hepatocellular carcinoma stratified by body mass index according to degree of alcohol consumption (A: Non-heavy drinkers < 60 g/d; B: Heavy drinkers \geq 60 g/d). Statistical analysis was performed using the Tukey-Kramer method. NS: Not significant; BMI: Body mass index.

as the core protein, cause oxidative damage by exposing the endoplasmic reticulum to oxidative stress^[19-21]. Hepatic oxidative stress is strongly associated with increased risk for HCC in patients with chronic HCV^[22]. Because oxidative stress is also caused by various host-related factors, it is expected to be influenced more strongly by host-related factors in HCV-infected patients than in those with HCV-negative liver disease. Indeed, we have previously reported that visceral fat accumulation was associated with greater insulin resistance in chronic HCV patients than in those with non-alcoholic fatty liver disease^[23]. Therefore, it is plausible that the association between earlier onset of HCC and increased BMI is due to the generation of hepatic oxidative stress.

An interesting aspect of our results is that underweight patients, defined as those with a BMI of $< 18.5 \text{ kg/m}^2$, tended to be older at HCC onset than patients within the

normal weight range (BMI 18.5-25 kg/m^2). Recently, Ohki *et al.*^[11] reported that patients with a BMI $< 18.5 \text{ kg/m}^2$ had the lowest risk of developing HCC due to chronic HCV infection among all BMI groups. In general, the mortality rate associated with cardiovascular disease or cancer is higher in underweight patients than in normal weight patients^[24,25]. Clearly, a larger cohort study is needed to investigate whether leanness confers a protective effect against hepatocarcinogenesis in HCV-infected patients.

Excessive alcohol consumption is also known to exacerbate hepatic oxidative stress and evoke liver fibrosis or HCC^[20,26]. In this study, there was no association between BMI and age at onset of HCC in heavy drinkers. We speculate that this group may include some patients who are malnourished and possibly losing weight.

Sex modulates the natural history of chronic liver disease. Previous studies have suggested that chronic HCV

infection progresses more rapidly in men than women, and that cirrhosis is predominately a disease of men and postmenopausal women^[27]. Shimizu *et al* suggested that estrogens protect against oxidative stress in liver injury and hepatic fibrosis^[28]. In this study, the effect of BMI on age at onset of HCC was more remarkable in men than women. We speculate two mechanisms to account for this difference: (1) estrogens mitigate oxidative stress or insulin resistance associated with obesity; and (2) subcutaneous fat accumulation is more dominant in obese women than visceral fat, which is known to produce several adipokines that cause insulin resistance^[29].

In addition, we examined factors associated with onset of HCC at an older age (≥ 80 years). In this analysis, ALT level was the only independent factor associated with hepatocarcinogenesis in HCV-infected patients at an age ≥ 80 years. It is well known that ALT levels are associated with liver inflammation and fibrosis progression, and Ishiguro *et al* recently reported that elevated ALT levels were strongly associated with the incidence of HCC, regardless of hepatitis virus positivity, in a large population-based cohort study^[30]. Therefore, lower ALT levels might indicate a slow course of progression of hepatic fibrosis or carcinogenesis.

A limitation of this study is that it was a cross-sectional observation, rather than a cohort follow-up study. Further studies are needed to confirm our results.

In conclusion, the results of the present study indicate that higher BMI, excessive alcohol consumption, and male sex are independent risk factors for onset of HCV-related HCC at an age of < 60 years. These results suggest that interventions to promote changes in the lifestyle of patients with chronic HCV may slow the progression of HCV infection to HCC.

ACKNOWLEDGMENTS

The authors would like to thank Yukie Watanabe and Chieko Ogawa for their assistance.

COMMENTS

Background

The incidence and mortality associated with hepatocellular carcinoma (HCC) have been increasing worldwide, and hepatitis C virus (HCV) infection plays an important role in the pathogenesis of HCC. However, the factors that influence the development of HCC in HCV-infected patients remain largely unknown. Previous studies have suggested that host factors, such as sex, alcohol consumption, smoking, diabetes mellitus, and obesity, are important risk factors for HCC. Meanwhile, it has been reported that HCV infection causes insulin resistance and leads to oxidative stress, potentiating fibrosis and hepatic carcinogenesis. Therefore, we hypothesized that body mass index (BMI) influences the onset age of HCC related to HCV infection.

Research frontiers

Many studies have indicated that obesity is an independent and a significant risk factor for HCC occurrence. Recently, several metabolic markers have been implicated in the development and progression of HCC.

Innovations and breakthroughs

This study indicated that higher BMI, heavy alcohol consumption, male sex, and high γ -glutamyl transpeptidase levels are independent risk factors for younger age at onset of HCV-related HCC. Interestingly, the underweight patients (BMI

$< 18.5 \text{ kg/m}^2$), tended to be older at HCC onset than patients within the normal weight range (BMI $18.5\text{--}25 \text{ kg/m}^2$).

Applications

The results of this study suggest that achieving an adequate body weight along with a reduction of alcohol intake in patients with chronic hepatitis C could help prevent hepatic carcinogenesis.

Peer review

The study was reasonably designed and well conducted, and the data support their conclusions.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Ma WH

Vitamin D deficiency in cirrhosis relates to liver dysfunction rather than aetiology

Mikkel Malham, Søren Peter Jørgensen, Peter Ott, Jørgen Agnholt, Hendrik Vilstrup, Mette Borre, Jens F Dahlerup

Mikkel Malham, Søren Peter Jørgensen, Peter Ott, Jørgen Agnholt, Hendrik Vilstrup, Mette Borre, Jens F Dahlerup, Department of Medicine V (Hepatology and Gastroenterology), Aarhus University Hospital, DK-8000, Aarhus C, Denmark

Author contributions: Malham M wrote the protocol, carried out the study and wrote the first draft of the manuscript; All authors contributed to the study design, data interpretation and analysis, preparation of the manuscript, and critical review; All authors read and approved the final manuscript.

Correspondence to: Mikkel Malham, MD, Department of Medicine V (Hepatology and Gastroenterology), Aarhus University Hospital, 44 Noerrebrogade, Building 1C, DK-8000, Aarhus C, Denmark. mikkel.malham@gmail.com

Telephone: +45-31121035 Fax: +45-89492740

Received: August 26, 2010 Revised: October 16, 2010

Accepted: October 23, 2010

Published online: February 21, 2011

to liver dysfunction rather than aetiology, with lower levels of vitamin D in alcoholic cirrhosis than in primary biliary cirrhosis.

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Key words: Alcoholic liver cirrhosis; Child-Pugh score; Primary biliary cirrhosis; Vitamin D deficiency

Peer reviewer: Lixin Zhu, MD, State University of New York, 3435 Main Street, 422 BRB, Buffalo, 14214 New York, United States

Malham M, Jørgensen SP, Ott P, Agnholt J, Vilstrup H, Borre M, Dahlerup JF. Vitamin D deficiency in cirrhosis relates to liver dysfunction rather than aetiology. *World J Gastroenterol* 2011; 17(7): 922-925 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i7/922.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.922>

Abstract

AIM: To examine the vitamin D status in patients with alcoholic cirrhosis compared to those with primary biliary cirrhosis.

METHODS: Our retrospective case series comprised 89 patients with alcoholic cirrhosis and 34 patients with primary biliary cirrhosis who visited our outpatient clinic in 2005 and underwent a serum vitamin D status assessment.

RESULTS: Among the patients with alcoholic cirrhosis, 85% had serum vitamin D levels below 50 nmol/L and 55% had levels below 25 nmol/L, as compared to 60% and 16% of the patients with primary biliary cirrhosis, respectively ($P < 0.001$). In both groups, serum vitamin D levels decreased with increasing liver disease severity, as determined by the Child-Pugh score.

CONCLUSION: Vitamin D deficiency in cirrhosis relates

INTRODUCTION

Patients with chronic liver disease have an increased risk for the development of osteoporosis and fractures, reduced muscle strength, an impaired inflammatory response, and malignancy^[1-3]. These conditions have also been associated with vitamin D deficiency^[4-6]. Vitamin D deficiency and osteomalacia have been described in chronic cholestatic liver disease, such as primary biliary cirrhosis (PBC)^[7]. However, the frequency of vitamin D deficiency, specifically in alcoholic liver cirrhosis (ALC), has not been well described. The limited available data suggest that there is a high frequency of vitamin D deficiency in patients with chronic liver disease^[8,9].

The main source of vitamin D in humans is the exposure of skin to sunlight. For further activation, vitamin D is hydroxylated in the liver to form 25-(OH) vitamin D

(25-OHD) and in the kidneys to form the active metabolite 1,25(OH)₂ vitamin D. The body stores of vitamin D are best reflected by the serum levels of 25-(OH)D^[10].

The aim of the present study was to describe the serum vitamin D status in a retrospective case series of patients with ALC compared to those with PBC. Patients with PBC were considered *a priori* to demonstrate a high incidence of vitamin D deficiency.

MATERIALS AND METHODS

We collected data from the medical records of all patients with a diagnosis of PBC or ALC who visited our outpatient clinic in 2005. A total of 205 patients were identified: 58 had PBC, and 147 had ALC. The study population comprised patients for whom vitamin D measurements had been completed and for whom the Child-Pugh status could be assessed (34 and 89 patients, respectively). In patients who had undergone serial vitamin D measurements, the first blood sample collected in 2005 was used. The vitamin D status was defined according to the following levels of 25-(OH)D: severe deficiency: 0-12.5 nmol/L, deficiency: 12.5-25 nmol/L, insufficiency: 25-50 nmol/L, and vitamin D replete: > 50 nmol/L^[11]. Data concerning previous and ongoing vitamin D supplementation were collected from the patients' medical records. To assess the severity of liver disease, the patients were scored according to the Child-Pugh classification. This score is based on the degree of encephalopathy, the presence of ascites, prothrombin time, and the serum levels of bilirubin, and albumin. The score ranges from 5 to 15 with increasing severity. Accordingly, the patients had either compensated liver disease (Class A, 5-6 points), moderate liver disease (Class B, 7-9 points), or severe liver disease (Class C, 10-15 points).

Techniques

Plasma 25(OH)D₂ and 25(OH)D₃ were analysed by isotope-dilution liquid chromatography-tandem mass spectrometry using an API3000 TM mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a method adapted from Maunsell *et al.*^[12]. The interassay variation coefficients for plasma 25(OH)D₂ were 8.5% at 23.4 nmol/L and 8.0% at 64.4 nmol/L, and for plasma 25(OH)D₃ these values were 9.6% at 24.8 nmol/L and 8.1% at 47.7 nmol/L.

Statistics

Non-parametric statistics were used for the descriptions, and the Mann-Whitney *U* test was employed for comparisons between groups. The association between two variables was assessed by the contingency coefficient *C*, and statistical significance was determined using the χ^2 test.

RESULTS

In the patients with ALC, 18% had a severe vitamin D deficiency. In comparison, none of the patients with PBC had such a deficiency. Similarly, in a comparison of patients

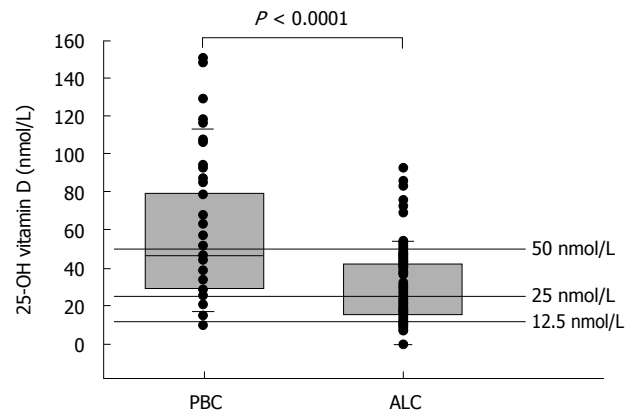


Figure 1 Vitamin D levels in the study group. Vitamin D levels in 37 patients with primary biliary cirrhosis and 89 patients with alcoholic liver cirrhosis. Patients with alcoholic liver cirrhosis demonstrated significantly lower overall vitamin D levels in comparison to patients with primary biliary cirrhosis ($P < 0.0001$, Mann-Whitney *U* test). PBC: Primary biliary cirrhosis; ALC: Alcoholic liver cirrhosis.

Table 1 Study group stratified according to the Child-Pugh class and the degree of vitamin D deficiency

Vitamin D (nmol/L)	Child-Pugh group		
	A	B	C
< 25	17	15	21
25-50	14	16	12
> 50	18	6	4

with ALC and PBC, vitamin D deficiency was identified in 37% *vs* 16% and vitamin D insufficiency was identified in 30% *vs* 41% of patients, respectively. Only 15% of patients with ALC were vitamin D replete in comparison to 40% of patients with PBC. The median 25-OHD blood concentration in ALC patients was 24 nmol/L, or 53% of the median serum level of 45 nmol/L in PBC patients ($P < 0.001$, Mann-Whitney *U* test) (Figure 1).

Four patients with ALC and 13 patients with PBC were receiving vitamin D supplementation at the time of blood sampling. Their vitamin D levels did not differ from those determined in patients who did not receive supplementation.

The distribution of Child-Pugh groups A, B, and C differed between ALC and PBC patients. Patients with ALC demonstrated more advanced disease (16 A, 36 B, and 37 C) compared to those with PBC (33 A, 1 B, and no C). In all the cirrhotic patients, there was an association between the Child-Pugh score and vitamin D status (contingency coefficient $C = 0.29$, $P < 0.05$, χ^2 test) (Table 1).

DISCUSSION

The vast majority (85%) of patients with ALC presented a compromised vitamin D status. The same was found in fewer than half of the patients with PBC (47%). This finding is in contrast to the standard clinical knowledge that vitamin D deficiency is expected in PBC. Further-

more, this marked vitamin D deficiency has never been demonstrated in a study population of this size.

Our study group included 60% of the cirrhotic patients who were seen at our clinic during 2005. This distribution does not introduce a selection bias because the vitamin D measurements were ordered without physician knowledge of the study purpose. Because the intensity of sunlight changes throughout the year, there might have been a seasonal difference in the vitamin D levels according to when the blood samples were drawn. However, patients were recruited throughout the year in both groups, and therefore, seasonal changes should not affect comparisons between the two groups.

The observed deficiency in vitamin D might be related to several causes: an impaired hepatic hydroxylation of vitamin D, dietary insufficiency, malabsorption, reduced hepatic production of vitamin D binding protein, and an impaired cutaneous production due to either reduced exposure to sunlight or jaundice^[9,13]. The observation that the deficiency was less pronounced in PBC patients suggests that bile acid-related lipid malabsorption is not the only mechanism involved in vitamin D deficiency. It seems plausible that the mechanism of vitamin D deficiency is multifactorial and differs between the two groups of cirrhotic patients. When the results were stratified according to the Child-Pugh class, an association was observed between vitamin D deficiency and the severity of liver disease. This association has never been demonstrated in such a large study population. Thus, the better preservation of vitamin D status in patients with PBC might be ascribed to the diminished severity of their liver disease, as assessed by their Child-Pugh scores. Based on this finding, one could hypothesise that the risk for vitamin D deficiency or insufficiency might be influenced more by the degree of liver dysfunction than by the aetiology of the liver disease. However, our study was not designed to elucidate the exact mechanism underlying the vitamin D deficiency. The purpose of the study was to emphasise the importance of monitoring the vitamin D status in all patients with cirrhosis, especially those with ALC for whom nutritional status has been a relatively neglected area of study.

Our results imply that vitamin D deficiency is highly prevalent in patients with ALC. Because this was a retrospective study, we cannot extrapolate the results to the general population of cirrhotic patients. However, these results indicate that the frequency and severity of vitamin D deficiency in ALC patients warrant greater attention, similar to the usual clinical practice in patients with PBC.

Although 17 of the study patients received vitamin D supplementation, this supplementation was clearly insufficient, as their vitamin D concentrations remained low. Thus, it appears that the vitamin D deficiency in these patients should be treated with higher doses of vitamin D than that used in standard clinical practice for repletion.

The risk for bone disease in cirrhotic patients justifies the use of routine vitamin D therapy. Furthermore, the patients might also benefit from correction of their

vitamin D status with respect to reduced muscle function, cancer risk, and immune impairment.

COMMENTS

Background

Patients with liver cirrhosis have an increased incidence of cancer, infections, osteoporosis, and decreased muscle strength. Vitamin D deficiency is associated with these complications in other patient groups and could be partially involved in the clinical complications related to cirrhosis.

Research frontiers

Vitamin D deficiency is a well reported complication in chronic cholestatic liver disease such as primary biliary cirrhosis. While the prevalence and treatment of this deficiency has been addressed in many articles over the last decades, little is known of the vitamin D status in alcoholic liver cirrhosis.

Innovations and breakthroughs

Recent studies imply that vitamin D deficiency is frequent in all patients with cirrhosis. The current study shows that vitamin D deficiency is more frequent and severe in patients with alcoholic liver cirrhosis than in patients with primary biliary cirrhosis. Furthermore, it indicates that the degree of liver dysfunction, rather than the aetiology of cirrhosis, dictates the risk of vitamin D deficiency.

Applications

This study emphasizes the importance of monitoring vitamin D levels in all patients with cirrhosis. However, further studies are needed to find the most favourable form of vitamin D supplementation for these patients.

Terminology

Primary biliary cirrhosis and alcoholic cirrhosis are two different diseases that cause cirrhosis of the liver. While primary biliary cirrhosis is a cholestatic, autoimmune disease, alcoholic liver cirrhosis is an alcohol-induced liver disease usually without cholestatic features. The Child-Pugh score assesses the prognosis in patients with cirrhosis and is also used to quantitate the degree of liver dysfunction.

Peer review

This brief article nicely demonstrated the association of the liver damage severity with the level of 25-hydroxy vitamin D. This is a very important report, as many doctors do not realize that liver damage could cause significant vitamin D deficiency.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Lin YP

Natural orifice transluminal endoscopic wedge hepatic resection with a water-jet hybrid knife in a non-survival porcine model

Hong Shi, Sheng-Jun Jiang, Bin Li, Deng-Ke Fu, Pei Xin, Yong-Guang Wang

Hong Shi, Bin Li, Pei Xin, Yong-Guang Wang, Institute of Minimally Invasive Medicine, Tongji University, Shanghai 200092, China

Hong Shi, Sheng-Jun Jiang, Bin Li, Deng-Ke Fu, Pei Xin, Yong-Guang Wang, Department of Minimally Invasive Surgery, Beijing Chuiyangliu Hospital, Beijing 100022, China

Author contributions: Shi H, Jiang SJ and Wang YG designed the study; Shi H wrote the first draft of the paper; all the authors contributed to the design and interpretation of the study and to the final manuscript.

Correspondence to: Yong-Guang Wang, MD, PhD, Department of Minimally Invasive Surgery, Beijing Chuiyangliu Hospital, No. 2, Chuiyangliu South Street, Chaoyang District, Beijing 100022, China. endowang@vip.sina.com

Telephone: +86-10-67718822 Fax: +86-10-67711960

Received: October 6, 2010 Revised: November 17, 2010

Accepted: November 24, 2010

Published online: February 21, 2011

Abstract

AIM: To explore the feasibility of a water-jet hybrid knife to facilitate wedge hepatic resection using a natural orifice transluminal endoscopic surgery (NOTES) approach in a non-survival porcine model.

METHODS: The Erbe Jet2 water-jet system allows a needleless, tissue-selective hydro-dissection with a pre-selected pressure. Using this system, wedge hepatic resection was performed through three natural routes (trans-anal, trans-vaginal and trans-umbilical) in three female pigs weighing 35 kg under general anesthesia. Entry into the peritoneal cavity was *via* a 15-mm incision using a hook knife. The targeted liver segment was marked by an APC probe, followed by wedge hepatic resection performed using a water-jet hybrid knife with the aid of a 4-mm transparent distance soft cap mounted onto the tip of the endoscope for holding up the desired plane. The exposed vascular and ductal structures were clipped with Endoclips. Hemostasis was applied to the bleeding

cut edges of the liver parenchyma by electrocautery. After the procedure, the incision site was left open, and the animal was euthanized followed by necropsy.

RESULTS: Using the Erbe Jet2 water-jet system, trans-anal and trans-vaginal wedge hepatic resection was successfully performed in two pigs without laparoscopic assistance. Trans-umbilical attempt failed due to an unstable operating platform. The incision for peritoneal entry took 1 min, and about 2 h was spent on excision of the liver tissue. The intra-operative blood loss ranged from 100 to 250 mL. Microscopically, the hydro-dissections were relatively precise and gentle, preserving most vessels.

CONCLUSION: The Erbe Jet2 water-jet system can safely accomplish non-anatomic wedge hepatic resection in NOTES, which deserves further studies to shorten the dissection time.

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Key words: Natural orifice transluminal endoscopic surgery; Hepatic resection; Water-jet; Hybrid knife; Triangulation

Peer reviewer: Chih-Chi Wang, MD, Department of Surgery, Chang Gung Memorial Hospital-Kaohsiung Medical Center, 123 Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan, China

Shi H, Jiang SJ, Li B, Fu DK, Xin P, Wang YG. Natural orifice transluminal endoscopic wedge hepatic resection with a water-jet hybrid knife in a non-survival porcine model. *World J Gastroenterol* 2011; 17(7): 926-931 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/926.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.926>

INTRODUCTION

Liver resection, a surgical procedure consisting of he-

patic parenchymal dissection as well as precise identification followed by control of intra/extra-hepatic vascular and biliary anatomy, is technically challenging due to the risk of massive bleeding during operation. Since excessive hemorrhage and subsequent blood transfusion are strongly associated with increased peri-operative morbidity and mortality, technical innovations have mainly focused on minimizing blood loss^[1]. Besides inflow occlusion and low central pressure used to prevent bleeding from inflow vessels and hepatic veins in the transaction surface since the early 20th century, the development of specific devices for separating hepatic parenchyma, such as the ultrasonic dissector, water jet, Harmonic scalpel, Ligasure, and Tissue-Link dissecting sealer, has also contributed to bloodless transection. A meta-analysis^[2] assessing the benefits and risks of current techniques of parenchymal transection showed that there were no significant differences in terms of the mortality, morbidity, markers of liver parenchymal injury or liver dysfunction in pairwise comparisons including cavitron ultrasound surgical aspirator, radiofrequency dissecting sealer, sharp dissection and hydro-jet. Among them, the water-jet dissector employs a pressurized jet of water to fragment the liver parenchyma tissue, with intact vascular and ductal structures, which can be ligated with staplers or clipped with titanium hemoclips, resulting in reduced blood loss, transfusion requirement, and biliary leak^[3].

High-pressure water-jet dissection technology was originally developed in the steel and glass industries, where ultra-precise cutting and engraving were considered as professional demands^[4]. Since introduced to medical application in 1982^[5], this technology (Hydro-Jet[®]; ERBE, Tuebingen, Germany) has been successfully employed in open and laparoscopic operations, achieving favorable results in precise, controllable tissue-selective (indicating water-rich tissue such as liver parenchyma) dissection with excellent visualization and minimal injury to the surrounding fibrous structures (such as ductal and vessel systems with a high content of collagen and elastin)^[6]. The above-mentioned Helix Hydro-Jet device with a rigid hand-held applicator is not designed with sufficient flexibility for natural orifice transluminal endoscopic surgery (NOTES) procedures, and can not be passed through a standard working channel of the current flexible endoscope because its outer-diameter is larger than the endoscopic operative channel. Now a new water-jet hybrid knife^[7] incorporating with high-pressure water-jet and radiofrequency may overcome this drawback. It has a smaller size, being easy to handle, and showing more preciseness, with almost linear correlation of pressure and dissection depth, and less foaming compared with the precursor model Helix Hydro-Jet^[5].

As is known, trans-luminal liver resection is technically demanding and its expansion has been lagged behind other NOTES procedures. Phee *et al*^[8] demonstrated for the first time how a dexterous master and slave trans-luminal endoscopic robot could efficiently perform the wedge hepatic resection without laparoscopic assistance. Unfortunately, this technology is still an unexplored field

in China. The aim of our study was to explore the safety and efficacy of a water-jet hybrid knife to facilitate wedge hepatic resection using a NOTES approach in a non-survival porcine model.

MATERIALS AND METHODS

Experimental design

This non-survival study evaluated the performance of the water-jet hybrid knife during NOTES procedure in a live porcine model. A pilot experiment in an isolated liver was conducted first, and followed by an open procedure in a 35-kg female porcine model. The formal study included three operations of wedge hepatic resection using NOTES and water-jet technology through three respective natural routes (trans-anal, trans-vaginal and trans-umbilical). The outcome measures were the time spent in performing a trans-visceral incision, the time spent in excising the liver segment, and the blood loss including oozing and brisk vascular hemorrhage, determined as blood accumulation in the suction device.

This study was conducted with prior approval by the Institutional Animal Care and Use Committee of Tongji University of China.

Experimental animal and instrument

Transluminal hepatic wedge hydro-dissection was performed in three 35-kg female pigs. The pigs were food deprived but allowed liquids for 24 h before the procedure. Urethral catheterization and warm saline enema were conducted immediately before surgery. The animals were then transferred to an operating table, and placed in supine position.

The water-jet hybrid knife (Erbe Elektromedizin) used in this study is a stainless-steel tube that incorporates a microcapillary with a diameter of 150 μm ^[7]. The flexible instrument has an outer diameter of 2.1 mm and a length of 2.20 m so that it can pass through the operating channels (diameter, 2.8 and 3.7 mm) of a forward-viewing dual-channel therapeutic endoscope (GIF-2T160; Olympus Medical Systems Corporation, Tokyo, Japan). The hybrid knife can be used for hydro-dissection, rinsing blood clot and rinsing for a better endoscopic view by water-jet application, as well as coagulation by radiofrequency application. The foaming with the use of the hybrid-knife can be scavenged by the suction mechanism of the endoscope. In NOTES procedure, a 4-mm transparent distance soft cap was mounted onto the tip of the endoscope for holding up the desired surface, subsequently avoiding the deviation in the direction of the water-jet. However, it was not used in the previous open procedure, because distraction (with surgical retractors) could allow the water-jet hybrid knife to effectively dissect the tissue by exposing the base of the cutting plane.

Rau *et al*^[6] found that a pressure of 30-40 bar was very effective to dissect normal human liver tissues, and the long-distance transmission attenuation was about 10%. Therefore, we set the pressure at 45 bar, which was proved to be effective in our pilot experiment and open operation.



Figure 1 Colostomy on anterior wall of rectal junction and sigmoid colon. At the beginning of trans-anal natural orifice transluminal endoscopic surgery procedure, entry into the peritoneal cavity was via a 15-mm linear incision using the hook knife (cutting width set at 6 units and cutting interval set at 1 unit). The ideal access point was the junction of rectum and sigmoid colon at a distance of 15-20 cm away from the anus.



Figure 2 Hydro-dissection of liver segment in natural orifice transluminal endoscopic surgery procedure. Hepatic parenchyma dissection was performed using the water-jet hybrid knife kept away from the tissue in a no-touch fashion and perpendicular to but not tangentially against the predetermined surface, keeping in a smooth, reproducible, back-and-forth waving motion. A 4-mm transparent distance soft cap was mounted onto the tip of the endoscope for holding up the desired surface, subsequently avoiding the deviation in the direction of the water-jet.

Other instruments used were as follows: a flexible sterile overtube (MD48618, Sumitomo Bakelite, Tokyo, Japan), a transparent distance flat soft cap (D-201-13404, Olympus), a hook knife (KD-620LR, Olympus), endoscopic hemostatic forceps (FD-410LR, Olympus), endoclips (HX-610-135L OLYMPUS, Olympus), a foreign forcep (FQ-46L-1, Olympus), APC probe (argon plasma coagulation, APC) (ERBE Elektromedizin), and the modular VIO generator (VIO 300D; Erbe Elektromedizin, Tübingen, Germany).

Experimental procedure

Anesthesia was induced with 5% isoflurane administered intravenously. The animal was then intubated with endotracheal tube, followed by general anesthesia with 1%-2% isoflurane. Throughout the operation, oxygen was administered to the animal at a flow rate according to oxygen saturation, and both pulse rate and oxygen

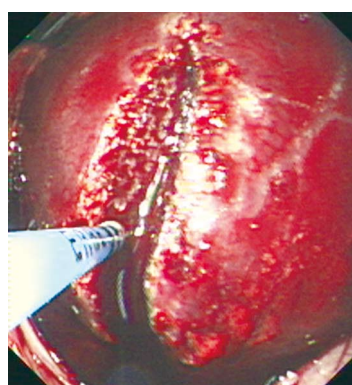


Figure 3 Hydro-dissection of liver segment in open procedure. Hepatic parenchyma dissection was performed using the water-jet hybrid knife in a similar natural orifice transluminal endoscopic surgery procedure, except that the 4-mm transparent distance soft cap was not used.

saturation were monitored continuously using the pulse oximeter clamped to the animal tongue. Then normal saline enema was administered to each animal. Residual stool would be removed with aggressive washing, and suctioning during endoscopic inspection.

At the beginning of the procedure, entry into the peritoneal cavity was *via* a 15-mm linear incision made by the hook knife (a cutting width was set at 6 units and cutting interval was set at 1 unit). The ideal access point was the abdominal site 1 cm away from the umbilicus in trans-umbilical route, the bottom of the vagina in trans-vaginal route, the junction of rectum and sigmoid colon at a distance of 15-20 cm away from the anus in trans-anal route (Figure 1). Then the endoscope with a 4-mm transparent distance soft cap mounted onto the tip of the endoscope beforehand was passed through the access to reach the peritoneum using the air inflation mechanism of the endoscope.

After the target liver segment was identified, hepatic parenchymal dissection with the water-jet hybrid knife was performed in the following steps (Figure 2), which were generally similar to those in the previous open operation except the assistance of manual retraction (Figure 3). The range to be separated was marked by an APC probe. The Glisson's capsule was scored 2-3 mm deep along the demarcated plane of transaction with the hook knife. Then hepatic parenchyma dissection was performed using the water-jet hybrid knife kept away from the tissue in a no-touch fashion. The tip of the knife was perpendicular to but not tangentially against the predetermined surface (this was achieved with a 4-mm transparent distance soft cap mounted onto the tip of the endoscope for holding up the desired surface, subsequently avoiding the deviation in the direction of the water-jet). A smooth, reproducible, back-and-forth waving motion was used. Minor slow oozing from the cutting surface was controlled using the same knife, the hook knife or APC probe to initiate bursts of coagulation. Visible intra-hepatic vascular and ductal structures were clipped with endoscopic hemoclips. Once the liver segment was completely free and after checking for hemostasis, the incision was slightly enlarged, then an

Table 1 Comparisons of three routes for natural orifice transluminal endoscopic surgery procedure

Items	Trans-umbilical route	Trans-anal route	Trans-vaginal route
Access position	Visually inspected at para-umbilical region	Verified by finger pressing	Located by surrounding anatomic landmarks
Time to complete a trans-visceral incision		About 1 min	
Time to reach peritoneum		About 2 min	
Liver exposure	Antero-lateral segments could be easily detected, while posterosuperior segments were hard to be explored		
Working platform	Unstable		Relatively stable
Time to hydro-dissection	Abandoned 1 h later	2 h	2 h and 40 min
Size of resected liver segment	No resected specimen was obtained due to failure in trans-umbilical hepatic resection	50 mm × 25 mm × 5 mm	45 mm × 30 mm × 7 mm
Bile leak		Not found	
Blood loss	100 mL	200 mL	250 mL
Injury to surrounding organs		Not occurred	

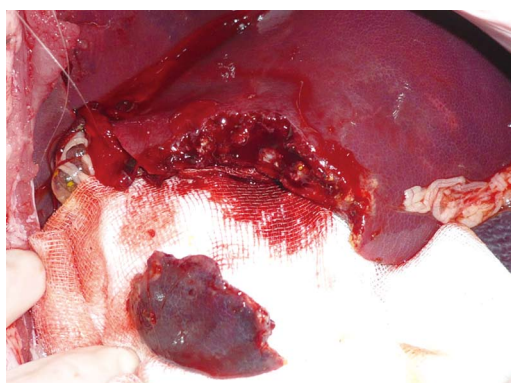


Figure 4 A resected liver segment compared with the reserved part. A resected liver segment was picked out with white gauze.

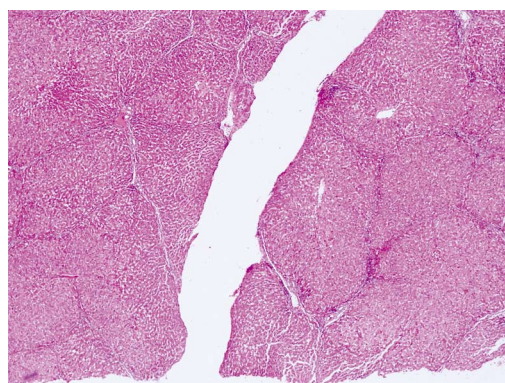


Figure 5 Microscopic findings of water-jet dissection in liver tissues (HE stain, ×40). A smooth and gentle cutting margin was presented. The cutting width at the bottom of the cut was similar to the dissection width at tissue surface, with little vessel damage.

endoscopic retrieval net was inserted through the endoscopic working channel and the specimen was introduced into the net and was retrieved intactly. After the procedure, the incision site was left open, and the animal was euthanized followed by necropsy.

Histopathological examination

Histologic examination was performed for all dissected specimens. The results were observed under microscope after hematoxylin and eosin staining based on the characteristics of the dissection margins, vessel preservation and dissection impact on the surrounding tissues. Thermal alterations such as edema and structural changes of different layers of the specimen were also microscopically analyzed.

RESULTS

It took 20 min to complete the excision of a liver segment 50 mm × 30 mm × 10 mm in size during the pilot experiment, and 45 min to complete the excision of a liver segment 45 mm × 25 mm × 10 mm in size during the open procedure. The blood loss was 100 mL in the open operation.

As for the NOTES procedure, using the Erbe Jet2 water-jet system, trans-anal and trans-vaginal wedge hepatic resections were successfully performed in two pigs without

laparoscopic assistance. Trans-umbilical attempt failed due to an unstable operating platform. Each incision for peritoneal entry took 1 min, and 2 h was spent on excision of the liver tissue, indicating a hugely time-consuming part of the entire procedure. There was neither hemodynamic nor pulmonary instability throughout the NOTES procedure, and target visualization within the peritoneum was always kept clear. No untoward incident such as injury to surrounding organs occurred, and the whole intra-operative blood loss ranged from 100 to 250 mL. Parenchymal bleeding from resection could be adequately controlled by electrocautery with the hybrid knife itself, the hook knife or the APC probe (Table 1, Figure 4). Since all the exposed ductal structures were successfully clipped with Endoclips, no bile leak from the remnant liver occurred.

There were relatively smooth and precise cutting margins in all histological preparations. The cutting width at the bottom of the cut was similar to the dissection width at tissue surface, with little vessel damage (Figure 5). Some thermal alterations were obtained due to intra-operative electrocautery (Figure 6).

DISCUSSION

To the best of our knowledge, this is the first study in

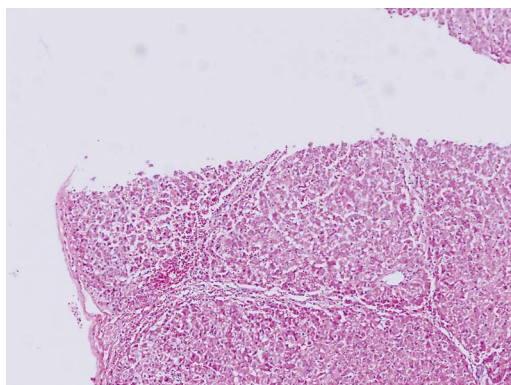


Figure 6 Thermal alterations due to intra-operative coagulation (HE stain, $\times 100$). Removal of the liver capsule could be seen in an example of thermal damage.

a non-survival porcine model evaluating the feasibility and safety of wedge hepatic resection merely using a NOTES approach, Erbe Jet2 water-jet technology and endoscopic instrument.

Since first described by Kalloo *et al*^[9], natural orifice transluminal endoscopic surgery (NOTES) has become the newest minimally invasive surgical procedure in contrast to open and laparoscopic technology. It involves passing flexible endoscopic systems through natural orifices (per-oral, trans-vaginal, trans-anal, trans-umbilical or trans-vesical routes), approaching target organs and performing intra-abdominal procedures. For the entry into the peritoneal cavity, a trans-luminal incision is mostly created by endoscopic needle knife followed by balloon dilation. However, in our study, it was achieved just in about 1 min *via* a hook knife, with the same desirable effect. The air-inflation mechanism of the endoscope was used to induce and maintain peritoneum, and the suction mechanism of the endoscope was used intermittently to avoid a high intra-abdominal pressure. Overall, there was neither hemodynamic nor pulmonary instability during NOTES, as described elsewhere^[10].

Similar to laparoscopic liver resection, NOTES hepatic procedures must confront one and the same Achilles' heel, difficulty in obtaining hemostasis. Given the facts that protection of blood vessels is essential to minimize hemorrhage and blood transfusion, and smooth dissection margins might minimize adhesion formation^[11], the water-jet hybrid knife was taken into consideration. Hydro-dissection was accomplished with the hybrid knife kept away from the tissue in a no-touch fashion and perpendicular to but not tangentially against the predetermined surface. Minor slow oozing from the cutting surface was controlled using the same knife, the hook knife or APC probe to initiate bursts of coagulation. Visible intra-hepatic vascular and ductal structures were clipped with endoscopic hemoclip. Certainly, the need for coagulation or clipping of individual vessels led to a prolonged operative time.

Current flexible endoscopes have significant limitations when used for complex therapeutic procedures. Stable platform and off-axis operation are often necessary for the NOTES. However, standard endoscopic shafts are too

flexible and prone to looping, if these unfavorable factors caused the failure in transumbilical endoscopic hepatic resection. As for triangulation of endoscopically deployed instruments to approach the same target, internal double channels are small and in close proximity, producing parallelism and limiting possible triangulating interactions^[12]. The operator interface parallelism does not allow satisfactory traction/countertraction for effective dissection of tissue and organs. To counteract the negative impact on dissection efficiency, a 4-mm transparent distance soft cap was mounted onto the tip of the endoscope for holding up the desired plane, subsequently avoiding the deviation in the direction of the water-jet. Unfortunately, its effect was limited due to the heavy weight of the porcine liver and the restricted field of view. As a result, excision of one piece of the same size from the porcine liver was more difficult in NOTES than in open procedure (more than 2 h was spent in NOTES, but only 45 min spent in open procedure).

Notably, non-anatomic wedge hepatic resection by a NOTES approach in either our or Phee's^[8] study is still at a primary stage. As NOTES using current endoscopic instruments is technically difficult to realize pedicle control with an intrahepatic Glissonian approach^[13], it is suitable only for superficial lesions of the liver mostly with the fine trabecular infrastructures and medium caliber structures. In order to achieve the same level of segment-based laparoscopic liver resection^[14], advance in NOTES technology still has a long way to go.

In conclusion, the water-jet hybrid knife with the capacity of selective vessel-sparing tissue dissection can safely accomplish non-anatomic wedge hepatic resection through a NOTES approach. At the same time, its efficiency may be discounted by endoscopic deficiencies: lack of surgical triangulation, unstable operating platform as well as transmission attenuation caused by long distance and endoscopic looping. Although this technology is only at its beginning stage, as the old saying goes: well begun is half done.

ACKNOWLEDGMENTS

We thank Dr. Jiang-Fan Zhu for his editorial assistance.

COMMENTS

Background

Liver resection is technically challenging due to the risk of massive bleeding during operation. Since the early 20th century, the development of specific devices for separating hepatic parenchyma has contributed to bloodless transection. Furthermore, trans-luminal liver resection is technically demanding and its expansion has been lagged behind other natural orifice transluminal endoscopic surgery (NOTES) procedures.

Research frontiers

Phee described for the first time how a dexterous master and slave transluminal endoscopic robot could efficiently perform the wedge hepatic resection without laparoscopic assistance. This technology is still an unexplored field in China.

Innovations and breakthroughs

This is the first study to evaluate the feasibility and safety of non-anatomic wedge hepatic resection in a non-survival porcine model using a NOTES approach, Erbe Jet2 water-jet technology and endoscopic instruments. The

study demonstrated that the water-jet hybrid knife with the capacity of selective vessel-sparing tissue dissection can safely accomplish non-anatomic wedge hepatic resection through a NOTES approach.

Applications

Currently, non-anatomic wedge hepatic resection using NOTES approach and water-jet technology is suitable only for superficial lesions of the liver mostly with the fine trabecular infrastructures and medium caliber structures.

Terminology

High-pressure water-jet dissection technology was originally developed in the steel and glass industries, where ultra-precise cutting and engraving were considered as professional demands^[4]. Since introduced to medical application in 1982^[5], this technology has been successfully employed in open and laparoscopic operations, achieving favorable results in precise, controllable tissue-selective dissection with excellent visualization and minimal injury to the surrounding fibrous structures (such as ductal and vessel systems with a high content of collagen and elastin).

Peer review

This is the study in a non-survival porcine model evaluating the feasibility and safety of wedge hepatic resection by using pure NOTES approach.

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S- Editor Tian L L- Editor Ma JY E- Editor Ma WH

Upregulated CD133 expression in tumorigenesis of colon cancer cells

Zhi-Li Yang, Qi Zheng, Jun Yan, Ye Pan, Zhi-Gang Wang

Zhi-Li Yang, Qi Zheng, Jun Yan, Ye Pan, Zhi-Gang Wang, Department of Surgery, Affiliated Sixth People's Hospital of Shanghai Jiao Tong University, Shanghai 200233, China

Author contributions: Yang ZL analyzed the CD133 expression in a panel of colon cancer cell lines and spheroid culture and drafted the manuscript; Zheng Q, Yan J and Pan Y participated in the study design and performed the RT-qPCR analysis; Wang ZG conceived the study and revised the manuscript.

Correspondence to: Zhi-Gang Wang, MD, Assistant Professor, Department of Surgery, Affiliated Sixth People's Hospital of Shanghai Jiao Tong University, Shanghai 200233, China. surlab@hotmail.com

Telephone: +86-21-64369181 Fax: +86-21-64701361

Received: August 7, 2010 Revised: November 12, 2010

Accepted: November 19, 2010

Published online: February 21, 2011

Abstract

AIM: To analyze the upregulated CD133 expression in tumorigenesis of primary colon cancer cells.

METHODS: Upregulated CD133 expression in tumorigenesis of colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) was analyzed by flow cytometry. Human colon cancer tissue samples were stained with anti-human CD133. SW620 cells were sorted according to the CD133 expression level measured by fluorescence-activated cell sorting. Spheroids of colorectal cancer cells were cultured with the hanging drop. Expression of CD133 and Lgr5 in spheroids of colorectal cancer cells and monolayer culture was detected by RT-qPCR. Spheroids of colorectal cancer cells were analyzed using anti-human CD133 with immunohistochemical staining.

RESULTS: CD133 antigen was expressed in colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) as well as in primary and metastatic human colon cancer tissues. However, the CD133 was differently expressed in these cell lines and tissues. The expression levels of CD133 and Lgr5 were significantly

higher in spheroids of parental, CD133^{hi} and CD133⁺ cells than in their monolayer culture at the mRNA level ($P < 0.05$). Immunohistochemical staining of spheroids of CD133⁺ cells showed that CD133 was highly expressed in colorectal cancer cell lines.

CONCLUSION: Upregulated CD133 expression plays a role in tumorigenesis colorectal cancer cells, which may promote the expression of other critical genes that can drive tumorigenesis.

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Key words: CD133; Colon cancer cells; Tumorigenesis; Cancer stem cells

Peer reviewer: Ioannis Kanellos, Professor, 4th Surgical Department, Aristotle University of Thessaloniki, Antheon 1, Pano-rama, Thessaloniki 55236, Greece

Yang ZL, Zheng Q, Yan J, Pan Y, Wang ZG. Upregulated CD133 expression in tumorigenesis of colon cancer cells. *World J Gastroenterol* 2011; 17(7): 932-937 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/932.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.932>

INTRODUCTION

CD133, also known as prominin-1, a transmembrane pentaspan protein, is originally described as a surface antigen specific for human hematopoietic stem and progenitor cells^[1,2]. Later, CD133 is recognized as a stem cell marker for other normal tissues of brain^[3], kidney^[4], prostate^[5], liver^[6], pancreas^[7], and skin^[8]. It has been increasingly reported that CD133 is a marker of putative cancer stem cells (CSC) in brain tumor^[9,10], prostate cancer^[11], colon cancer^[12-14], lung cancer^[15], hepatocellular carcinoma^[16], melanoma^[17], ovarian cancer^[18], and pancreatic cancer^[19]. Accordingly, CD133 has been referred to as "the molecule of the moment"^[20].

It has been recently shown that CD133 expression is broadly distributed in primary colon cancer cells including cancer stem cells, both CD133⁺ and CD133^{hi} metastatic colon cancer cells initiate tumors^[21-23]. However, whether CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown.

In the present study, upregulated CD133 expression in several colorectal cancer cell lines as well as in human primary and metastatic colon cancer tissue samples was analyzed. SW620 cell line was sorted using CD133 antigen. Spheroids of parental, CD133⁺ and CD133^{hi} cells were cultured with the hanging drop. Expressions of CD133 and Lgr5 were detected in spheroids of colorectal cancer cells. CD133 was widely expressed in human colorectal cancer cell lines as well as in primary and metastatic colon cancer tissues and upregulated CD133 expression was detected in spheroids of colorectal cancer cells, indicating that upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis.

MATERIALS AND METHODS

Cell lines and cell culture and tissue samples

Human colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 10 μ mol/L thioglycerol, 12.5 U insulin, 0.5 mg hydrocortisone, and 30mg penicillin G/0.05 g streptomycin. Colorectal cancer cells were cultured at 37°C in a humidified atmosphere containing 10% CO₂. CD133 expression was detected in formalin-fixed, paraffin-embedded primary and metastatic colorectal cancer tissue samples from Affiliated Sixth People's Hospital of Shanghai Jiaotong University. The study was approved by the Ethics Committee of Affiliated Sixth People's Hospital of Shanghai Jiaotong University.

Fluorescence-activated cell sorting

Single-cell suspensions were stained with antibodies against human CD133 (AC133, 1:40) and human CD133/1 and CD133/2(1:10, APC conjugated, Miltenyi Biotech, Germany). Dead cells, cell debris, doublets and aggregates were excluded by forward and side scattering and pulse-width gating. Colorectal cancer cells (1×10^5) were stained in an eppendorf tube. Primary antibody was incubated for 45 min on ice and second antibody (anti-mouse Alexa488, 1:400) was incubated for 30 min on ice in the dark. Flow cytometry analysis was carried out on a fluorescence-activated cell sorting (FACS) caliber (BD). Colorectal cancer cells (1×10^6) were prepared for sorting, stained with human CD133/1 (1:10, APC conjugated, Miltenyi Biotech) and 1 μ g/mL propidium iodide (PI) to exclude dead cells during sorting. The cells were sorted using FACS Aria (BD). Matched isotype antibodies were applied in parallel as controls.

Colon spheroids were culture with hanging drop

SW620 colorectal cancer cells and their sorted CD133⁺ and CD133^{hi} cells were prepared as a single cell suspension. The cells were counted and diluted in RPMI1640

containing 20% FBS and antibiotics to a concentration of 500 cells per 20 μ L/drop in a sterile basin. The lid was lifted, inverted and placed on top of the dish containing 10 mL PBS. An 8-channel pipette was used to make rows of 20 μ L drops on the up-turned inner surface of the tissue culture dish lid. The drops were incubated at 37°C in an atmosphere containing 10% CO₂ for 10 d.

Immunohistochemistry

Frozen sections of the spheroids of colorectal cancer cells were fixed in acetone at -20°C for 10 min and rehydrated in PBS. Endogenous peroxidase was inactivated by immersing the sections in 0.3% hydrogen peroxide for 20 min. The primary antibody for frozen sections of the spheroids of colorectal cancer cells and paraffin-embedded sections of colorectal cancer tissue samples was a mouse anti-human monoclonal CD133/2 (1:40, Miltenyi Biotech, Germany) and a rabbit anti-human polyclonal CD133 (1:100, Abcam, England), respectively. The sections were incubated overnight at 4°C in a humidified chamber, then with biotinylated secondary antibody (VECTASTAIN ABC kit, Vector Laboratories) for 30 min at room temperature. Each section was incubated with the VECTASTAIN ABC reagent for 30 min at room temperature. The sections were developed using the DAB (Vector Laboratories) as the substrate and then counterstained with hematoxylin. The negative control was performed by incubating samples with PBS.

Quantification of CD133 expression by quantitative polymerase chain reaction

Total RNA was isolated from cultured colorectal cancer cells and their spheroids using the RNeasy extraction kit (GE Healthcare) and reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems) according to their manufacturer's instructions, respectively. Relative quantitative polymerase chain reaction (PCR) was performed on a 7300 fast real-time PCR system (Applied Biosystems) using SYBR green PCR master mix (Applied Biosystems). The human-specific intron spanning primer pairs for CD133 were provided by QIAGEN (Catalog number: QT00075586). The sequences of primer pairs used for GAPDH and Lgr5 are CAATGACCCCTTCATTGACC (forward) and TGATGACAAGCTTCCCGTTC (reverse), and CTTTCAACCTCAGCGTCTTC (forward) and TTTCCCGCAAGACGTAAGTTC (reverse), respectively. PCR was performed for 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specificity of PCR products was tested according to the dissociation curves. Relative values of transcripts were calculated using the equation: $2^{-\Delta\Delta C_t}$, where ΔC_t is equal to the difference in threshold cycles for target and reference.

Statistical analysis

Results were expressed as mean \pm SD for three repeated individual experiments in each group. Statistical analyses were conducted using the SPSS software (version 10.0).

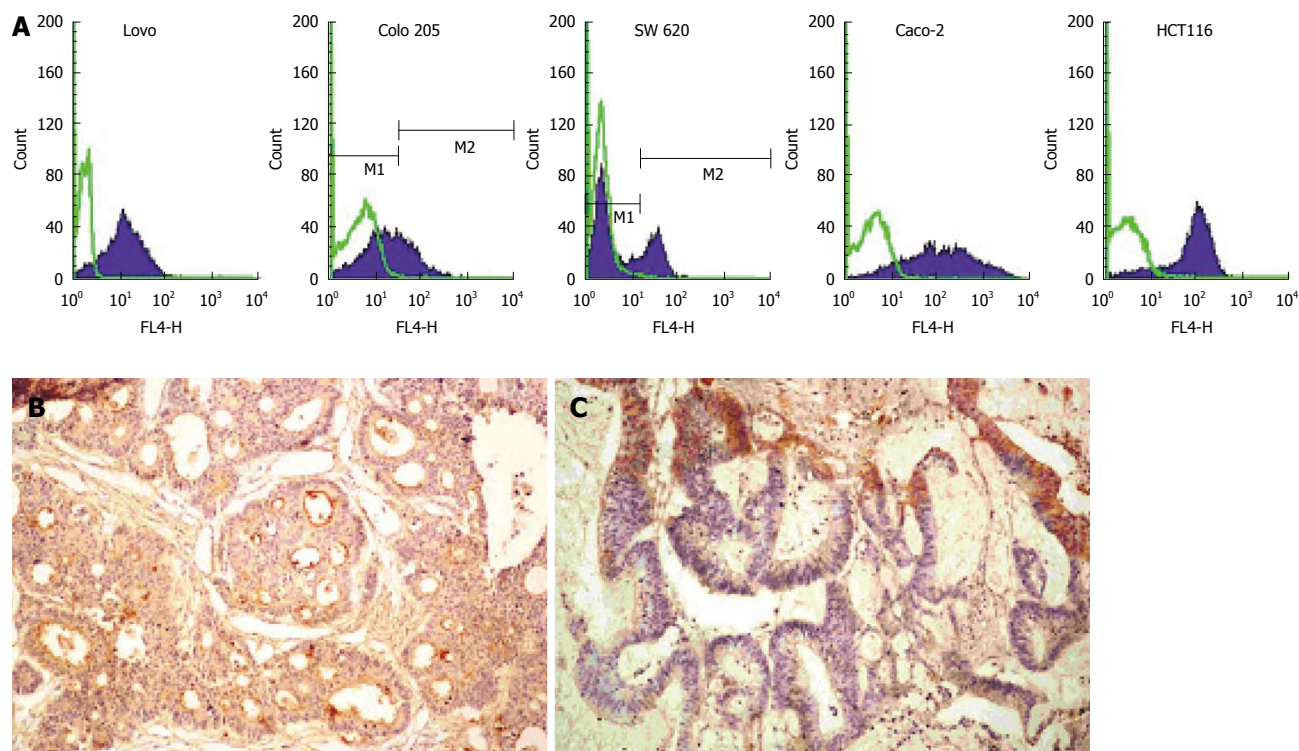


Figure 1 Fluorescence-activated cell sorting showing CD133 expression in different colorectal cancer cell lines (A), CD133 staining of human primary colorectal cancer tissue (B) and metastatic colorectal cancer tissue (C) (Original magnification $\times 100$). Brown indicates positive staining.

Correlation between sample groups and molecular variables was assayed with paired *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

CD133 expression in colon cancer cell lines and human colon cancer tissues

CD133 antigen was expressed in all colorectal cancer cell lines with a difference of 30%-95% (Figure 1A). CD133 in human colorectal cancer tissue samples was stained with polyclonal antibody. CD133 expression was detected in 18 of the 20 primary cancer tissue samples, exclusively on the membrane of the vast majority of colorectal cancer gland cells (Figure 1B), and in 9 of the 10 metastatic colorectal cancer tissue samples with positive staining in cytoplasm of cancer cells (Figure 1C).

CD133 expression in spheroids of sorted colorectal cancer cell subpopulations

To minimize the contamination between the sorted CD133⁺ and CD133⁻ cells, a high CD133 expression cell subpopulation (CD133^{hi}) and a CD133⁻ cell subpopulation sorted from the SW620 cells could be persistently passed. CD133 antigen was stably expressed in the monolayer culture (Figure 2A). To mimic the tumorigenesis of colorectal cancer cells *in vivo*, spheroids of the sorted cells were cultured with hanging drop. The parental, CD133^{hi} and CD133⁻ cells could grow into spheroids. CD133 expression was upregulated in spheroids of CD133⁻ cells. Although the CD133 expression rate was not changed,

the mean fluorescence intensity (MFI) was significantly increased in spheroids of CD133^{hi} cells, and the CD133 expression rate and MFI were significantly increased in spheroids of parental cells detected by FACS assay (Figure 2B). Immunohistochemical staining of CD133 antigen was observed in spheroids of CD133⁻ cells (Figure 2C). The CD133 gene expression level was significantly higher in spheroids of SW620, CD133^{hi} and CD133⁻ cells than in their monolayer culture at the mRNA level (4.224 ± 0.063 *vs* 2.680 ± 0.117 , 3.653 ± 0.061 *vs* 1.325 ± 0.044 , 8.746 ± 0.029 *vs* 3.761 ± 0.065 , $P < 0.05$) (Figure 2D).

Lgr5 expression in spheroids of sorted colorectal cancer cell subpopulations

Lgr5 expression was analyzed by RT-qPCR in order to observe the role of the expression of other colon stem cell genes in tumorigenesis of colorectal cancer cells. The results showed that the Lgr5 expression level was significantly higher in spheroids of parental, CD133^{hi} and CD133⁻ cells than in their monolayer cells (5.942 ± 0.091 *vs* 4.003 ± 0.039 , 6.611 ± 0.214 *vs* 3.645 ± 0.046 , 5.910 ± 0.035 *vs* 3.903 ± 0.083 , $P < 0.05$) (Figure 3).

DISCUSSION

Whether CD133 antigen can be used as a marker of colorectal cancer stem cells is still controversial. The focus is that CD133 expression is not restricted to just a small number of colorectal cancer cells. In this study, the CD133 expression was upregulated in colorectal cancer cell lines and primary or metastatic colorectal cancer tissue

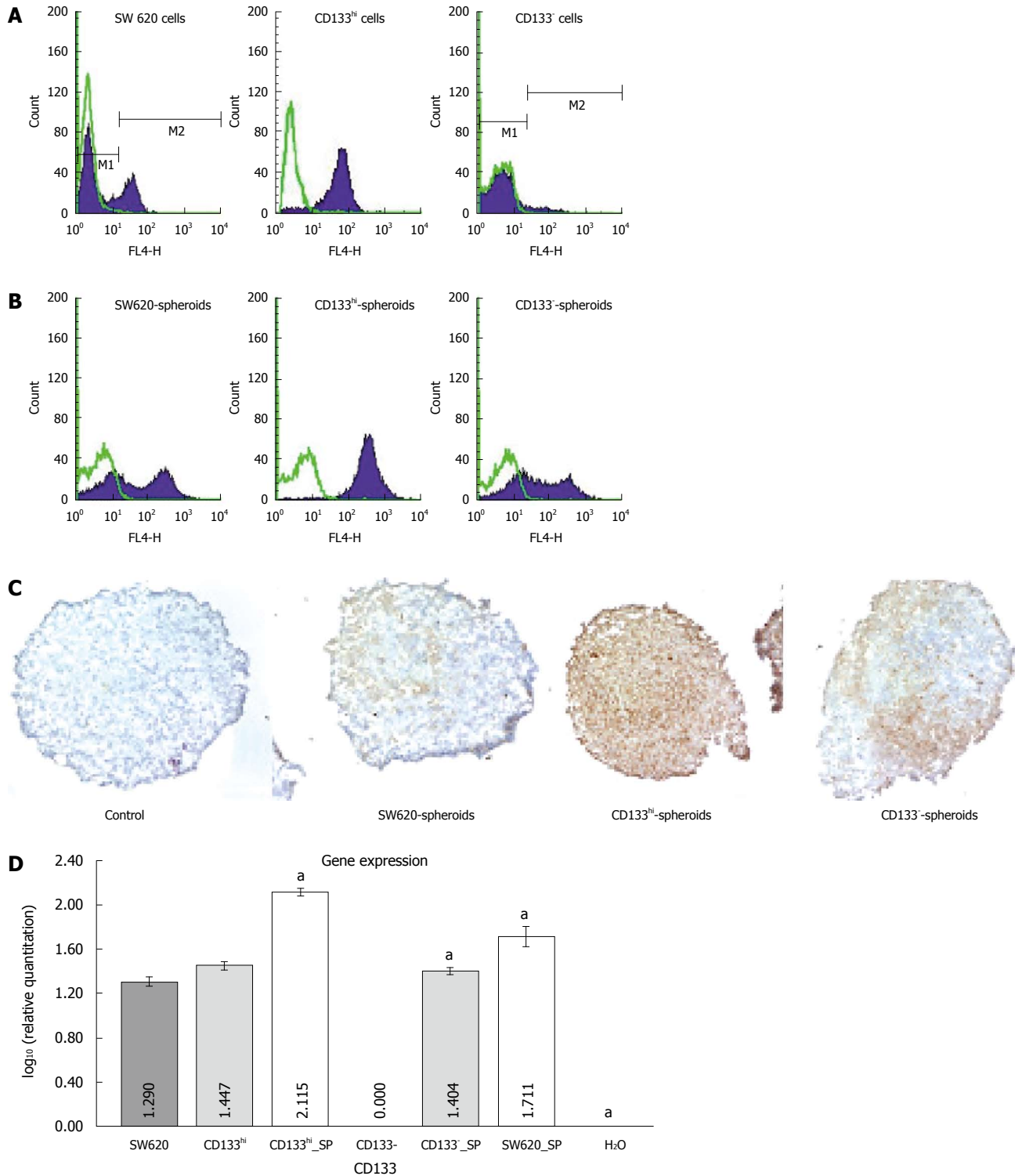


Figure 2 Fluorescence-activated cell sorting showing CD133 expression in SW620, CD133⁻ and CD133^{hi} cells (A) and in their spheroids (B), CD133 staining in spheroids of SW620, CD133⁻ and CD133^{hi} cells (original magnification $\times 100$, brown indicates positive staining) (C), and reverse transcription-polymerase chain reaction showing CD133 expression in SW620, CD133⁻ and CD133^{hi} cells and their spheroids. ^a $P < 0.05$ vs monolayer cells. SP: Spheroid.

samples, showing that CD133 antigen can be expressed in colorectal cancer cell lines with a difference of 30%-95%. CD133 expression was detected in 18 of the 20 primary colorectal cancer tissue samples, exclusively on the membrane of a large number of colorectal cancer gland cells, and in 9 of the 10 metastatic colorectal cancer tissue samples with a positive staining in cytoplasm of colorec-

tal cancer cells, which is consistent with the reported findings^[21-23]. The different CD133 expression levels in colorectal cancer cell lines may be related to the different glycosylation to the mask specific epitopes of CD133 antigen in colorectal cancer cell differentiation^[24]. Therefore, our data indicate that CD133 is commonly expressed in colorectal cancer cells.

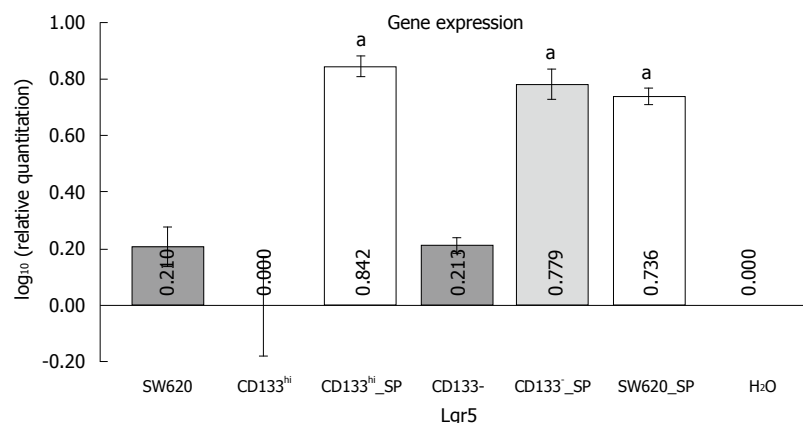


Figure 3 Quantitative reverse transcription-polymerase chain reaction showing Lgr5 expression in SW620, CD133^{hi} and CD133^{hi} cells and their spheroids. ^a*P* < 0.05 vs monolayer cells. SP: Spheroid.

To investigate whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, SW620 cell line containing two cell subpopulations (CD133^{hi}, CD133⁻) was selected and sorted using CD133 antigen, the spheroids of parental, CD133^{hi} and CD133⁻ cells were cultured with the hanging drop *in vitro*, which is based on the natural disposition of cells to aggregate without the need for polymer scaffolds such as matrigel, polyglycolic acid or microporous supports to achieve homogeneous multicellular tumor spheroids^[25]. The spheroids represent a popular *in vitro* 3D tissue structure that mimics *in vivo* tumor tissue organization and microenvironment^[26,27]. In the present study, CD133^{hi} and CD133⁻ cells could be cultured into their spheroids, CD133 expression was upregulated in spheroids of CD133⁻ cells. Although the CD133 expression was not changed, the mean fluorescence intensity (MFI) was significantly increased in spheroids of CD133^{hi} cells as detected by FACS assay. Immunohistochemical staining of CD133 antigen was observed in spheroids of CD133⁻ cells, indicating that CD133 antigen expression is upregulated in spheroids of CD133⁻ and CD133^{hi} cells. Further analysis revealed that the CD133 gene expression level was significantly higher in spheroids of SW620, CD133^{hi} and CD133⁻ cells than in their monolayer culture at the mRNA level, suggesting that the upregulated expression of CD133 including protein and gene plays a role in tumorigenesis of colorectal cancer cells.

Since the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, whether CD133 protein supports the growth of colorectal cancer is a subject that should be actively studied. As CD133 by itself may lack of a functional role in initiation of tumors and metastasis of human colorectal cancer^[28,29], it has an impact on the survival of colorectal cancer patients^[22,29]. It has been recently demonstrated that prominin 1 (also called CD133)-marked mouse intestinal stem cells are susceptible to neoplastic transformation^[30], possibly due to the fact that upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells. In this study, the expression level of Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5), also known as Gpr49, a colon stem cell marker

gene^[31], was significantly higher in spheroids of parental, CD133^{hi} and CD133⁻ cells than in their monolayer cells.

In conclusion, the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, which may be related to the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells. Further study is needed to confirm the present results *in vivo*.

ACKNOWLEDGMENTS

The authors thank Francesca Walker and Hui-Hua Zhang (Ludwig Institute, Melbourne) for their assistance to cell experiments and FACS studies, and You-Fang Zhang (Ludwig Institute, Melbourne) for his support in immunohistochemical studies.

COMMENTS

Background

It has been recently shown that CD133 expression is broadly distributed in primary colorectal cancer cells, and not restricted to cancer stem cells. Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown.

Research frontiers

It has been increasingly reported that CD133 is a marker of putative cancer stem cells (CSC) in some cancers. However, it has been recently shown that CD133 expression is broadly distributed in primary colon cancer cells and not restricted to cancer stem cells, and both CD133⁻ and CD133^{hi} metastatic colorectal cancer cells initiate tumors. Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown. In this study, the upregulated CD133 expression was found to play a role in tumorigenesis of colorectal cancer cells.

Innovations and breakthroughs

Recent reports have shown that whether CD133 antigen can be used as a marker of colorectal cancer stem cells is controversial. This is the first study to report the role of upregulated CD133 expression in tumorigenesis of colorectal cancer cells. Furthermore, our *in vitro* studies suggested that the upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells.

Applications

Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells was studied, the results may help to solve the controversy on CD133 antigen as a marker of colorectal cancer stem cells.

Terminology

CD133, also known as prominin-1, a transmembrane pentaspan protein, is originally described as a surface antigen specific for human hematopoietic stem

and progenitor cells. Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5), also known as Gpr49, is a colon stem cell marker gene.

Peer review

The authors detected the expression of CD133 in a panel of colorectal cancer cell lines and human colorectal cancer tissue samples. The expression of CD133 and Lgr5 in spheroids of the sorted colorectal cancer cell subpopulations suggests that the upregulated expression plays a role in tumorigenesis of colorectal cancer cells, which may promote the expression of other critical genes that can drive tumorigenesis. The results are interesting.

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Transplantation of microencapsulated umbilical-cord-blood-derived hepatic-like cells for treatment of hepatic failure

Fang-Ting Zhang, Hui-Juan Wan, Ming-Hua Li, Jing Ye, Mei-Jun Yin, Chun-Qiao Huang, Jie Yu

Fang-Ting Zhang, Hui-Juan Wan, Ming-Hua Li, Jing Ye, Mei-Jun Yin, Chun-Qiao Huang, Jie Yu, Central Laboratory, Peking University Shenzhen Hospital, Shenzhen 518036, China
 Author contributions: Zhang FT and Yu J designed the research; Zhang FT, Wan HJ, Li MH, Yin MJ, Ye J and Huang CQ performed the research; Zhang FT analyzed data; Zhang FT wrote the paper; Yu J edited the paper.

Supported by Guangdong Natural Science Foundation (9151 030002000008) and Shenzhen Science and Technology Planning Priority Program (JH200205270412B, 200808001, 2008 01012)

Correspondence to: Jie Yu, PhD, Central Laboratory, Peking University Shenzhen Hospital, 1120 Lian Hua Road, Shenzhen 518036, China. yujie007@hotmail.com

Telephone: +86-755-83923333 Fax: +86-755-83923333

Received: July 19, 2010 Revised: October 20, 2010

Accepted: October 27, 2010

Published online: February 21, 2011

Abstract

AIM: To investigate intraperitoneal transplantation of microencapsulated hepatic-like cells from human umbilical cord blood for treatment of hepatic failure in rats.

METHODS: CD34⁺ cells in umbilical cord blood cells were isolated by magnetic cell sorting. In the *in vitro* experiment, sorted CD34⁺ cells were amplified and induced into hepatic-like cells by culturing with a combination of fibroblast growth factor 4 and hepatocyte growth factor. Cultures without growth factor addition served as controls. mRNA and protein levels for hepatic-like cells were analyzed by reverse transcription-polymerase chain reaction, immunohistochemistry and immunofluorescence. In the *in vivo* experiment, the hepatic-like cells were encapsulated and transplanted into the abdominal cavity of acute hepatic failure (AHF) rats at 48 h after D-galactosamine induction of acute hepatic failure. Transplantation with PBS and unencapsulated hepatic-like cells served as controls. The mortality rate, hepatic pathological changes and serum

biochemical indexes were determined. The morphology and structure of microcapsules in the greater omentum were observed.

RESULTS: Human albumin, alpha-fetoprotein and GATA-4 mRNA and albumin protein positive cells were found among cultured cells after 16 d. Albumin level in culture medium was significantly increased after culturing with growth factors in comparison with culturing without growth factor addition ($P < 0.01$). Compared with the unencapsulated group, the mortality rate of the encapsulated hepatic-like cell-transplanted group was significantly lower ($P < 0.05$). Serum biochemical parameters, alanine aminotransferase, aspartate aminotransferase and total bilirubin in the encapsulated group were significantly improvement compared with the PBS control group ($P < 0.01$). Pathological staining further supported these findings. At 1-2 wk post-transplantation, free microcapsules with a round clear structure and a smooth surface were observed in peritoneal lavage fluid, surviving cells inside microcapsules were found by trypan blue staining, but some fibrous tissue around microcapsules was also detected in the greater omentum of encapsulated group by hematoxylin and eosin staining.

CONCLUSION: Transplantation of microencapsulated hepatic-like cells derived from umbilical cord blood cells could preliminarily alleviate the symptoms of AHF rats.

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Key words: Microencapsulation; Hepatic-like cells; Umbilical cord blood cells; CD34 antigen; Alginate; Acute hepatic failure

Peer reviewer: Toshihiro Mitaka, MD, Professor, Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University, 060-8556 Sapporo, Japan

Zhang FT, Wan HJ, Li MH, Ye J, Yin MJ, Huang CQ, Yu J. Transplantation of microencapsulated umbilical-cord-blood-derived hepatic-like cells for treatment of hepatic failure. *World*

J Gastroenterol 2011; 17(7): 938-945 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/938.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.938>

INTRODUCTION

Substantial efforts have been made with regard to cell transplantation as an effective supporting system for hepatic failure and assisted therapies. However, immunological rejection has always been an important problem for cell transplantation. Alginate-poly-L-lysine-alginate (APA) microcapsules have proven to be effective in protecting enclosed target cells from immune rejection following transplantation into experimental animals, thereby eliminating the problems of immunosuppressive therapy^[1-3].

Extensive studies have also been conducted on the core of this therapy, namely the cell sources. The investigated cells have included liver stem cells, embryonic stem cells, human umbilical cord blood (UCB) cells and bone marrow stem cells. Human UCB cells have some advantages that other cells do not have. The frequencies of UCB hematopoietic stem/progenitor cells exceed those from bone marrow and peripheral blood. In our previous study, we confirmed the differentiation of mononuclear cells (MNCs) from human UCB into hepatocytes in three different ways, namely co-culture with injured liver cells, growth factor-assisted culture, and MNC transplantation in animal models of liver injury^[4]. In the present study, we found that CD34⁺ cells derived from human UCB could be converted into hepatic-like cells that generate hepatocyte lineage cells. Furthermore, we encapsulated the hepatic-like cells using an alginate method and transplanted them into acute hepatic failure (AHF) rats to evaluate the effects of encapsulated hepatic-like cell transplantation.

MATERIALS AND METHODS

Isolation and identification of CD34⁺ cells

UCB (more than 80 samples) from full-term deliveries were obtained from the Obstetrics Department of Peking University Shenzhen Hospital. UCB cells were harvested after written informed consent was obtained. The study protocol was approved by the Ethics Committee of Peking University Shenzhen Hospital. MNCs were isolated from the UCB samples by density-gradient centrifugation at 2000 r/min for 35 min using Ficoll-Hypaque (Huaqing, Shanghai, China). CD34⁺ subpopulations were isolated using a Miltenyi Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The specific steps were as follows: (1) isolated MNCs were resuspended in a final volume of 300 μ L of PBS that contained 5 g/L bovine serum albumin (BSA); (2) 100 μ L of FcR Blocking Reagent and 100 μ L of CD34 Micro Beads per 1×10^8 total cells were sequentially added, mixed well and incubated for 30 min in a refrigerator at 4°C; (3) cells were passed through a magnetic column twice and purified; and (4) CD34⁺ cells were collected, resuspended in

Table 1 Primers used for reverse transcription-polymerase chain reaction

Gene	Primer (5'-3')		Amplicon (bp)
	Forward primer	Reverse primer	
ALB	CTTTCAAAGCAT-GGGCAGTAG	GCAGCAGCACGA-CAGAGTAA	411
GATA-4	ACCTGGGACTTG-GAGGATAG	GACAAGGACATCTT-GGGAAA	250
AFP	TGAGCACTGTTG-CAGAGGAG	CTGAGACAG-CAAGCTGAGGA	308

ALB: Albumin; AFP: α -fetoprotein.

100 μ L PBS, incubated with 10 μ L CD34-phycoerythrin for 10 min at 4°C and identified by flow cytometry.

Differentiation in vitro

Freshly isolated CD34⁺ cells were primarily cultured in Dulbecco's modified Eagle's medium - low glucose (DMEM-LG, Gibco, Carlsbad, CA, USA), amplified for 3-5 d with a combination of 12.5 μ g/mL thrombopoietin (TPO) (R&D Systems, Minneapolis, MN, USA), 50 ng/mL stem cell factor (SCF) (R&D Systems) and 50 ng/mL Flt-3 (R&D Systems); then induced into hepatic-like cells by culturing in DMEM-LG that contained 50 mL/L fetal bovine serum (Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin, 4.7 μ g/mL linoleic acid, 1×10^{-4} mol/L L-ascorbic acid 2-P supplemented with 100 ng/mL fibroblast growth factor (FGF)4 (R&D Systems) and 20 ng/mL hepatocyte growth factor (HGF; Sigma, St. Louis, MO, USA). CD34⁺ cells were incubated in 24-well plates at 37°C in a 5% CO₂ atmosphere. Culture medium was replaced every 3 d. Cultured cells were collected after 8 and 16 d. Cultures without growth factors served as controls.

Total mRNA isolation and reverse transcription-polymerase chain reaction

Total mRNA was extracted from collected cells using Trizol (Mrcgene, Cincinnati, OH, USA). mRNA was reverse-transcribed and the resulting cDNA was amplified using the primer sets shown in Table 1 and a RobusT I reverse transcription-polymerase chain reaction (RT-PCR) Kit (Finnzymes, Espoo, Finland). Reverse transcriptase reaction was run at 48°C for 45 min and PCR was initiated with pre-denaturation at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, annealing at 58°C for 30 s and extension at 72°C for 30 s, with 72°C for 7 min for final extension. The PCR products were separated on a 1.2% agarose gel.

Immunocytochemistry for CD34⁺ cells

Cytospins prepared from cells were fixed with 4% paraformaldehyde and 0.15% picric acid in PBS at room temperature for 20 min, then permeabilized and blocked with 10% goat serum and 0.1% Triton X-100 in PBS at room temperature for 10 min. The cells were sequentially incu-

bated with a mouse anti-human albumin antibody (R&D Systems) for 30 min, a biotinylated peroxidase-conjugated secondary antibody (Zymed, South San Francisco, CA, USA) for 10 min, and diaminobenzidine for 10 min. Between the above steps, cells were washed with 0.1 mol/L PBS that contained 1 g/L BSA.

Albumin determination

Culture media were collected for the quantitative determination of human albumin by ELISA using a Human Albumin ELISA Kit (Alpha Diagnostics International, San Antonio, TX, USA) according to the manufacturer's instructions.

Cell encapsulation

Cells collected after 16 d induction were washed with PBS and resuspended in the alginate. The alginate-cell mixture was passed through a microcapsule generator and extruded into 40 mL 1.1% CaCl_2 solution. The airflow rate was adjusted for the regulation of the microcapsule diameter between 300 and 800 μm . The capsules and CaCl_2 solution were then transferred to 50-mL conical tubes. After removal of the supernatant, the capsules were gently mixed with the wash solution and allowed to settle for 2 min. Before transplantation, a few drops of encapsulated cells were placed on a slide, stained with 0.4% Trypan blue, covered with a cover glass and lightly pressed to force cells out of the microcapsules. Numbers of living cells were counted and expressed as percentages.

Induction of AHF and cell transplantation

Sprague-Dawley rats were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). The Scientific Committee at Peking University Shenzhen Hospital approved the use of animals for experimental purposes. Forty-eight hours before transplantation, the Sprague-Dawley rats (weight: 180–250 g) were intraperitoneally injected at 1.4 g/kg with a 10% D-galactosamine solution in normal saline. On the day of the experiment, microencapsulated cells at a density of 2×10^6 cells/mL were prepared and transplanted into the abdominal cavity of rats. Transplantation with PBS only or unencapsulated hepatocyte-like cells were performed for the establishment of control groups. As UCB samples are not delivered on the same day, animal experiments were carried out by batch and the transplantation of cells performed also on different days. The mortality rate, hepatic pathological changes and serum biochemical indexes were determined.

AHF rats grouping

We obtained total 135 AHF rats 48 h after injection of D-galactosamine. They were divided into three groups on the day of the transplantation. Namely, encapsulated group (transplantation with encapsulated hepatic-like cells, $n = 55$), unencapsulated group (transplantation with unencapsulated hepatic-like cells, $n = 40$), PBS group (transplantation with PBS, $n = 40$). Among these, 76 AHF rats were determined for hepatic pathological changes and

serum biochemical indexes (encapsulated group, $n = 36$; unencapsulated group, $n = 20$; PBS group, $n = 20$). The remaining 59 rats were determined for mortality rate (encapsulated group, $n = 19$; unencapsulated group, $n = 20$; PBS group, $n = 20$).

Histology

The liver and greater omentum from all three groups were fixed in 4% buffered formaldehyde overnight. After paraffin embedding, 4–5- μm thick serial sections were stained with hematoxylin and eosin (HE) and observed under the light microscope.

Statistical analysis

Data were expressed as the mean \pm SD. Mortality rate analysis was determined by Fisher's exact test. Serum biochemical index statistical analysis was performed by ANOVA using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Differences with P values < 0.05 were considered statistically significant.

RESULTS

Differentiation of CD34⁺ cells into hepatic-like cells

Approximately 3×10^5 – 9×10^5 /mL sorted cells were obtained using the CD34 immunomagnetic bead method, and 91% of them expressed CD34 by flow cytometry analysis (Figure 1). CD34⁺ cells were firstly amplified 20-fold by a combination of TPO, SCF and Flt-3, and then they were cultured with HGF and FGF4. At 16 d, they developed larger volumes, richer cytoplasts, and binucleated structures, as observed under a Hoffman microscope (Figure 2). The RT-PCR showed no human albumin, α -fetoprotein (AFP) and GATA-4 mRNA expression in CD34⁺ cells before the induction procedure. The expression of albumin and GATA-4 mRNA increased with the culture time after the addition of growth factors, whereas the amount of AFP mRNA expression peaked after 8 d and reduced at 16 d (Figure 3). Cells that expressed albumin and AFP were verified by immunocytochemical staining and ELISA (Figures 2 and 4). The percentage of albumin- and AFP-positive cells at 16 d was 30% and 24%, respectively. The albumin product in culture medium was significantly increased after culturing with HGF and FGF4 in comparison with control groups ($P < 0.01$).

Cell encapsulation and transplantation

The APA microencapsulation technique was used to encapsulate hepatic-like cells. The percentage of living cells was $> 80\%$, as determined by trypan blue staining. The AHF animal model was successfully established using Sprague-Dawley rats by the injection of D-galactosamine. Pathological section of the AHF liver revealed that the structure of the hepatic lobules was destroyed and the hepatic cord was disordered, with large areas of denatured and necrotic hepatocytes, and infiltrating lymphocytes were found on the portal area at 48 h after injection. On the day of the experiment, microencapsulated cells at a density of 2×10^6 cells/mL were prepared and transplanted.

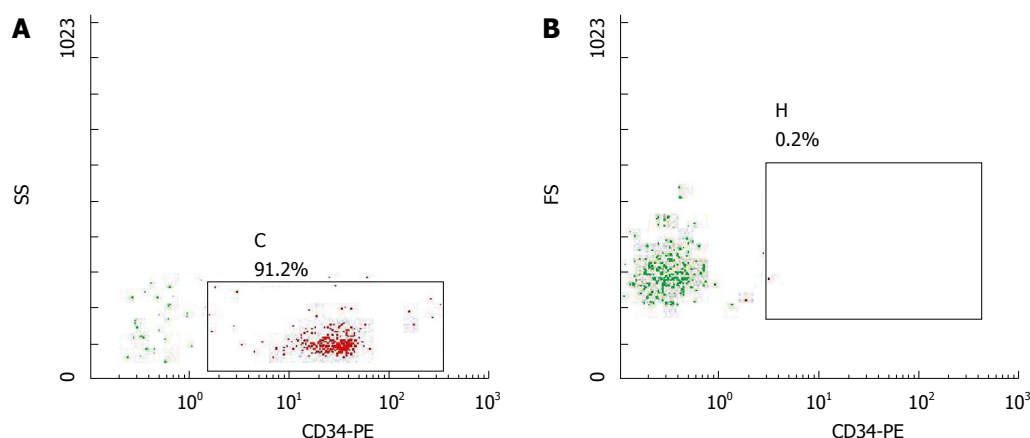


Figure 1 FACS determination of CD34⁺ cells. A: Purity of CD34⁺ cells; B: Homotypic control cells.

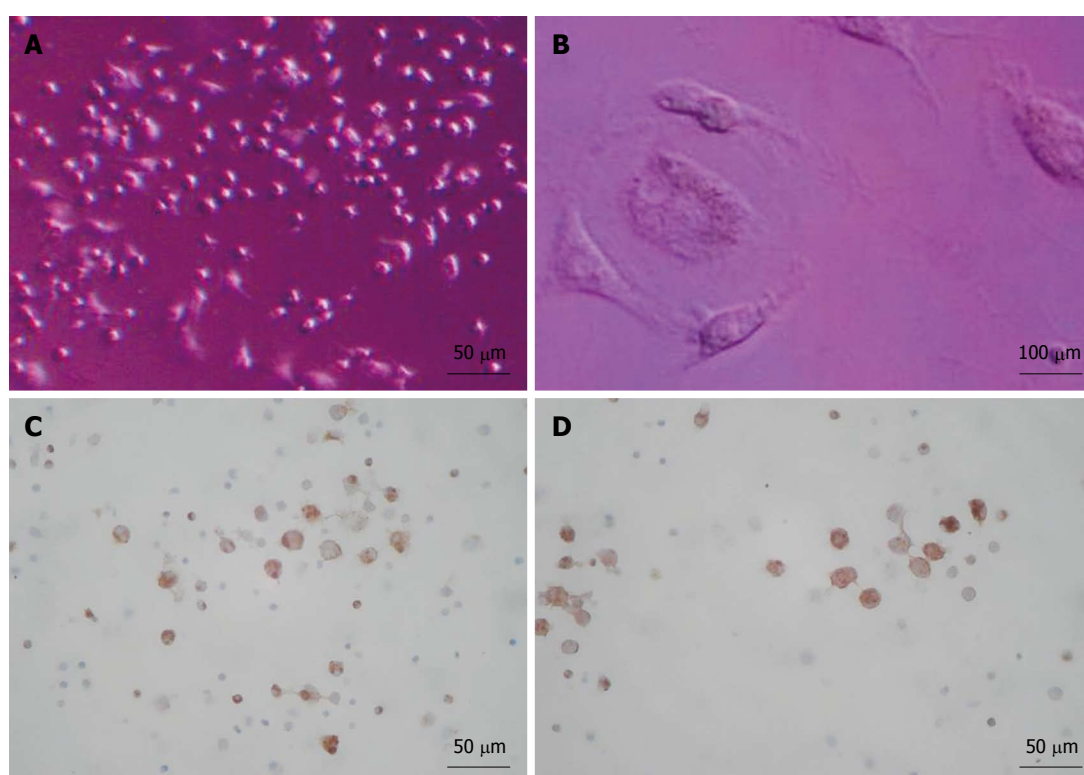


Figure 2 Cell culture and analyses. A: After 16 d; B: A binucleated cell; C, D: Positive staining for albumin (C) and α -fetoprotein (D) after 16 d of induction.

ed into the abdominal cavity of AHF rats. The mortality rate and hepatic pathological changes were determined. At 48 h after transplantation, HE staining of the encapsulated group revealed that the hepatic lobules were still intact; denaturation was the major change in hepatocytes and the area of necrosis nidus was small, and congestion and hemorrhage were almost undetectable (Figure 5). The mortality rate at 48 h after transplantation in three groups was 42.1% (encapsulated group), 65% (unencapsulated group) and 75% (PBS group), respectively. Compared with the unencapsulated group, the mortality rate of the encapsulated group was significantly lower ($P < 0.05$). In addition, the serum biochemical indexes of ALT, AST and total bilirubin in the microencapsulated group differed significantly from those in the PBS group ($P < 0.01$)

at 48 h after transplantation, but there were no differences between the encapsulated and the unencapsulated group (Table 2). At 1-2 wk post-transplantation, free microcapsules with a round clear structure and a smooth surface were observed in peritoneal lavage fluid, surviving cells in microcapsules were found by trypan blue staining, but some fibrous tissues around microcapsules were also detected in the greater omentum of encapsulated group by HE staining (Figure 6).

DISCUSSION

With the continued increase in people with hepatic failure from cirrhosis and hepatocarcinoma, cell transplantation as an effective therapy is becoming a matter of concern

Table 2 Changes in serum biochemical indexes at different times

	48 h after injection D-GaIN	48 h after transplantation			7 d after transplantation		
	All 3 groups	Encapsulated group	Unencapsulated group	PBS group	Encapsulated group	Unencapsulated group	PBS group
ALT (U/L)	3242.3 ± 2403.24	93.93 ± 63.45 ^b	126.1 ± 54.35	245.9 ± 67.87	42.25 ± 11.86	45.07 ± 10.56	47.27 ± 11.08
AST (U/L)	4237.20 ± 1372.07	168.87 ± 89.33 ^b	275.7 ± 52.74	439.7 ± 133.01	162.6 ± 54.29	124.52 ± 24.61	114.83 ± 16.50
TBIL (μmol/L)	5.57 ± 1.86	1.73 ± 1.01 ^a	2.23 ± 1.98	3.50 ± 1.23	1.90 ± 0.52	2.72 ± 0.96	3.72 ± 1.18

Data are shown as means ± SD. ^a $P < 0.05$; ^b $P < 0.01$, in comparison with PBS group. TBIL: total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

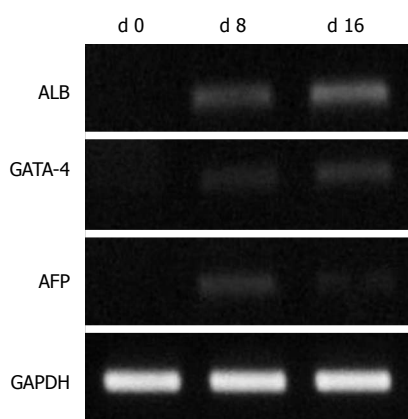


Figure 3 Reverse transcription-polymerase chain reaction analysis of umbilical cord blood CD34⁺ cells cultured *in vitro* d 0, d 8 and d 16. ALB: Albumin; AFP: α -fetoprotein.

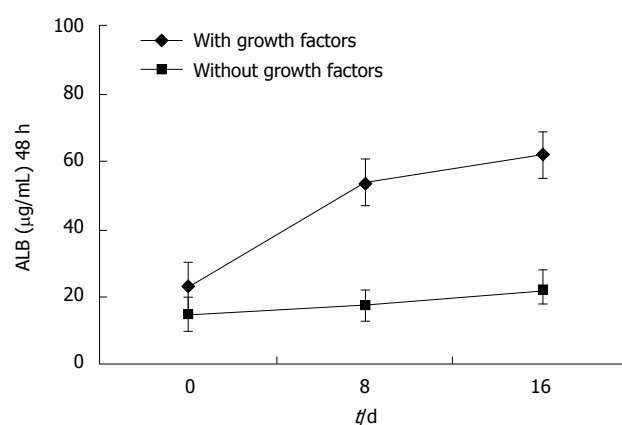


Figure 4 Determination of albumin expression by Enzyme-Linked Immunosorbent Assay. ALB: Albumin.

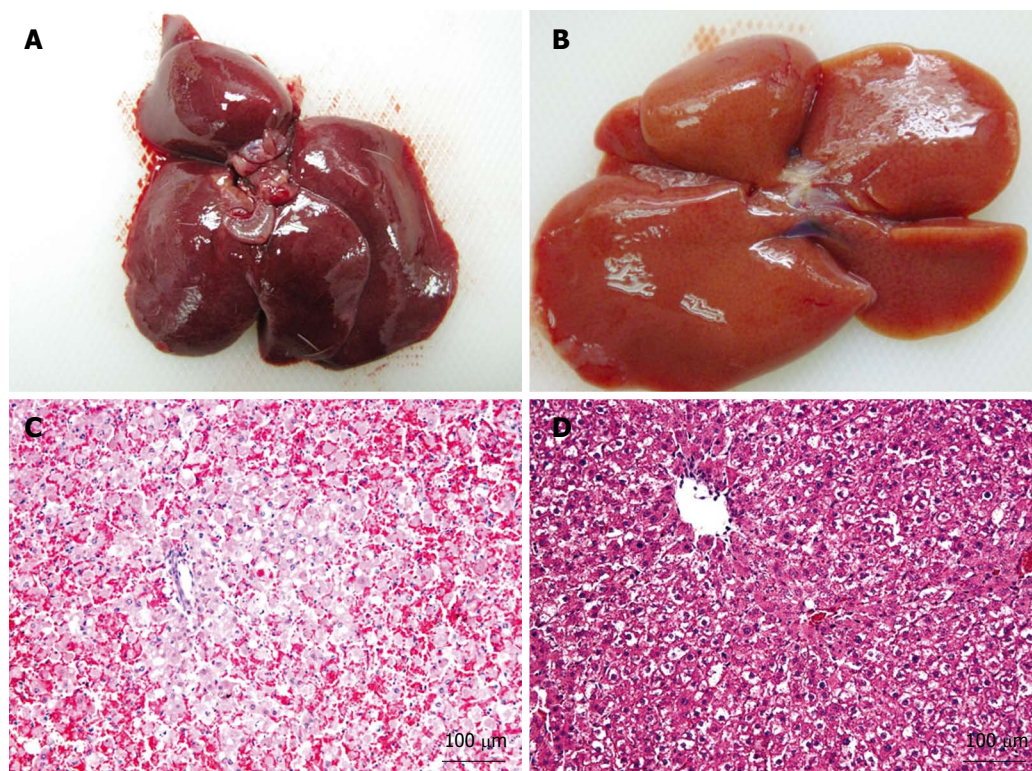


Figure 5 Pathological changes in the livers of acute hepatic failure rats. A: Liver at 48 h after injection of D-galactosamine; B: Liver at 48 h after microcapsule transplantation; C: HE staining of the liver shown in section (A); D: HE staining of the liver shown in section (B).

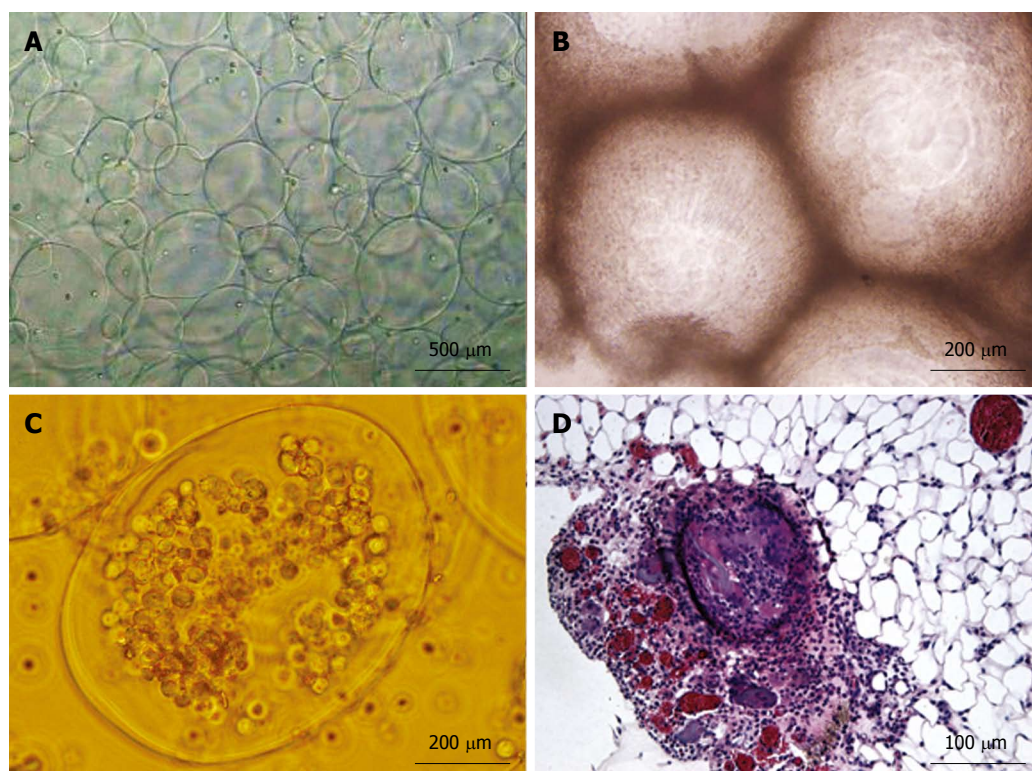


Figure 6 Encapsules observation. A: Microcapsules created by the Alginate-poly-L-lysine-alginate microencapsulation method; B: Microcapsule masses in the peritoneal lavage fluid; C: Free microcapsules in the peritoneal lavage fluid; D: HE staining shows microcapsules in the greater omentum.

for more scientists. Cell transplantation could offer metabolic support when liver function is damaged, and extend the waiting time for a liver donor^[5,6]. Hepatic cell transplantation via the peritoneum or spleen has shown good prospects in clinical and animal experiments. However, the cell sources for transplantation and the requirement for long-term immunosuppression have caused stagnation in this field.

There have been some intriguing studies that have described adult stem cells displaying plasticity in recent years. These studies have led us to consider that using adult stem cells might cure diseases such as AHF^[7,8]. Human UCB cells are enriched in hematopoietic stem/progenitor cells that exceed those in the bone marrow and peripheral blood. In comparison with bone marrow stem cells, UCB stem cells are even more immature and with lower immunogenicity. In our previous study, we confirmed that the conversion of UCB MNCs into hepatocytes by three different ways, namely co-culture with injured liver cells, growth-factor-assisted culture, and MNC transplantation in AHF animal models^[4]. In the present study, we explored the possibility that CD34⁺ cells derived from human UCB could be converted into hepatic-like cells. At present, the curative effect of hepatic-like cells derived from CD34⁺ cells in the bone marrow has already been confirmed by *in vivo* animal experiments^[9-12]. This showed that an AHF model was initially set up using immunodeficient mice, and CD34⁺ cells enriched by immunobeads were injected through the tail vein or portal vein into the model animals. Expression of differentiation markers of donor cells in

recipient livers at different times after transplantation was determined by fluorescence *in situ* hybridization, immunohistochemistry and molecular biological techniques. It was found that stress-induced signals, such as increased expression of stromal-cell-derived factor 1, matrix metalloproteinase-9 and HGF, recruits human CD34⁺ progenitors with hematopoietic and/or hepatic-like potential to the liver of NOD/SCID mice^[13]. Furthermore, another study has confirmed that FGF, leukemia inhibitory factor, SCF, HGF, FGF4 and oncostatin M contribute to the proliferation and/or differentiation of hepatic cells in different ways, and that combinations of these factors, especially HGF and FGF4, are necessary for human UCB cells to convert into albumin-producing cells^[14].

With a combination of HGF and FGF4, we have established a 16-d culture system to induce CD34⁺ cell differentiation. The culture system with HGF and FGF4 displays the capability to convert the CD34⁺ cells from human UCB into cells with hepatocyte phenotypes, as confirmed by RT-PCR, immunohistochemical staining, and ELISA. Moreover, the positive ratio of albumin-containing cells by immunocytochemical staining was about 30%, which is consistent with the study of Kakinuma *et al*^[14]. All these indicate that after proliferation and differentiation, we could obtain many transplantable hepatic-like cells.

Although the lower immunogenicity of UCB stem cells has advantages in heterogenic transplantation, untreated UCB cells can sometimes cause serious immune rejection. How to resolve this problem is therefore a key point for further studies. Microencapsulation offers a

possibility to overcome the difficulty. This technique uses microcapsules such as APA microcapsules to coat target cells or organs, and is beneficial for heterogenic transplantation because its biocompatible and semi-permeable membranes are capable of intercepting substances with molecular weights above 11×10^4 . Since Lim *et al.*^[15] first presented the concept of bio-microcapsules in 1980, artificial cell microcapsules as an effective barrier system for immunoprotection have been successfully applied in diabetes, parkinsonism, spinal cord injury, and peripheral nerve regeneration^[15,16].

Our study examined coated hepatic-like cells derived from UCB by the APA microencapsulation technique. The obtained microcapsules exhibited a good smooth surface and integrated appearance. Furthermore, living cells inside the microcapsules were $> 80\%$ as determined by trypan blue staining. The mortality rate of AHF rats transplanted with microencapsulated hepatic-like cells significantly decreased in comparison with AHF rats transplanted with unencapsulated cells. In addition, there were significantly better outcomes in serum biochemical indexes such as ALT, AST and total bilirubin in the encapsulated group than in the PBS group, but no differences were observed between the encapsulated and the unencapsulated groups. Liver pathological staining supported these findings. The reason why the latter two groups showed no difference requires further exploration, although it is possibly related to the lower number of encapsulated cells. There have been some studies to support the notion that microcapsules provide the encapsulated cells with a good living space, and can significantly increase their survival time, therefore, we could theoretically reduce the number of transplanted cells^[17]. Our data suggest that the transplantation of microencapsulated hepatic-like cells could offer a metabolic support to AHF rats in the short term, but it is not sufficient to interrupt or repair the damage of the recipient hepatocytes.

In our study, the pathological staining clearly showed liver recovery at 7 d after induction of AHF with D-galactosamine. At 2 wk post-transplantation, the morphological form of free microcapsules could be observed in the peritoneal lavage fluid, and showed round clear structures and smooth surfaces, and some microcapsule fragments were observed as well. HE staining revealed that some microcapsules attached to the greater omentum exhibited lymphocyte invasion surrounded with fibrous tissues. Although transplantation of microencapsulated hepatic-like cells could preliminarily alleviate the symptoms of AHF rats, their short lifespan and varying stability are still problems for the further use of the technique. The improvement in the airflow encapsulation system might be considered to yield sufficient uniformity in the size of microcapsules^[18].

Transplantation of microencapsulated cells could provide a temporary metabolic support to AHF patients and/or be a transitional treatment, because its mechanism is not only related to the immunosuppressive and substitution effects of the transplanted cells, but is also associated

with liver repair promoted by the transplanted cells. This new approach could provide a potential alternative for severe liver diseases.

COMMENTS

Background

With the continued increase in people with hepatic failure from cirrhosis and hepatocarcinoma, cell transplantation could offer metabolic support when liver function is damaged, and extend the waiting time for a liver donor. However, the cell sources for transplantation and the requirement for long-term immunosuppression have caused stagnation in this field.

Research frontiers

Alginate-poly-L-lysine-alginate (APA) microcapsules have been proved effective in protecting enclosed target cells from immune rejection following transplantation into experimental animals. Many studies have been conducted on the cell sources such as liver stem cells, embryonic stem cells, umbilical cord blood (UCB) cells and bone marrow stem cells.

Innovations and breakthroughs

The research team led by Professor Yu has established an artificial cell microcapsules platform, which is based on APA microcapsule technology and stem cell differentiation, to study the therapeutic effects of intraperitoneal transplantation of microencapsulated hepatic-like cells derived from UCB cells on AHF in rats. The effective immunoprotectivity of artificial cell microcapsules has been observed in this study, which suggests that the transplantation of microencapsulated hepatic-like cells could offer a metabolic support to AHF rats in the short term, but it is not yet sufficient to interrupt or repair the damage of the recipient hepatocytes.

Applications

Transplantation of microencapsulated cells could provide a temporary metabolic support to AHF patients and/or be used as a transitional treatment. This new approach could provide a potential alternative for severe liver diseases.

Terminology

UCB was obtained from full-term deliveries at the Obstetrics Department of Peking University Shenzhen Hospital. Hepatic-like cells were induced from UCB CD34⁺ cells by culturing with FGF4 and HGF. Alginate-poly-L-lysine-alginate microcapsules have biocompatibility and semi-permeable membranes, and can intercept substances with molecular weights $> 1.1 \times 10^5$.

Peer review

Zhang *et al.* reported that CD34⁺ cells sorted from human UCB cells were cultured for 16 d in a specific medium and could differentiate into hepatocyte-like cells. When the hepatocyte-like cells were encapsulated by alginate and intraperitoneally transplanted into rats with galactosamine-induced AHF, the number of surviving rats increased compared to that of control rats at 2 d after transplantation. Although the differentiation of CD34⁺ cells derived from UCB to hepatocyte-like cells has been reported, it is interesting to use the peritoneal injection of alginate-encapsulated hepatocyte-like cells for the alleviation of AHF. If the preserved UCB cells are used for the treatment of AHF and related diseases, it will be beneficial to the patients.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Primary clear cell carcinoma in the liver: CT and MRI findings

Qing-Yu Liu, Hai-Gang Li, Ming Gao, Xiao-Feng Lin, Yong Li, Jian-Yu Chen

Qing-Yu Liu, Ming Gao, Xiao-Feng Lin, Yong Li, Jian-Yu Chen, Department of Radiology, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China

Hai-Gang Li, Department of Pathology, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China

Author contributions: Liu QY designed the study and wrote the manuscript; Gao M, Li Y and Chen JY contributed to the analysis and interpretation of data; Li HG performed the pathological analysis; Lin XF contributed to the statistical analysis.

Correspondence to: Qing-Yu Liu, PhD, Department of Radiology, The Second Affiliated Hospital of Sun Yat-sen University, 107 Yan Jiang Xi Road, Guangzhou 510120, Guangdong Province, China. liu.qingyu@163.com

Telephone: +86-20-81332243 Fax: +86-20-81332702

Received: August 29, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: February 21, 2011

Abstract

AIM: To retrospectively analyze the computed tomography (CT) and magnetic resonance imaging (MRI) appearances of primary clear cell carcinoma of the liver (PCCCL) and compare the imaging appearances of PCCCL and common type hepatocellular carcinoma (CHCC) to determine whether any differences exist between the two groups.

METHODS: Twenty cases with pathologically proven PCCCL and 127 cases with CHCC in the Second Affiliated Hospital of Sun Yat-sen University were included in this study. CT or MRI images from these patients were retrospectively analyzed. The following imaging findings were reviewed: the presence of liver cirrhosis, tumor size, the enhancement pattern on dynamic contrast scanning, the presence of pseudo capsules, tumor rupture, portal vein thrombosis and lymph node metastasis.

RESULTS: Both PCCCL and CHCC were prone to occur in patients with liver cirrhosis, the association rate of liver cirrhosis was 80.0% and 78.7%, respectively ($P >$

0.05). The mean sizes of PCCCL and CHCC tumors were (7.28 ± 4.25) cm and (6.96 ± 3.98) cm, respectively. Small HCCs were found in 25.0% (5/20) of PCCCL and 19.7% (25/127) of CHCC cases. No significant differences in mean size and ratio of small HCCs were found between the two groups ($P = 0.658$ and 0.803 , respectively). Compared with CHCC patients, PCCCL patients were more prone to form pseudo capsules (49.6% vs 75.0%, $P = 0.034$). Tumor rupture, typical HCC enhancement patterns and portal vein tumor thrombosis were detected in 15.0% (3/20), 72.2% (13/18) and 20.0% (4/20) of patients with PCCCL and 3.1% (4/127), 83.6% (97/116) and 17.3% (22/127) of patients with CHCC, respectively. There were no significant differences between the two groups (all $P > 0.05$). No patients with PCCCL and 2.4% (3/127) of patients with CHCC showed signs of lymph node metastasis ($P > 0.05$).

CONCLUSION: The imaging characteristics of PCCCL are similar to those of CHCC and could be useful for differentiating these from other liver tumors (such as hemangioma and hepatic metastases). PCCCLs are more prone than CHCCs to form pseudo capsules.

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Key words: Clear cell carcinoma; Hepatocellular carcinoma; Pathology; Magnetic resonance imaging; Computed Tomography; X-ray

Peer reviewers: C Triantopoulou, MD, PhD, Head of Radiology Department, Konstantopouleio general Hospital, 3-5, Agias Olgas street, 14233 N. Ionia, Athens, Greece; Hiroshi Yoshida, Associate Professor, Department of Surgery, Nippon Medical School Tama Nagayama Hospital, 1-7-1 Nagayama, Tama-city, Tokyo, 206-8512, Japan

Liu QY, Li HG, Gao M, Lin XF, Li Y, Chen JY. Primary clear cell carcinoma in the liver: CT and MRI findings. *World J Gastroenterol* 2011; 17(7): 946-952 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/946.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.946>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. It can be classified according to its histological architecture or cytological features. HCC includes various cytological types; the less common ones are clear cell type, spindle cell type, giant cell type, small cell type and squamous cell type^[1,2]. Primary clear cell carcinoma of the liver (PCCCL) is rare, with a frequency varying between 2.2% and 6.7% among HCCs reported in the published literatures^[3,4]. Due to the accumulation of glycogens and/or fats, the PCCCL cell cytoplasm is clear to hematoxylin-eosin staining. PCCCL may pose a diagnostic dilemma even with histological evaluation because the morphology of PCCCL cells is similar to that of extrahepatic clear cell tumors, such as clear cell cancers of the kidneys, adrenal glands, ovaries, thyroid, endometrium, uterine cervix, and vagina^[5,6]. PCCCLs should be differentiated from metastatic clear cell cancer because their treatment strategies and prognoses are quite different. The prognosis of PCCCL is generally considered better than that of the common type of HCC (CHCC)^[3,7,8].

Computed tomography (CT) and magnetic resonance imaging (MRI) are important examinations for the detection and characterization of liver tumors^[9,10]. To our knowledge, the imaging features of PCCCL have rarely been reported in the English literature^[11]. The purpose of this study was to describe the CT and MRI findings of PCCCL and compare them to CHCC to determine whether any differences exist between the two groups.

MATERIALS AND METHODS

Patients

Between January 2005 and August 2009, a total of 570 patients with primary HCC underwent hepatectomy at the Second Affiliated Hospital of Sun Yat-sen University. Twenty (3.5%) of these patients had pathologically confirmed PCCCL. The participants of this study included 20 patients with PCCCL and 127 patients with CHCC (randomly selected from the other 550 cases of primary HCC). No patient had received preoperative treatment, such as interventional therapy or chemotherapy.

Of the 20 patients with PCCCL, 14 had right upper abdominal pain, two complained of fatigue and four were asymptomatic. All patients with PCCCL were positive for HBsAg, and two were positive for anti-hepatitis C virus-IgG. The serum concentration of α -fetoprotein (AFP) was 5.8-68 787.0 $\mu\text{g/L}$ for PCCCL patients, with a median of 149.9 $\mu\text{g/L}$. Of the 20 patients with PCCCL, 17 were AFP-positive ($> 25 \mu\text{g/L}$).

Pathologic examinations were retrospectively reviewed by an experienced pathologist. According to diagnostic criteria generally accepted by pathologists in China, PCCCL was diagnosed when clear cells accounted for more than 50% of the tumor^[1,3,4,12].

Imaging protocols

CT or MRI examinations were performed no more than 5

days before hepatectomy. Thirteen patients with PCCCL and 73 patients with CHCC underwent dynamic CT examination using a spiral CT scanner (HiSpeed NX/I; GE Medical Systems, Milwaukee, WI) or a multi-detector CT scanner (Sensation 64; Siemens Medical Solutions, Erlangen, Germany). The scan parameters were as follows: 5-7 mm slice thickness reconstructions, 120-kV, 220-400 mA current, 25 cm field of view, and 256×256 matrix. Scans began at the dome of the diaphragm and proceeded in a caudal direction. After pre-contrast CT scans, the patients underwent dynamic contrast-enhanced scans. A bolus injection of 80-100 mL of non-ionic contrast medium (Iopamidol, Bracco, Milano, Italy) with a concentration of 350 mg I/mL was given via the antecubital vein at a rate of 3.5 mL/s. Images of the hepatic arterial phase (HAP), portal venous phase (PVP) and equilibrium phase (EP) were obtained at 25 s, 70 s and 120 s, respectively, after the injection of contrast agent.

Seven patients with PCCCL and 54 patients with CHCC underwent MRI studies with a 1.5-T MR unit (Gyrosan Intera, Philips Medical System, Best, the Netherlands). Unenhanced MR images included T1-weighted images with a water-selective excitation technique (FFE, TR 218ms, TE 4.9 ms, flip angle of 80, one acquisition) and turbo spin-echo T2-weighted images with fat saturation (TR 1600 ms, TE 70 ms, TSE Factor 24, three acquisitions). Five patients with PCCCL and 43 patients with CHCC underwent dynamic contrast-enhanced MR scans using a high-resolution turbo spin-echo sequence (TR 5.3 ms, TE 1.4 ms, flip angle of 40, 3.0-mm slice thickness, no gap, one acquisition) via a power injector; contrast agent was administered at a rate of 2.5 mL/sec. HAP, PVP and EP scans were obtained at 20, 60, and 110 s, respectively. The other 13 patients (2 with PCCCL and 11 with CHCC) received manual injections of gadopentetate dimeglumine (Magnevist, Bayer Schering, Berlin, Germany) at a dose of 0.1 mmol/kg; post-contrast T1-weighted images were obtained at PVP (60-80 s after injection) with the same scanning parameters as the pre-contrast T1W scan. Regardless of the technique employed, axial and coronal images were acquired with 5.0-mm slice thickness.

Image interpretation

The CT and MRI images were retrospectively analyzed by two radiologists who have 10 and 15 years of experience in diagnosing abdominal diseases. Neither radiologist was aware of the patients' clinicopathological data. Reviews were performed jointly and by consensus. The presence of liver cirrhosis, tumor size, the enhancement pattern on dynamic contrast scanning, the presence of pseudocapsule, tumor rupture, portal vein thrombus, and lymph node metastasis were recorded. A typical HCC enhancement pattern was defined as early enhancement at HAP and rapid contrast medium washout at PVP or EP with hypo-attenuation/intense signal or iso-attenuation/intense signal^[9,10].

Statistical analysis

Differences in mean age and tumor size were assessed

with an independent-samples *t* test. Differences in the frequencies of liver cirrhosis, tumor capsule formation, tumor rupture, typical enhancement pattern, portal vein tumor thrombus and lymph node metastases between the two groups were compared using the Chi-squared test or Fischer's exact test. A *P* value of 0.05 or less was considered significant. Statistical analysis was performed using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

The male-to-female ratio was 4.0:1 in the PCCCL group and 6.1:1 in the CHCC group. The mean age was 52.00 ± 10.09 years (range, 29-66 years) in the PCCCL group and 51.82 ± 13.20 years (range, 19-83 years) in the CHCC group. There were no statistical differences between the two groups regarding sex or age ($P = 0.733$ and $P = 0.953$, respectively).

Table 1 summarizes the imaging features observed in patients with PCCCL and patients with CHCC. Both PCCCL and CHCC were prone to occur in patients with liver cirrhosis, with a rate of 80.0% and 78.7%, respectively. The mean sizes of PCCCLs and CHCCs were 7.28 ± 4.25 cm (range, 2.0-15.9 cm), and 6.96 ± 3.98 cm (range, 1.0-17.0 cm), respectively. Small HCCs with diameters ≤ 3.0 cm were found in 25.0% (5/20) of PCCCL cases and 19.7% (25/127) of CHCC cases. No statistically significant differences in mean size or ratio of small HCC were found between the two groups ($P = 0.658$ and 0.803 , respectively). Compared with CHCCs, PCCCLs were more prone to form pseudo capsules, with a rate of 49.6% and 75.0%, respectively ($P = 0.034$). Pseudo capsules showed hypo-attenuation/intensity haloes on pre-contrast scans and rim enhancement after contrast administration (Figures 1 and 2).

A higher percentage of tumor rupture was found in patients with PCCCL (15.0%, 3/20) than in patients with CHCC (3.1%, 4/127); however, there was no significant difference between the two groups ($P > 0.05$). Of the 20 PCCCL cases, three showed tumor ruptures. The ruptured tumors were 15.9 cm, 10.9 cm and 9.3 cm in diameter and were located at the periphery of the liver with protruding contours. Two cases presented as discontinuities of the liver surface on CT scan (Figure 1). The remaining case presented a local hematoma at the rupture site on MRI, which appeared as mixed iso-/hypo-intense signals on T1WI and hypo-intense signals on T2WI with no enhancement after injection of contrast agent.

Typical HCC enhancement patterns were noted in 72.2% (13/18) of PCCCLs and 83.6% (97/116) of CHCCs; however, no significant difference was found between the two groups ($P > 0.05$) (Figures 1 and 3). The other five PCCCL cases showed atypical CT features on dynamic scan: two cases showed minimal enhancement and remained hypo-attenuated at HAP and PVP, while the other three cases showed gradual contrast enhancement during the portal phase.

Four patients (20.0%) with PCCCL had portal vein tumor thrombosis: one located at the left branch of the portal vein, one at the right branch, and one at the right

Table 1 Characteristics of clear cell hepatocellular carcinoma in the liver

Parameters	PCCCL (<i>n</i> = 20)	CHCC (<i>n</i> = 127)	<i>P</i> value
Sex			0.733
Male	16	109	
Female	4	18	
Liver cirrhosis			1.000
Positive	16	100	
Negative	4	27	
Tumor diameter (cm)			0.803
≤ 3.0	5	25	
> 3.0	15	102	
Capsule formation			0.034
Positive	15	63	
Negative	5	64	
Rupture			0.053
Positive	3	4	
Negative	17	123	
Typical enhancement pattern			0.399
Positive	13	97	
Negative	5	19	
Portal vein tumor thrombus			1.000
Positive	4	22	
Negative	16	105	
Lymph node metastases			1.000
Positive	0	3	
Negative	20	124	

PCCCL: Primary clear cell carcinoma of the liver; CHCC: Common type of hepatocellular carcinoma.

anterior branch and main portal vein. Compared with CHCC patients, PCCCL patients showed a slightly higher incidence of portal vein tumor thrombosis (17.3% and 20.0%, respectively); however, there was no significant difference between the two groups ($P > 0.05$). No PCCCL patients and 2.4% (3/127) CHCC patients showed sign of lymph node metastasis ($P > 0.05$).

DISCUSSION

PCCCL is a specific and rare subtype of primary HCC. The reported incidence of PCCCL is 0.4%-37%; inconsistent diagnostic criteria may be responsible for the variable reports^[1,3,4,7,8,12,13]. Lai *et al*^[7] suggested that the diagnosis of PCCCL could be made even when the proportion of clear cells was $< 30\%$, while Buchanan *et al*^[8] suggested that PCCCL should be diagnosed when the proportion of clear cells was $> 30\%$. Most studies diagnosed PCCCL when the proportion of clear cells was $> 50\%$ ^[1,3,4,12]. Using this criteria, PCCCL only accounts for 2.2%-6.7% of all resectable HCCs in most reports^[3,4]. Among the 570 cases of primary HCC resected in our hospital, only 3.5% patients had PCCCL. The clear cell development is presumed to involve metabolic disorders and abnormalities of sugar metabolism^[14,15].

The clinicopathological presentations of PCCCL were different from those of CHCC. The rates of hepatitis C infection and capsule formation were higher in PCCCL patients than in those with CHCC; however, no remarkable differences in patients' age, sex, AFP-positive rate or

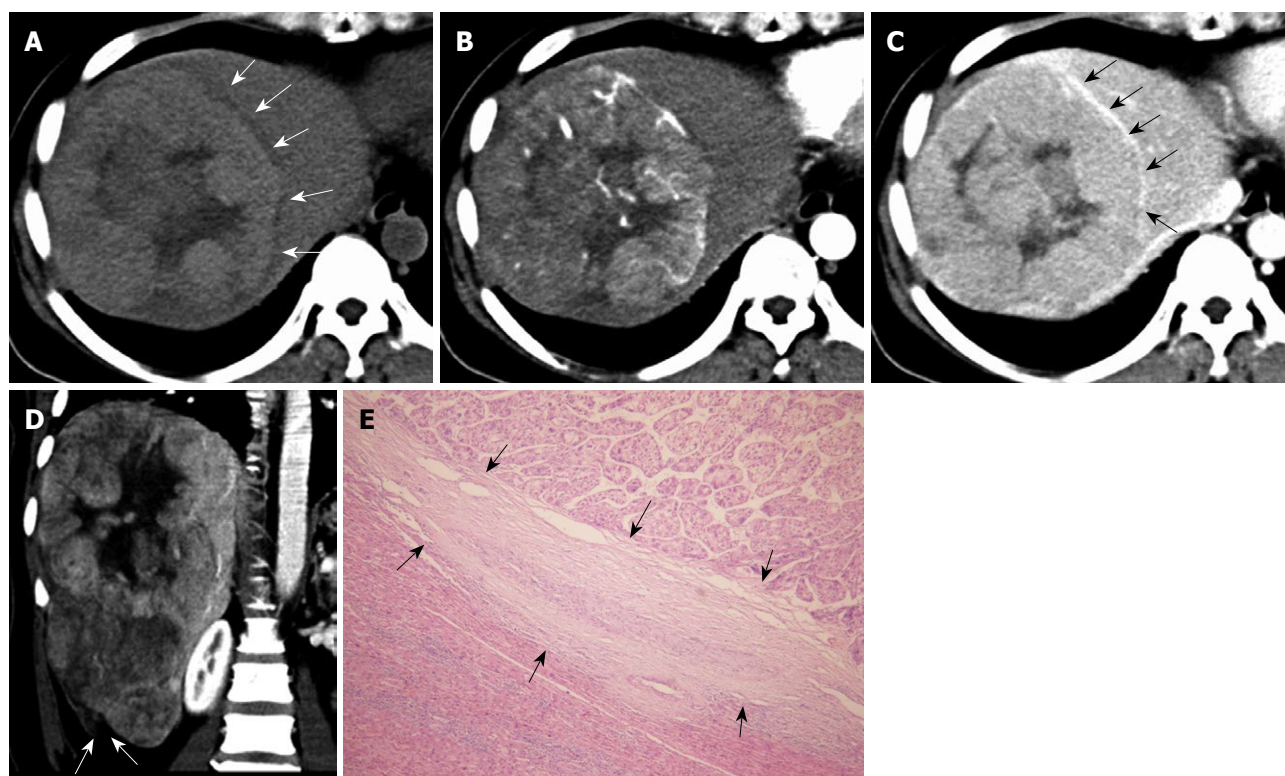


Figure 1 Primary clear cell carcinoma of the liver in a 47-year-old woman. A: On pre-contrast computed tomography scan, the mass shows slight hyper-attenuation with a hypo-attenuation halo (arrows); B: At hepatic arterial phase, the mass shows early enhancement; C: At the equilibrium phase, the mass presents hypo-attenuation with rim enhancement (arrows); D: At portal venous phase, the reconstructed coronal image shows the mass with a discontinuous liver capsule (arrows) at Segment VI, indicating tumor rupture, which was surgically confirmed; E: Pathologically, the mass shows a pseudocapsule (arrows) (HE, $\times 100$).

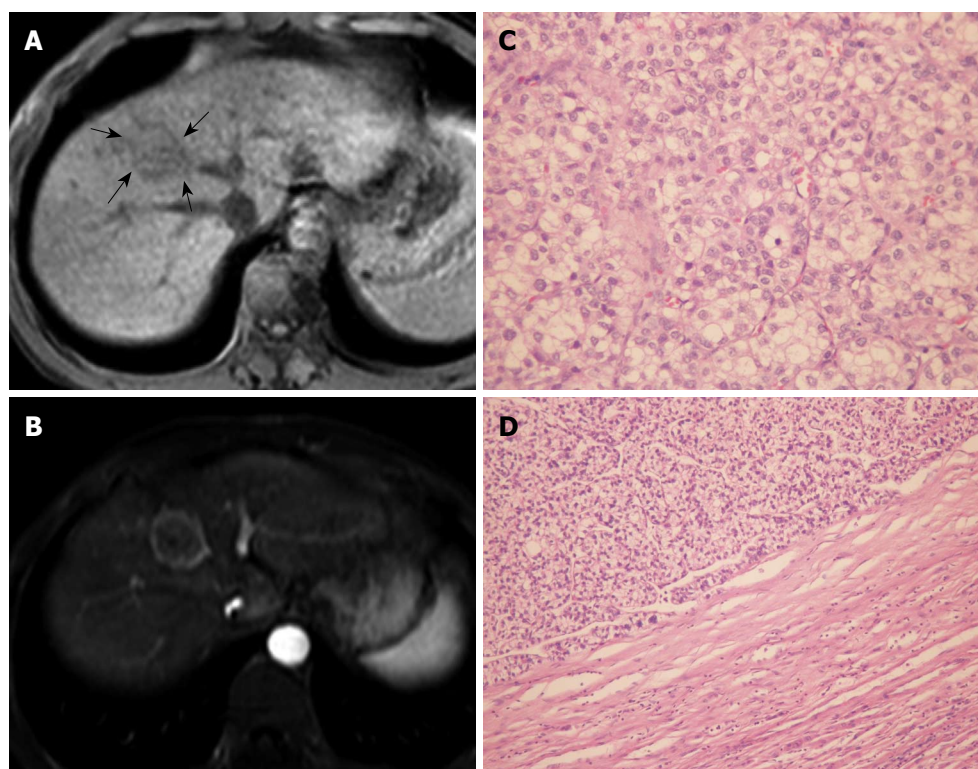


Figure 2 Primary clear cell carcinoma of the liver in a 29-year-old man. A: On T1WI, the mass shows slightly hypo-intense signals (arrows); B: At portal venous phase, the mass presents with rim enhancement (pseudocapsule); C: Pathologically, the mass is mainly composed of clear cells (HE, $\times 200$); D: Pathologically, the mass shows a pseudocapsule (HE, $\times 100$).

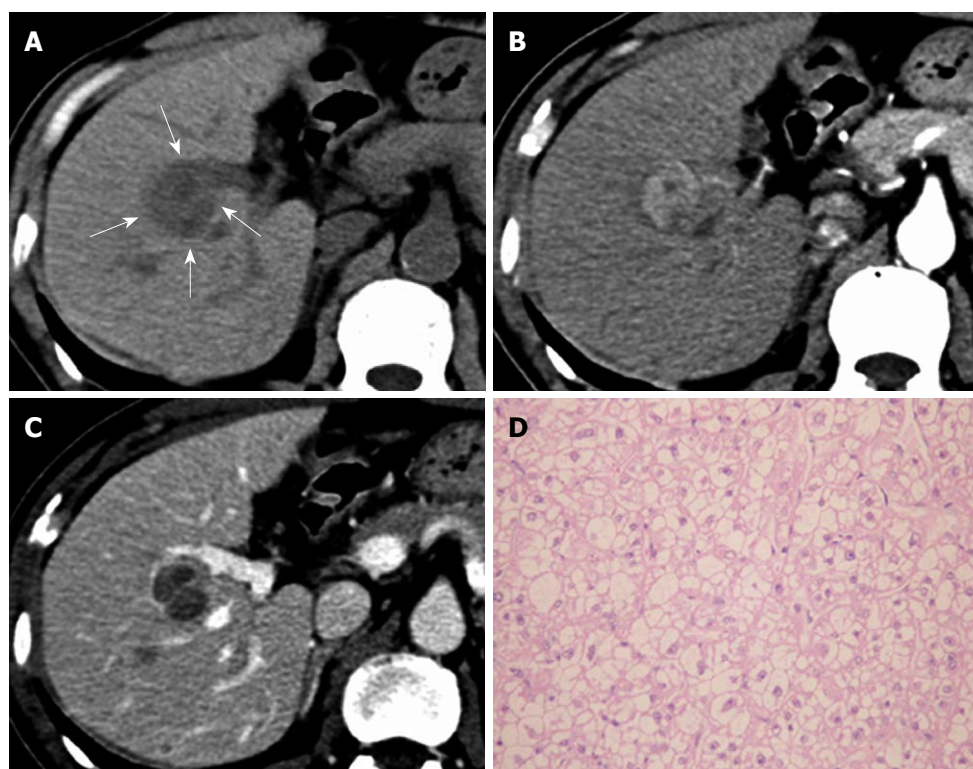


Figure 3 Primary clear cell carcinoma of the liver in a 62-year-old man. A: On pre-contrast computed tomography scan, the mass shows hypo-attenuation (arrows); B: At hepatic arterial phase, the mass shows early enhancement; C: At portal venous phase, the mass shows hypo-attenuation and thin rim enhancement (pseudo-capsule); D: Microscopically, the mass is mainly composed of clear cells (HE, $\times 200$).

the location, number, size and grade of tumors were observed between the two groups^[3]. Both tumor types were prone to occur in patients with hepatitis B, mostly on the basis of liver cirrhosis^[3]. PCCCL had a better prognosis than CHCC, mainly related to capsule formation, vascular invasion, preoperative liver function and clear cell proportion^[3,4,12]. Surgical resection is an effective treatment for patients with PCCCL^[3,4,7].

The presence of clear cells and fatty changes characterizes well-differentiated HCC in the early stage, and their ratio is presumed to decrease as the tumor enlarges^[15]. In 1999, Monzawa *et al*^[16] analyzed the pathologic and imaging changes of well-differentiated HCC; and found that some well-differentiated HCCs showed clear cell formation and/or fatty changes, which presented as high echo on ultrasound and hyper-intense signals on T1WI. However, in their study, the proportion of clear cells in the recruited HCC was less than 10%, or only 10%-50%, which did not meet the diagnosis criteria for PCCCL. In 2008, Takahashi *et al*^[11] described CT, MR and angiographic findings of PCCCL in a woman with a normal liver. To our knowledge, no further research on the imaging manifestations of PCCCL has been conducted.

Pseudocapsule formation (consisting mainly of peritumoral hepatic sinusoids and/or fibrosis) is an important gross pathologic feature of HCC. Pseudocapsule indicates a relatively positive prognosis after tumor resection^[17]. Liu *et al*^[3] found a higher ratio of pseudocapsule formation in PCCCL than in CHCC microscopically (88.4% *vs* 68.0%, $P < 0.05$); and pseudocapsule formation might be related

to a relatively lower degree of malignancy and a better prognosis for PCCCL. CT and MRI are reliable imaging examinations for the detection of HCC pseudo capsules. The pseudocapsule presents as rim enhancement on dynamic contrast scanning, and MRI is more sensitive than CT in identifying pseudocapsule^[17-19]. Among the 20 cases of PCCCL in our study, 15 (75.0%) had pseudocapsule, all of which were confirmed pathologically. The percentage of pseudocapsule formation was higher in PCCCL patients than in CHCC patients ($P < 0.05$).

Because of hypervascular blood supply, typical HCC showed early enhancement at HAP, and rapid contrast medium washout at PVP or EP with hypo-attenuation/intense signal or iso-attenuation/intense signal^[9,10]. Among the 18 PCCCL cases in our study that underwent dynamic contrast CT or MRI examination, 13 presented a typical HCC enhancement pattern, indicating that the tumor is rich of blood supply. The enhancement pattern of PCCCL is not different from that of CHCC ($P > 0.05$). This imaging characteristic may be useful in differentiating PCCCL from other liver tumors, such as hemangioma and hepatic metastases. The other five PCCCL cases presented atypical enhancement on dynamic CT scans: two cases showed minimal enhancement with hypo-attenuation at HAP and PVP, indicating hypovascularity, and three cases showed gradual contrast enhancement during the portal phase, which may be attributable to the difference in blood supply (such as existence of small arteriportal shunts), tumor differentiation or liver cirrhosis background^[20,21].

Spontaneous rupture of HCC is usually life-threatening

ing but relatively uncommon, with a reported incidence of 3%-15%^[22]. CT is a valuable imaging technique for diagnosing HCC ruptures. The imaging findings include: discontinuity or disruption of the liver capsule adjacent to the liver mass and hematoma with hyper-attenuation at the rupture site. The enucleation sign is a specific sign for diagnosing HCC rupture^[23,24]. To our knowledge, no report on PCCCL rupture is available for review. Among the 20 PCCCL cases in our study, only three had tumor rupture: two showed discontinuity of the liver capsule on CT scans, and the other showed a hematoma at the rupture site on MRI, with iso-/hypo-intense signals on T1WI and hypo-intense signals on T2WI.

Portal vein thrombosis, the characteristic growth pattern of HCC, occurs in 12.5%-39.7% of HCC patients^[25]. Liu *et al*^[3] reported that the microscopic vascular invasion rates are similar between PCCCL and CHCC (53.4% *vs* 65.0%, $P > 0.05$). In our study, the incidence of macroscopic portal vein tumor thrombus in PCCCL and CHCC detected on imaging examination was not significantly different ($P > 0.05$). Portal vein invasion was an independent risk factor for the prognosis of patients with PCCCL^[12].

Chemical shift imaging is valuable for characterizing lesions with a mixture of water and fat^[26]. Renal clear cell carcinomas usually contain fat, and present focal and diffused signal loss on chemical shift imaging. This imaging technique is helpful for differentiating renal clear cell carcinoma from other types of renal cancer^[27,28]. The cell morphology of PCCCL is similar to that of renal clear cell carcinoma, with cytoplasmic accumulation of glycogens and/or fat. The signal reduction of HCC during chemical shift imaging may help identify intratumoral fatty components and confirm a diagnosis of PCCCL^[2].

In summary, the imaging characteristics of PCCCL are similar to those of CHCC, including early enhancement and rapid washout of contrast agent on dynamic contrast scans, and presence of portal vein thrombus or tumor rupture. These imaging features may help differentiate PCCCL from other liver tumors, such as hemangioma and hepatic metastases. Pseudocapsule formation is more likely to occur in PCCCL than in CHCC and may be related to PCCCL's relatively lower degree of malignancy and better prognosis.

COMMENTS

Background

Primary clear cell carcinoma of the liver (PCCCL) is a specific and rare subtype of primary hepatocellular carcinoma (HCC), with a frequency varying between 2.2% and 6.7% among HCCs in the published literatures. PCCCL may pose a diagnostic dilemma even with histological sections because the morphology of PCCCL cells is similar to that of metastatic clear cell tumors. As a result of the paucity of cases, available data about its imaging findings are limited.

Research frontiers

Imaging modalities [computed tomography (CT) and magnetic resonance imaging (MRI)] are important for the detection and characterization of liver tumors. The imaging characteristics of common type hepatocellular carcinoma (CHCC) are well documented; for example, CHCC is usually associated with liver cirrhosis, typical enhancement pattern on dynamic contrast scanning (early enhancement at hepatic arterial phase and rapid contrast medium washout at portal venous phase or equilibrium phase) and the presence of pseudocapsule. However, the imaging features of PCCCL have not been unequivocally addressed. This study clarifies the CT or MRI findings of PCCCL.

Innovations and breakthroughs

The authors presented 20 surgically confirmed PCCCL cases and retrospectively analyzed their imaging findings. This study revealed that the imaging characteristics of PCCCL are similar to those of CHCC. PCCCLs are more likely to form pseudo capsules than CHCCs.

Applications

With a better understanding of the imaging features of PCCCL, further investigations should determine how to use imaging modalities, especially MRI, to differentiate PCCCL from CHCC or metastatic clear cell cancer. Chemical shift imaging with an MR scanner may help detect lipid component in the cytoplasm of clear cells in PCCCL.

Terminology

PCCCL is a rare variant of HCC. Due to the accumulation of large amounts of glycogen and/or lipids that are dissolved by routine histological processing (hematoxylin-eosin staining), the cytoplasm of PCCCL cells is clear. PCCCL can be diagnosed when the tumor cells are predominantly or wholly composed of clear cell cytoplasm (a proportion of clear cells > 50%). The prognosis of PCCCL is generally considered better than that of the CHCC.

Peer review

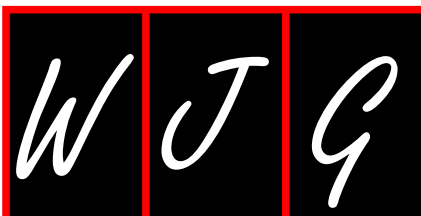
It is a well written paper, with interesting results.

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S- Editor Tian L L- Editor Ma JY E- Editor Lin YP



ACKNOWLEDGMENTS

Acknowledgments to reviewers of World Journal of Gastroenterology

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Shahab Abid, Dr., Associate Professor, Department of Medicine, Aga Khan University, Stadium Road, PO Box 3500, Karachi 74800, Pakistan

Hussein M Atta, MD, PhD, Department of Surgery, Faculty of Medicine, Minia University, Mir-Aswan Road, El-Minia 61519, Egypt

Huijie Bian, Professor, Vice-Director, Department of Cell Biology/Cell Engineering Research Center, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China

Alberto Biondi, Dr., PhD, Department of Surgery, 1st Surgical Division, Catholic University of Rome, Largo A. Gemelli 8, Rome 00168, Italy

Hoon Jai Chun, MD, PhD, AGAF, Professor, Department of Internal Medicine, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, 126-1, Anam-dong 5-ga, Seongbuk-gu, Seoul 136-705, South Korea

Laurie DeLeve, Dr., University of Southern California Keck School of Medicine, 2011 Zonal Avenue-HMR603, LA 90033, United States

AM El-Tawil, MSc, MRCS, PhD, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor, Birmingham, B15 2TH, United Kingdom

Giammarco Fava, MD, Department of Gastroenterology, Università Politecnica delle Marche, Ancona, via Gervasoni 12, 60129 Ancona, Italy

Fritz Francois, Dr., Assistant Dean for Academic Affairs and Diversity, Assistant Professor of Medicine, New York University School of Medicine, 423 E. 23rd St. Room 1132N, New York, NY 10010, United States

Beata Jolanta Jabłońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

Waliul Khan, MBBs, PhD, Assistant Professor, Department of Medicine, McMaster University, Room 3N5D, Health Science Center, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

Hong Joo Kim, MD, Professor, Department of Internal Medicine,

Sungkyunkwan University Kangbuk Samsung Hospital, 108, Pyung-Dong, Jongro-Ku, Seoul, 110-746, South Korea

Kirk Ludwig, M.D., Associate Professor of Surgery, Chief of Colorectal Surgery, Department of Surgery, Medical College of Wisconsin, 9200 West Wisconsin Avenue, Milwaukee, Wisconsin, WI 53226, United States

Eli Magen, Allergy and Clinical Immunology, Medicine B, Barzilai Medical Center, Ashdod 77456, Israel

Ricardo Marcos, Ph.D., Lab Histology and Embryology, Institute of Biomedical Sciences Abel Salazar, ICBAS, Lg Prof Abel Salazar, 2, Porto, 4099-003, Portugal

Luca Morelli, MD, UO, Dr., Anatomy and Histology, Ospedale S. Chiara, Largo Medaglie d'Oro 9, Trento, 38100, Italy

Tor C Savidge, PhD, Associate Professor, Department of Gastroenterology & Hepatology, Galveston, TX 77555, United States

Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PAN-SINI, 5, Naples 80131, Italy

Cesare Tosetti, MD, Department of Primary Care, Health Care Agency of Bologna Via Rosselli 21, 40046 Porretta Terme (BO), Italy

Evangelos Tsiambas, MD, PhD, Cytopathologist, Lecturer in Molecular Cytopathology, Department of Pathology, Medical School, University of Athens, Ag Paraskevi Attiki, 15341, Greece

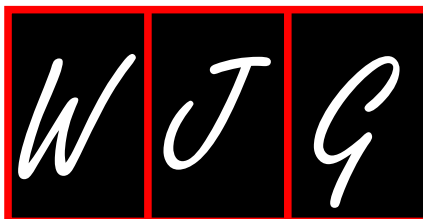
Masahito Uemura, MD, Associate Professor, Third Department of Internal Medicine, Nara Medical University, Shijo-cho, 840, Kashihara, Nara 634-8522, Japan

Lea Veijola, Consultant Gastroenterologist, Herttoniemi Hospital, Health Care of City of Helsinki, Kettutie 8, Helsinki, 00800, Finland

Steven D Wexner, MD, Professor of Surgery, The Cleveland Clinic Foundation Health Sciences Center of the Ohio State University, and Clinical Professor, Department of Surgery, Division of General Surgery, University of South Florida College of Medicine, 21st Century Oncology Chair in Colorectal Surgery, Chairman Department of Colorectal Surgery, Chief of Staff, Cleveland Clinic Florida, 2950 Cleveland Clinic Boulevard, Weston, FL 33331, United States

George Y Wu, Professor, Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, United States

Satoshi Yamagiwa, MD, PhD, Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata, 951-8510, Japan



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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Name of journal

World Journal of Gastroenterology

Serial publication number

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Issue with no volume

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No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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World Journal of Gastroenterology®

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February 28, 2011



Published by Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
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ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

ISSN 1007-9327 (print)
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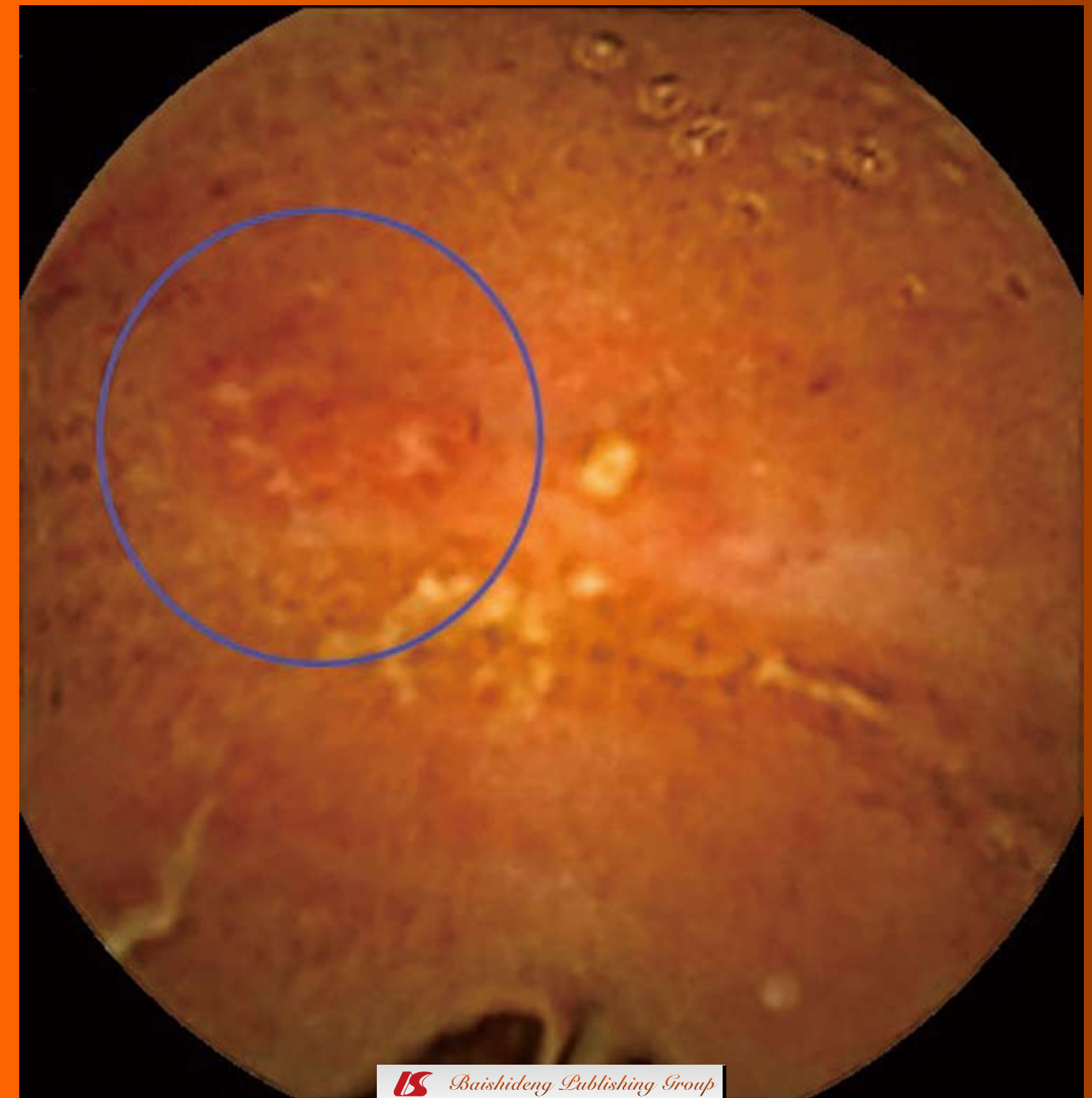
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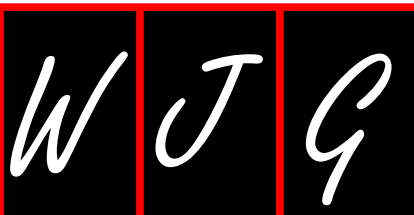
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<http://www.wjgnet.com/1007-9327/full/v17/i8/1030.htm>

AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited, Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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SUBSCRIPTION
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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
February 28, 2011

SERIAL PUBLICATION NUMBER
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

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Pancreaticobiliary reflux in patients with a normal pancreaticobiliary junction: Pathologic implications

Marcelo A Beltrán

Marcelo A Beltrán, Department of Surgery, La Serena Hospital, La Serena, IV Región 1700000, Chile

Author contributions: Beltrán MA solely contributed to this paper.

Correspondence to: Marcelo A Beltrán, MD, Department of Surgery, La Serena Hospital, La Serena, IV Región 1700000, Chile. beltran_01@yahoo.com

Telephone: +56-51-485923 Fax: +56-51-333382

Received: September 19, 2010 Revised: December 3, 2010

Accepted: December 10, 2010

Published online: February 28, 2011

Abstract

Knowledge on pancreaticobiliary reflux in normal pancreaticobiliary junction and its pathologic implications has experienced tremendous progress during the last few years. This editorial reviews the current knowledge on this condition and its pathological implications on gallbladder diseases. The following aspects were defined appropriate for discussion: (1) Evidence of carcinogenesis associated with pancreaticobiliary reflux; (2) Evidence of pancreaticobiliary reflux in normal pancreaticobiliary junction; and (3) Evidence of sphincter of Oddi (SO) dysfunction as a cause of pancreaticobiliary reflux in normal pancreaticobiliary junction. The articles reviewed were selected and classified according to five levels of evidence: Level I, meta-analysis double-blind randomized clinical trials, Level II, cohort non-blinded studies and non-randomized clinical trials, Level III, good quality case-control studies and non-randomized cohort studies, Level IV, case series and poor quality case-control studies, and Level V, case report articles and experts' opinion. Evidence levels II, III, IV and V were found to support biliary carcinogenesis associated with pancreaticobiliary reflux in normal and abnormal pancreaticobiliary junction. The same levels of evidence were found to support the common occurrence of pancreaticobiliary reflux in normal pancreaticobiliary junction, and SO dysfunction as the most plausible cause of

this condition. Although an important body of research has been published regarding pancreaticobiliary reflux in normal pancreaticobiliary junction and its clinical significance, the current evidence does not fully support what has been suggested. Studies with evidence level I have not been undertaken. This is a fascinating subject of study, and if finally supported by evidence level I, the importance of this condition will constitute a major breakthrough in biliary pathology.

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Key words: Biliary tract diseases; Biliary tract motility disorders; Pancreaticobiliary junction; Pancreaticobiliary reflux; Sphincter of Oddi

Peer reviewer: Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle, WA98111-0900, United States

Beltrán MA. Pancreaticobiliary reflux in patients with a normal pancreaticobiliary junction: Pathologic implications. *World J Gastroenterol* 2011; 17(8): 953-962 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/953.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.953>

INTRODUCTION

The reflux of pancreatic enzymes into the biliary tract has been associated with proliferative changes of the biliary epithelium, hyperplasia and carcinoma^[1-3]. This sequence of events, caused by pancreaticobiliary reflux (PBR), has been extensively studied in patients with biliary tract anomalies such as anomalous pancreaticobiliary junction and choledochal cysts^[4-19]. Recently, it has been recognized that PBR is a phenomenon occurring in normal pancreaticobiliary junction (NPBJ)^[20-29]. It has also been associated with gallbladder carcinoma^[30-38], and it has been suggested that it could play a role in gallstone formation through inflammatory changes in the gallbladder mucosa^[20,29,36]. The

pathophysiology and clinical importance of this phenomenon is now being elucidated, and attributes the occurrence of PBR to biliary tree motility disorders involving the sphincter of Oddi (SO), a theory which remains to be definitively proved^[27,29,36]. The purpose of this review is to discuss the current knowledge on PBR in NPBj and its pathologic implications.

SUBJECTS OF DISCUSSION

The following aspects were defined appropriate for discussion on the current knowledge and pathologic implications of PBR in NPBj: (1) Evidence of carcinogenesis associated with PBR; (2) Evidence of PBR in normal NPBj; and (3) Evidence of SO dysfunction as a cause of PBR in NPBj.

LEVELS OF EVIDENCE

The articles reviewed for this report were selected and classified according to five levels of evidence^[38,39]: (1) Level I, meta-analysis double-blind randomized clinical trials; (2) Level II, cohort non-blinded studies and non-randomized clinical trials; (3) Level III, good quality case-control studies and non-randomized cohort studies; (4) Level IV, case series and poor quality case-control studies; and (5) Level V, case report articles and experts' opinion.

OTHER SOURCES

In order to provide an adequate background, a PubMed search with the MeSH terms: pancreaticobiliary reflux in normal pancreaticobiliary junction and SO pathophysiology and dysfunction was performed. Articles in English language concerning pancreaticobiliary reflux in pancreaticobiliary maljunction, diagnosis and implications of a long common channel, and SO dysfunction were identified and reviewed regardless of their level of evidence.

CARCINOGENESIS ASSOCIATED WITH PANCREATICOBILIARY REFLUX: WHAT IS CURRENTLY KNOWN?

Most of the current knowledge on gallbladder carcinogenesis associated with PBR comes from studies on patients with anomalous pancreaticobiliary junction; consequently this part of the review deals with patients with gallbladder cancer and anomalous pancreaticobiliary junction. A good level of evidence has been collected on this subject and is detailed in Table 1. The reflux of pancreatic juice plays an important role in gallbladder carcinogenesis, and this fact has been recognized for more than 60 years^[2,9]. Gallbladder carcinoma associated with PBR was first described in patients with choledochal cysts^[2,4,40]. In these cases, the presence of an anomalous pancreaticobiliary junction in more than 96% of these patients^[42-46]; causes reflux of pancreatic juice into the dilated or non-

Table 1 Articles from "Carcinogenesis associated to pancreaticobiliary reflux: What is currently known?" – Levels of evidence in anomalous pancreaticobiliary junction

Author	Level of evidence
Kimura <i>et al</i> ^[2] , 1985	IV
Kinoshita <i>et al</i> ^[4] , 1984	IV
Mizuno <i>et al</i> ^[6] , 1996	II
Chao <i>et al</i> ^[7] , 1999	IV
Matsumoto <i>et al</i> ^[8] , 2003	IV
Funabiki <i>et al</i> ^[9] , 2009	IV
Kamisawa <i>et al</i> ^[10] , 2009	IV
Tsuchida <i>et al</i> ^[12] , 2003	IV
Hanada <i>et al</i> ^[13] , 1999	III
Obara <i>et al</i> ^[14] , 1999	IV
Hanada <i>et al</i> ^[15] , 1999	III
Seki <i>et al</i> ^[16] , 2005	III
Funabiki <i>et al</i> ^[17] , 1997	IV
Wistuba <i>et al</i> ^[18] , 1999	IV
Hanada <i>et al</i> ^[19] , 1999	IV
Iwai <i>et al</i> ^[40] , 1992	IV
Lipsett <i>et al</i> ^[41] , 1994	IV
Lenriot <i>et al</i> ^[42] , 1998	IV
Singham <i>et al</i> ^[43] , 2009	IV
Todani ^[44] , 1997	IV
Todani <i>et al</i> ^[45] , 1994	V
Akiyama <i>et al</i> ^[46] , 1998	V
Yamato <i>et al</i> ^[47] , 1999	III
Ichikawa <i>et al</i> ^[48] , 2004	III

dilated cystic common bile duct and into the gallbladder, where active pancreatic enzymes concentrate leading to chronic inflammation with associated mucosal malignant changes: hyperplasia, metaplasia, dysplasia, and eventually carcinoma *in-situ* and invasive carcinoma^[6,9,12,16-19,40,43-45]. In patients with gallbladder cancer, an incidence of 8.7% to 16.7% of anomalous pancreaticobiliary maljunction has been found^[17,45]. Gallbladder carcinoma has an incidence of between 8.4% and 24.6% in patients with anomalous pancreaticobiliary junction^[14,17]. Gallbladder carcinoma has been reported to be more frequently associated with gallbladder carcinoma in pancreaticobiliary maljunction without dilatation of the biliary tract than in pancreaticobiliary maljunction with dilatation of the biliary tract, with a reported incidence ranging from 41% to 90%^[6,9,14,17,19], whereas in the dilated type the incidence is about 10%^[14]. In addition, the incidence of epithelial hyperplasia is significantly higher in gallbladders without common bile duct dilatation (91%) than in those with common bile duct dilatation (38%)^[7,19]. A plausible explanation for the differences between dilated and non-dilated common bile duct in anomalous pancreaticobiliary junction, is that refluxed pancreatic juice stagnates in the gallbladder in the non-dilated type consequently injuring the biliary mucosa, and in the case of the dilated bile duct, pancreatic juice stagnates in the dilated common bile duct injuring the ductal biliary mucosa. Pancreaticobiliary maljunction outside the duodenum causes two-way regurgitation: pancreatic juice refluxes into the bile duct, or bile regurgitates into the pancreatic duct^[44], however, pancreatic duct hydrostatic pressure is higher within the pancreatic

duct^[10,42], consequently the reflux of pancreatic juice into the common bile duct is a more frequent phenomenon^[10]. Refluxed bile, undergoes stasis in the gallbladder accumulating and causing inflammation of the mucosa which suffers multiple cellular and molecular changes finally leading to carcinoma^[6,13,15,16,47,48].

Epithelial changes

Metaplasia and hyperplasia are often found in gallbladder epithelia of patients with PBR, while they are seldom found in control epithelia^[7,14,17]. Hyperplastic and metaplastic changes in the gallbladder of these patients are commonly found^[9,17], and papillary hyperplasia, mainly low-grade hyperplasia, is commonly seen in patients with PBR, and has been associated with gallbladder cancer^[16]. However, others suggest that high-grade hyperplasia is more frequent in patients with pancreaticobiliary maljunction and a non-dilated common bile duct^[9,14]. Epithelial hyperplasia of the gallbladder is an early characteristic change of the gallbladder mucosa in patients with PBR, with an incidence ranging from 38.5% to 87%; higher than the incidence of epithelial hyperplasia in patients without PBR. Moreover, epithelial hyperplasia is more frequent in patients with non-dilated common bile duct and pancreaticobiliary maljunction (91%) than in patients with a dilated choledochus and pancreaticobiliary maljunction (38%)^[9,14]. Gallbladder carcinoma in patients with PBR is habitually found in people over 50 years-old^[36,46], more frequently in women than in men, occurring mainly in the gallbladder fundus, and with the atypia of the gallbladder epithelium becoming less marked nearest the common bile duct^[46]. The pathogenesis of epithelial hyperplasia of the gallbladder mucosa in PBR can be explained by an increased hydrostatic pressure in the biliary tract secondary to the reflux of pancreatic juice, and an increased concentration of bile cholesterol secondary to biliary stasis in the gallbladder acting as a stimulus for the development of hyperplastic epithelium^[14].

Cellular kinetics

The mixture of bile acids and pancreatic activated enzymes present in PBR contains carcinogens, which are mutagenic, and cause injury to DNA which shows aneuploid or polyploid patterns, accelerating the cell cycle of the biliary epithelium of patients with PBR^[14,17]. Other specific molecular changes demonstrated in the mucosal cells of patients with PBR related to increased cellular kinetics are: elevation of the labeling indices of bromodeoxyuridine demonstrating a significantly greater nuclear S-phase fraction; elevation of the proliferative cell nuclear antigen; elevation of Ki-67 labeling index (Ki-67LI); an increase in the mean number of argyrophilic nucleolar organizer regions per nucleus; an increase in the activity of the ornithine decarboxylase, the rate-limiting enzyme in the biosynthesis of polyamines; and increased expression of the transforming growth factor- α (TGF- α) which regulates cell proliferation^[9,14,17-19]. Of interest, was the finding of overexpression of mucin core protein (MUC1)

in hyperplastic and dysplastic epithelia of the gallbladder mucosa and carcinomatous lesions in PBR, this anomaly was found even in noncancerous epithelium reflecting an altered phenotype of epithelial cells, and suggesting that the PBR itself might be related to carcinogenesis^[9,47].

Gene mutations: K-ras, p53, and others

The prevalence of K-ras mutation ranged from 0% to 58% in gallbladder hyperplasia, and from 5% to 100% in gallbladder carcinoma in patients with PBR^[12-14,18,19]. Point mutations of the K-ras oncogene in codon 12 (specific point mutation of GGT -Gly- to GAT -Asp- transition, only found in gallbladder carcinoma with PBR), and codon 13 of exon 1 in the gallbladder epithelium have been identified^[7,9,13,14,17,19]. Gene mutations of K-ras range from 5% to 100% in invasive gallbladder carcinomas and from 15% to 73% in dysplastic gallbladder lesions associated with PBR^[13,18]. In addition, the overexpression of p53 tumor suppressor gene located on the short arm of chromosome 17 (17p) in 57.1% patients with gallbladder carcinoma has been identified^[9]. Specific point mutations were found at codons 207, 212 and 217 on exons 5 to 8 in 31% to 80% patients with gallbladder cancer and PBR^[8,9,15,17,18]; p53 overexpression or mutations have not been detected in the noncancerous hyperplastic or dysplastic region adjacent to the cancer region, these observations suggest that p53 overexpression or mutations may be related to the transition from premalignancy to malignancy in the carcinogenesis of gallbladder mucosa^[12,17-19]. However, p53 mutations have also been detected in noncancerous biliary epithelium (38.5%) in patients with PBR, supporting the involvement of p53 gene mutations in biliary epithelium carcinogenesis in PBR^[15]. Finally, loss of heterozygosity of p53 has been detected in 72% patients with gallbladder carcinoma and PBR^[8,9,15]. In patients with PBR, p53 overexpression was detected in cancer but not in hyperplasia, indicating that it may be a late event in gallbladder carcinogenesis^[9,14]. Within the gallbladder, and along the biliary tract, chronic severe inflammation from PBR destroys the protective mucin-producing epithelial cells, and this repeated process of destruction/regeneration of biliary epithelium, leads to the known sequence of hyperplasia, metaplasia, dysplasia and carcinoma^[16]. K-ras mutations and overexpression of p53, are present in malignant, precancerous dysplastic and chronically inflamed bile ducts in patients with choledochal cysts and abnormal pancreaticobiliary junction without choledochal dilatation, this suggests that the reflux of active pancreatic enzymes causes these cellular and molecular alterations and that the biliary epithelium of patients with PBR should be considered as an epithelium with high carcinogenic potential^[7,9,15,16,44]. Mutations in tumor suppressor genes p14ARF, p16INK4a, and p16INK4/CDK2 have been frequently found in gallbladder cancer in PBR, and also in PBR without gallbladder cancer^[9]. Other genetic changes described in patients with gallbladder cancer and PBR involve inactivation of the

CDKN2 gene (80%), allelic loss at 8p22 locus (44%), DCC (18q21) deletions (31%), allelic loss at 17q13 at the *TP53* gene, allelic loss at 9p21 at the *p16^{Ink4a}/CDKN2* gene, and 5q21 loss of heterozygosity near the APC gene (22%); these mutations are considered early events in the pathogenesis of gallbladder carcinoma^[18]. Overexpression of Bcl2, an inhibitor of apoptosis found in mitochondria membranes, has been found in the non-cancerous portion of gallbladders in PBR and in the gallbladder carcinoma of these patients; this was regarded as an early event causing carcinogenesis in PBR^[19,48]. Telomerase activity is increased in gallbladder cancer only in patients with PBR, but is also increased in the noncancerous gallbladder mucosa of these patients, and in the epithelia of noncancerous patients with PBR^[48]. Microsatellite instability (MSI) was detected in 80% of gallbladder cancerous lesions, in 87.5% of dysplastic lesions and 0% of hyperplastic lesions in patients with PBR, contributing to carcinogenesis in these patients^[9]. Finally, mRNA indices in metaplastic and hyperplastic gallbladder epithelia in PBR are significantly increased, signaling metaplasia and hyperplasia as precancerous lesions^[9].

Carcinogens

Some bile acid fractions such as lithocholic and deoxycholic acids, deconjugated bile acids and β -glucuronidase, and active pancreatic enzymes in bile such as amylase, lipase, trypsin, elastase I, and phospholipase A₂ promote carcinoma under conditions of infection, inflammation, bile stasis, decreased trypsin inhibitors, and the presence of enterokinase^[6,7,9,17,19]. Moreover, phospholipase A₂ hydrolyzes lecithin into lysolecithin which is harmful to the mucosal barrier injuring the cell membrane^[6,7,9,17,47]. Other secondary bile acids, mainly taurodeoxycholic acid, may play a role in carcinogenesis, although this hypothesis is not completely accepted^[9]. Also, some amino acids acting as mutagenic substances, such as glycine, tyrosine and phenylalanine, have been found in large quantities in PBR^[6,9,17].

Cytokines and growth factors

Akiyama reported high levels of serum interleukin-6 (IL-6), TGF- α , and hepatocyte growth factor in a patient with gallbladder carcinoma and pancreaticobiliary maljunction. Also high levels of IL-6 and TGF- α were found in the same patient, suggesting a relationship between these molecules and chronic inflammation of the biliary tract in PBR^[46]. Other investigators have found overexpression of TGF- α , β -catenin, cyclinD1 and COX-2 in hyperplastic gallbladder mucosa of patients with PBR^[8,9,12].

Gallbladder cancer in normal pancreaticobiliary junction

Recently, PBR in patients with NPBj has been identified and has been associated with gallbladder cancer. A good level of evidence was found in published articles dealing with this subject, and is detailed in Table 2. The first reports on patients with NPBj and PBR associated with

Table 2 Articles from "Carcinogenesis associated to pancreaticobiliary reflux: What is currently known?" – Levels of evidence in normal pancreaticobiliary junction

Authors	Level of evidence
Sai <i>et al</i> ^[22] , 2002	III
Horaguchi <i>et al</i> ^[26] , 2008	III
Sai <i>et al</i> ^[30] , 2005	V
Sai <i>et al</i> ^[31] , 2005	III
Itoi <i>et al</i> ^[32] , 2005	III
Sai <i>et al</i> ^[33] , 2006	V
Sai <i>et al</i> ^[34] , 2006	III
Inagaki <i>et al</i> ^[35] , 2005	V
Beltrán <i>et al</i> ^[36] , 2007	II
Sakamoto <i>et al</i> ^[37] , 2009	III

gallbladder cancer were almost anecdotal case reports or case series^[22,30,33,35]. The reported incidence of gallbladder cancer in reports of series of cases, ranged from 6.5% to 50%^[22,26,31,34,36]. The levels of pancreatic enzymes, mainly amylase, have been reported to be extremely high in patients with gallbladder cancer and PBR in NPBj, compared to patients with benign gallbladder diseases within the same study^[22,26,31,34,36]. Although there has been no explanation as to why amylase levels were highly elevated in these cases, a strong correlation was found between gallbladder cancer and higher levels of amylase in the gallbladder bile of patients with PBR and NPBj^[26,30,33,36]. In patients with higher amylase levels, thickening of the gallbladder mucosa was a significant manifestation, and histological examination showed a high incidence of metaplastic changes compared to patients with lower levels of amylase^[26,36,37]. Markers of increased cellular kinetics, such as Ki-67LI, were found to be highly elevated in patients with higher amylase levels compared to those with lower amylase levels in patients with gallbladder cancer^[26,36]. Moreover, studies on non-cancerous epithelium of patients with PBR and NPBj, have found an increased Ki-67LI, COX-2 expression and overexpression, and mutations of *K-ras* gene at codon 12 with an increased and statistically significant frequency in patients with higher amylase levels^[31,32]; and increased Li-67LI in patients with hyperplastic or dysplastic gallbladder epithelium without gallbladder cancer, with a higher mean Li-67LI in dysplastic epithelium compared to metaplastic epithelium^[31]. Dysplasia and hyperplasia are frequently found in patients with PBR and NPBj, the incidence has been reported to be between 46% and 50%^[31,34]; also, intestinal metaplasia has been found in 16.8% of patients with extremely high levels of amylase and NPBj^[37]. These findings suggest that besides PBR, higher levels of pancreatic enzymes in bile constitute a risk factor for gallbladder cancer, and that the sequence of hyperplasia–metaplasia–dysplasia–carcinoma seen in patients with abnormal pancreaticobiliary junction might be similar for patients with NPBj and PBR^[30–34,36]. Overexpression of *p53* gene has not been frequently found in gallbladder cancer of patients with PBR associated with NPBj^[32], while only a few articles have reported this genetic marker as positive in patients

with gallbladder cancer^[30]. This fact is in contrast with the frequent identification of *p53* abnormalities in gallbladder carcinoma of patients with pancreaticobiliary maljunction. It has been assumed that the pathophysiological changes leading to gallbladder carcinoma in patients with NPBJ and PBR parallel the pathophysiology described in studies on patients with anomalous pancreaticobiliary junction and gallbladder carcinoma^[22,31,33,34,36].

Summary

Gallbladder carcinogenesis, in patients with PBR, is a multifactorial and multistage dynamic process involving multiple genetic changes and proliferative inductions of the gallbladder mucosa induced by the reflux of active pancreatic enzymes into the biliary tree and pooling in the gallbladder lumen. After many years of investigation and multiple publications on the subject, the precise processes of gallbladder carcinogenesis secondary to PBR are not yet clear. Gallbladder cancer in anomalous pancreaticobiliary junction is associated with a non-dilated common bile tract, in which stasis of the refluxed pancreatic juice occurs in the gallbladder and causes the variety of changes previously reviewed, leading to gallbladder cancer. The pathophysiology of gallbladder cancer in NPBJ probably resembles this well-known condition.

PANCREATICOBILIARY REFLUX IN NORMAL PANCREATICOBILIARY JUNCTION: WHAT IS CURRENTLY ACCEPTED?

The reflux of pancreatic enzymes into the biliary tract and its role in gallbladder diseases has been studied since the first half of the twentieth century^[50]. However, despite some early elegant published studies^[20], PBR occurring in NPBJ has only recently become accepted^[21-24,27,29,36]. Normal pancreaticobiliary junction was defined as the union of the common bile duct and the main pancreatic duct inside the duodenal wall where the SO surrounds them with muscular fascicles which regulate the flow of bile and pancreatic juice^[50,51]. According to this definition, reflux of pancreatic juice into the biliary tract in patients with a NPBJ can only be explained by an improper functioning of the SO^[29,34,36,52]. Most studies on PBR in NPBJ have addressed the potential relationships with gallbladder cancer and have been previously discussed. In this section we will discuss other aspects referring to benign gallbladder diseases related to PBR, diagnostic methods and normal values (Table 3).

Methods employed to diagnose pancreaticobiliary reflux in normal pancreaticobiliary junction

A variety of methods has been employed to demonstrate PBR. Some investigators have used radioimmunoassay of biliary trypsin in bile samples taken directly from a T tube inserted directly into the common bile duct after sur-

Table 3 Articles from “Pancreaticobiliary reflux in normal pancreaticobiliary junction: What is currently accepted?”

Author	Level of evidence
Anderson <i>et al</i> ^[20] , 1979	IV
Sai <i>et al</i> ^[21] , 2002	V
Sai <i>et al</i> ^[22] , 2003	III
Vracko <i>et al</i> ^[23] , 2003	III
Itokawa <i>et al</i> ^[24] , 2004	III
Kamisawa <i>et al</i> ^[25] , 2006	III
Horaguchi <i>et al</i> ^[26] , 2008	III
Xian <i>et al</i> ^[28] , 2009	II
Beltrán <i>et al</i> ^[29] , 2010	II
Sai <i>et al</i> ^[30] , 2005	V
Sai <i>et al</i> ^[31] , 2005	III
Itoi <i>et al</i> ^[32] , 2005	III
Sai <i>et al</i> ^[33] , 2006	V
Sai <i>et al</i> ^[34] , 2006	III
Beltrán <i>et al</i> ^[36] , 2007	II
Hjorth E. ^[49] , 1947	IV
Paulsen <i>et al</i> ^[50] , 2002	II
Toouli <i>et al</i> ^[51] , 1999	IV
Vracko <i>et al</i> ^[52] , 1994	II
Vracko <i>et al</i> ^[53] , 2000	III
Vracko <i>et al</i> ^[54] , 2006	II
Ko <i>et al</i> ^[55] , 2005	II

gery^[20,52]. A widely used method has been the bile sample taken directly from the gallbladder at the time of cholecystectomy and pancreatic amylase and lipase determination by colorimetric or enzymatic methods^[20,23,24,29,36,53]. Many researchers have measured pancreatic enzymes in bile using samples taken directly from the bile duct in patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) and analyzing the samples with enzymatic methods or Western-blotting tests^[24-28,30-34,36,54]. Others have employed magnetic resonance cholangiopancreatography (MRCP) with secretin injection to stimulate the SO to indirectly show reflux of pancreatic juice into the biliary tract^[21,22,24,25,27]. However, of these methods, the sampling of bile by ERCP seems to be an inadequate method to demonstrate PBR because in order to take the sample of bile, the SO must necessarily be disrupted, potentially causing reflux of enzymes into the bile duct and gallbladder and consequently invalidating the sample and the method. Secretin injection MRCP is an indirect and unspecific method with low sensitivity and specificity to prove PBR^[22,24], and in patients who should be submitted to surgery, it seems to be a rather unnecessary preoperative study. Taking bile samples from an indwelling T tube is probably an inadequate method to prove PBR because in order to insert the T tube, the biliary tract must have been manipulated and have suffered surgical trauma with instrumentation of the SO, consequently invalidating the method. We believe that the most accurate method to measure PBR is directly sampling the bile from the gallbladder during cholecystectomy, before any manipulation in the triangle of Calot area or over the common bile duct has been performed^[29,36].

WHICH ARE “NORMAL” LEVELS OF PANCREATIC ENZYMES IN BILE?

No one has determined and validated normal values or levels of pancreatic enzymes in bile^[1,27,36]. However, most researchers use as a reference the normal values of pancreatic enzymes in plasma to discriminate patients with PBR from those without the considered anomalous reflux of pancreatic juice into the biliary tract^[22-24,27,29,36,52,53]. The argument favoring this assumption is that any patient with bile levels of pancreatic enzymes within the normal plasma value does not have PBR, considering that the presence of pancreatic enzymes in bile within the normal plasma value depends on the normal hepatic filtration ratio which was determined at 0.30 to 0.70 for trypsin^[23,52]. In a recent investigation, we determined that patients without gallstones have minimal or no levels of pancreatic enzymes in their gallbladders^[29]. Consequently, we may argue that “normal” values of pancreatic enzymes in bile should be close to zero.

Acute cholecystitis

Anderson *et al*^[20] determined the concentration of pancreatic enzymes, amylase and lipase, in bile obtained from the gallbladder in 70 patients and found highly elevated levels of amylase in 87% cases and lipase in 66%, they concluded that the reflux of pancreatic enzymes may initiate chronic inflammatory changes in the gallbladder and could play a role in gallstone formation and in the pathogenesis of some cases of acute cholecystitis. This premise, was also followed by Josef Vracko who investigated the role of PBR in acute cholecystitis^[23,54]. Vracko *et al*^[23,54] postulated that endoscopic sphincterotomy releasing the common channel outlet obstruction could initially improve the course of acute cholecystitis in elderly patients, reducing the risk of biliary sepsis, and delaying surgery until their conditions were improved in order to undergo elective surgery at a later time and avoid emergency surgery. Pancreatic enzymes were found to be extremely elevated in patients with initial edematous acute cholecystitis compared with patients with late gangrenous cholecystitis and patients with chronic symptomatic cholelithiasis; suggesting that in some patients, acute cholecystitis could be initiated by the reflux of pancreatic enzymes into the gallbladder due to SO dysfunction or an obstructing gallstone in the papilla of Vater or both^[23]. Another interesting finding was that in patients with gangrenous cholecystitis, pancreatic enzymes were lower and comparable to patients with chronic cholecystitis; this phenomenon was explained as a consequence of the consumption of pancreatic enzymes by extensive damage of the gallbladder wall, including vascular damage, coagulation, fat necrosis, and intramural hemorrhage^[23]. Consequently, although not absolutely proven, the role of PBR in patients with acute cholecystitis might be related to a sudden functional or mechanical obstruction of the SO

leading to an excessive reflux of active injurious pancreatic enzymes into the common bile duct and gallbladder, initiating a cascade of events ultimately leading to acute cholecystitis. This theory might find its clinical application in the proposal of endoscopic sphincterotomy in the early course of acute cholecystitis within the first 72 h after the onset of symptoms, as an alternative to more invasive surgery in elderly frail patients, improving the clinical course and allowing time for conservative treatment or delayed elective surgery^[23,55].

Gallstone formation

Pancreaticobiliary reflux causes chronic inflammation and injury of the biliary tract mucosa, particularly of the gallbladder mucosa where bile concentrate and pancreatic enzymes pooling reaches high levels^[22,24,25,29,31,36]. High levels of pancreatic enzymes have been found within the whole spectrum of gallbladder diseases, benign and malignant, including acute and chronic cholecystitis, common bile duct stones, gallbladder polyps, and gallbladder cancer^[20,22,24,26,29,36,37]. This suggests that PBR has a role in the whole spectrum of gallbladder diseases^[20,24,27,29,36]. Initial and chronic inflammatory changes of the gallbladder mucosa induced by active pancreatic enzymes could play a role in gallstone formation^[20,27,29,36]. The reflux of pancreatic enzymes causes chronic inflammation of the gallbladder mucosa^[20-29,36,53,54]. Chronic inflammation of the gallbladder mucosa modifies the hepatic bile in ways other than the reabsorption of fluids and electrolytes, with the addition of total proteins such as mucin and albumin which increase the nucleation time leading to the formation of biliary sludge, microlithiasis and ultimately gallstones^[20,27,55]. It has been suggested that motility disorders of the gallbladder and biliary tree; including spastic episodes of the SO that could be influenced by gender, hormones and genetic predisposition, and associated with PBR leading to biliary tract and gallbladder mucosa chronic injury; could play a role in the etiology of gallstones, chronic gallbladder disease and ultimately gallbladder cancer, constituting only different continuous stages of a common pathologic entity^[29].

Summary

The occurrence of PBR in patients with a NPBJ seems to be a pathologic condition occurring in benign and malignant biliary diseases such as acute and chronic gallstone cholecystitis and gallbladder cancer. This reflux plays a role in acute and chronic inflammation of the gallbladder epithelium, gallstone formation, acute cholecystitis and carcinogenesis. To study PBR in normal pancreaticobiliary junction, a variety of indirect and direct methods have been employed; however, none seem to be the ideal method. A sample of bile directly from the gallbladder during surgery would be the best currently available method for this purpose. The so-called normal levels of pancreatic enzymes in bile would be close to zero.

Table 4 Articles from “Pancreaticobiliary reflux in normal pancreaticobiliary junction: What is currently suspected?”

Author	Level of evidence
Sai <i>et al</i> ^[22] , 2003	III
Vracko <i>et al</i> ^[23] , 2003	III
Kamisawa <i>et al</i> ^[27] , 2008	IV
Sai <i>et al</i> ^[34] , 2006	III
Beltrán <i>et al</i> ^[36] , 2007	II
Iwai N, <i>et al</i> ^[40] , 1992	IV
Paulsen <i>et al</i> ^[50] , 2002	II
Vracko <i>et al</i> ^[51] , 1994	II
Todani <i>et al</i> ^[52] , 1994	IV
Kamisawa <i>et al</i> ^[53] , 2009	III
Kamisawa <i>et al</i> ^[58] , 2010	II
Kamisawa <i>et al</i> ^[59] , 2002	II
Kamisawa <i>et al</i> ^[60] , 2007	III
Toouli <i>et al</i> ^[61] , 1982	II
Carr-Locke <i>et al</i> ^[62] , 1981	III
Csendes <i>et al</i> ^[63] , 1979	II
Boyden EA ^[64] , 1937	IV
Yokohata <i>et al</i> ^[65] , 2000	IV
Tanaka M ^[66] , 2002	IV

PANCREATICOBIILIARY REFLUX IN NORMAL PANCREATICOBIILIARY JUNCTION: WHAT IS CURRENTLY SUSPECTED?”

This section will deal with the most plausible cause of PBR in patients with NPBJ (Table 4).

What is a normal pancreaticobiliary junction?

Pancreaticobiliary maljunction has been defined as a union of the pancreatic and biliary ducts which is located outside the duodenal wall forming a markedly long common channel^[56,57]. Consequently, NPBJ must, necessarily, be located inside the duodenal wall where the sphincteric mechanism provided by the muscular fascicles, of which the SO is composed, can influence the normal antegrade flow of bile and pancreatic juice.

Long common channel and its implications

A long common channel has been regarded as an intermediate variant of pancreaticobiliary maljunction, others have also named it a high confluence of pancreaticobiliary ducts^[58,59]. Some authors, argument that a long common channel is included within the SO^[27,57-60]; however, it is not plausible that this long common channel would be under the complete influence of the SO, consequently this would be the cause of PBR in these cases. Most authors agree that the normal length of the common channel generally tends to be less than 10 mm in adults and 4 mm in infants^[50,56], consequently a longer common channel should be considered an abnormal variant. However, other authors have suggested that a common channel should be considered long when it is equal or larger than 5 mm^[58]. A long common channel is not under the complete influence of the SO, no high-pressure area has been found in

the area of the common channel of the pancreaticobiliary junction in patients with anomalous pancreaticobiliary junction or a long common channel, which confirms that the SO does not fully extend to the area of the pancreaticobiliary junction in these cases^[41,60], consequently pancreaticobiliary and biliopancreatic reflux is a common occurrence^[60]. The most important clinical significance of a long common channel is that, as well as in patients with anomalous pancreaticobiliary maljunction, patients with a long common channel showed significant PBR related to gallbladder cancer^[22,58,59].

SO: Its role in pancreaticobiliary reflux in normal pancreaticobiliary junction

The human bile duct lacks a contractile muscle layer^[52]. Therefore, the flow of bile and pancreatic juice towards the duodenum is a consequence of gallbladder activity and intraductal bile and pancreatic duct pressures, which depend on the production of bile and pancreatic juice, respectively, and is regulated by the SO. The normal sphincter activity directs the flow of bile and pancreatic juice towards the duodenum by antegrade phasic contractions^[61], consequently, in patients with PBR and NPBJ, the only plausible explanation for reflux of pancreatic juice into the biliary tract is an anomaly in the normal function of the SO causing a functional obstruction to the normal flow and retrograde contractions favoring the reflux of pancreatic juice into the biliary tract^[23,27,29,34,36,52-54], because the intraluminal pressure of the pancreatic duct is higher than the intraluminal pressure of the common bile duct^[51,62,63].

SO: How it functions?

The SO is a smooth muscle structure measuring approximately 1 cm in length which is situated at the junction of the bile duct, pancreatic duct and duodenum^[51,66]. The SO normally produces high-pressure phasic antegrade contractions that are superimposed on a modest basal pressure, the phasic contractions propel small volumes of either bile or pancreatic juice into the duodenum^[51,56,65,66]. In man, most flow occurs between the phasic contractions^[51,65,66]. The SO function is influenced by a number of neural stimuli, circulating hormones such as cholecystokinin, and duodenal activity secondary to ingestion of food^[51]. During fasting, the SO demonstrates regular phasic contractile activity which alternates with the interdigestive motor activity of the duodenum^[51,65]. During duodenal phases I and II, the SO contracts at the rate of 2 to 4 contractions per minute without a quiescent phase; approaching duodenum phase III, the SO also increases its contractile activity concluding at the same time, this pattern repeats over the same period as duodenal activity^[51,65,66]. The fact that the SO continues to contract during the quiescence of duodenal phase I supports the independent nature of SO motility. This pattern of function demonstrated by the SO, supports indirectly the theory regarding the cause of PBR in NPBJ, which states that spasms of the SO not related to the migrating myo-

electric complex is the cause of this phenomenon^[23,27,36,65]. SO dysfunction is suspected clinically based on the established Rome II Diagnostic Criteria^[67,68]. To study SO dysfunction, less invasive procedures to rule-out other biliary tract conditions should be considered first, such as liver function blood tests and abdominal ultrasound^[67]. Other more specific, although non-invasive methods, such as the Morphine-Prostigmin Provocative Test or Nardi Test which has a low sensitivity and specificity, the Quantitative Hepatobiliary Scintigraphy Test which has not been fully accepted due to controversial and imprecise criteria to diagnose SO dysfunction, and Magnetic Resonance Cholangiopancreatography with secretin stimulation could also be employed^[67,68]. Other invasive methods such as endoscopic ultrasonography, intraductal ultrasonography, and endoscopic cholangiography have been used, and although they can give indirect evidence of the presence of SO dysfunction, their results are nonspecific^[67,68]. Currently, the gold standard to study and diagnose SO dysfunction is SO manometry^[51,61,62,64,66-68]. The cause of SO dysfunction is unknown at the present time; however, we suggest that this is probably due to congenital motility anomaly traits.

Summary

SO dysfunction is the most plausible cause of PBR in patients with NPBj.

CONCLUSION

Although an important body of research has been published regarding PBR in NPBj and its clinical significance, the current evidence does not fully support what has been suggested. Studies with evidence level I have not been undertaken. This is a fascinating subject of study, and if finally supported by evidence level I, the importance of PBR in NPBj will constitute a major breakthrough in biliary pathology.

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S- Editor Tian L L- Editor Webster JR E- Editor Sun H

Current treatment of rectovaginal fistula in Crohn's disease

Yan-Fei Zhu, Guo-Qing Tao, Ning Zhou, Chen Xiang

Yan-Fei Zhu, Guo-Qing Tao, Ning Zhou, Chen Xiang, Department of General Surgery, Wuxi People's Hospital of Nanjing Medical University, No. 299, Qingyang Road, Wuxi 214023, Jiangsu Province, China

Author contributions: Zhu YF and Tao GQ designed the research; Zhu YF wrote the paper; Zhou N and Xiang C searched the references and analyzed them.

Correspondence to: Yan-Fei Zhu, MD, Department of General Surgery, Wuxi People's Hospital of Nanjing Medical University, No. 299, Qingyang Road, Wuxi 214023, Jiangsu Province, China. zhuyanfei_2002@163.com

Telephone: +86-510-85350091 Fax: +86-510-82828435

Received: September 22, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: February 28, 2011

Abstract

Rectovaginal fistula (RVF) continues to be the most difficult perianal manifestation of Crohn's disease to treat. This devastating and disabling complication has a significant impact on patients' quality of life and presents unique management challenges. Current therapeutic approaches include many medical therapeutics and surgical treatments with a wide range of success rates reported. However, current evidence is lacking to support any recommendation. The choice of repair depends on various patient and disease factors and basic surgical tenets. In this article, we review the current options to consider in the treatment of Crohn's-related RVF, and try to evaluate their effects on fistulae closure and quality of life.

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Key words: Rectovaginal fistula; Crohn's disease; Advancement flap; Treatment; Relapse

Peer reviewers: Paola De Nardi, MD, Department of Surgery, Scientific Institute San Raffaele Hospital, Via Olgettina 60, Milan, 20132, Italy; Ronald S Chamberlain, MD, MPA, Chairman and Surgeon-in-Chief, Professor of Surgery, UMDNJ, Saint Barnabas Medical Center, 94 Old Short Hills Road, Livingston, NJ 07945, United States

Zhu YF, Tao GQ, Zhou N, Xiang C. Current treatment of rectovaginal fistula in Crohn's disease. *World J Gastroenterol* 2011; 17(8): 963-967 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i8/963.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.963>

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder with a variable clinical course that may occur anywhere in the alimentary tract, characterized by a tendency to form fistulas with adjacent structures. In addition, CD is the second most common cause of rectovaginal fistula (RVF) after obstetrical trauma. In general, the cumulative incidence of all perianal fistulas in CD is 50% after the age of 20, and up to 9% of these are RVFs. The incidence of Crohn's RVF is thought to be proportionate to the frequency and severity of large-bowel inflammation^[1,2]. Although representing less than 10% of anal fistulas, RVF may cause significant clinical distress and social embarrassment. Common symptoms include dyspareunia, perianal pain, vaginal irritation, and poor hygiene leading to recurrent genitourinary infections^[3].

Accurate assessment is essential for planning management. The treatment of fistulas is based on the severity of symptoms, the anatomic location, the number and complexity of tracts and the presence of stricturing intestinal disease. Treatment options range from observation to medical therapeutics to the need for surgical intervention. Various surgical options have been suggested for RVF repair with a wide range of success rates, such as direct repair, fistulotomy, fibrin glue instillation, endorectal or vaginal advancement flap, abdominal procedures with colorectal or coloanal anastomosis, and epiploplasty. However, none has been universally accepted as the procedure of choice. The recurrence rates ranged from 25% to 50%^[2-4]. In this article, we review the current options to consider in the treatment of Crohn's-related RVF and their effects on quality of life.

TREATMENT CHOICE

The management involves an organized and detailed workup to accurately make the diagnosis and then implement the appropriate treatment. Only a combination of advanced imaging, physical examination, and clinical experience will afford the surgeon the opportunity to precisely identify the location and cause of this problem. There are no evidence-based randomized controlled trials for the appropriate management of RVFs. More focused studies targeting these patients with the use of combined medical and surgical therapy are necessary^[1,4].

Medical treatment of Crohn's RVF

Treatment of RVF is mainly surgical. However, medical therapy does have a role in the treatment of Crohn's RVF. Over the years, attempts at healing Crohn's RVF by medical treatment have been met with failure. The principles of medical therapy have been aimed at the treatment of the underlying active disease and include the use of antibiotics, corticosteroids, immunosuppressives and infliximab.

Up to now, there have been no randomized, double-blind, placebo-controlled studies to support the use of antibiotics and corticosteroids in healing Crohn's RVF. These treatments are not favorable, with low rates of long-term symptomatic control and unacceptable high rates of recurrence. Besides, some local therapies, including warm sitz baths, antidiarrheal medications and so on, might provide minor relief from symptoms but are not curative measures^[5]. More and more surgeons used them as adjuncts to surgical treatment of Crohn's RVF, despite the lack of clinical trials to establish their efficacy.

Antibiotics were found to display only short-term benefit for patients^[6]. Some studies reported that more than 80% RVFs required complete fistula closure when cyclosporine was used in high doses intravenously. However, about one third of patients relapsed when therapy was switched from intravenous to oral. Furthermore, cyclosporine was associated with toxicity, especially nephrotoxicity in long-term therapy. Tacrolimus had a mechanism similar to cyclosporin, and preliminary reports showed fistula healing rates of up to 64% when combined with azathioprine, but further studies should be done to evaluate the efficacy^[7]. The introduction of anti-tumor necrosis- α (TNF- α) therapy has been a major advance in the treatment of fistulizing CD, and has completely altered treatment strategies for perianal disease^[8,9]. About 25 women with Crohn's RVF were enrolled in the *post hoc* subset analysis of the ACCENT II (A CD Clinical trial Evaluating infliximab in a New long-term Treatment regimen in patients with fistulizing CD). After infusions of infliximab at weeks 0, 2, and 6, 60.7% and 44.8% of RVFs were closed at weeks 10 and 14. The duration of RVF closure was longer in the infliximab 5 mg/kg maintenance group than in the placebo group. They concluded that infliximab was effective in short-term closure of RVF, and maintenance treatment was more effective than placebo in prolonging RVF closure^[10]. Parsi and his colleagues^[11] studied the differences in response to infliximab among

patients with different types of Crohn's fistulas. The total closure rate for all external Crohn's fistulas was 78%, but the closure rate for Crohn's RVF was only 14% at 4 to 6 wk follow-up. Higashi *et al.*^[12] reported that the frequency of neoplasia did not differ according to whether infliximab was used or not, but it was necessary to be careful in terms of long-term administration and to select patients in whom efficacy could be expected.

So far, medically induced fistula healing is rare with basic therapeutic approaches and is only short lived, with no challenging results of infliximab treatment for Crohn's RVF, which might due to a poorly vascularized rectovaginal septum^[4,10].

Surgical treatment of Crohn's RVF

RVF in CD continue to be a challenging problem. A multistep approach has been recommended to treat patients with Crohn's-related RVF, in which medical treatment and drainage of local sepsis are the first initial steps to alleviate their discomfort before definitive surgical intervention is attempted^[1,13,14]. Many reports believed that the presence of acute perianal sepsis needed to be separately addressed prior to any attempts to repair RVF. This may require surgical drainage accompanied by placement of a loose draining seton^[15-17]. Subsequently, various surgical options have been suggested for RVF repair. Among them are direct repair, fistulotomy, fibrin glue instillation, endorectal or vaginal advancement flap, abdominal procedures with colorectal or coloanal anastomosis, and epiplooplasty^[18-20]. There are no guidelines concerning optimal therapeutic approaches. Endoanal mobilization techniques such as the advancement flap technique were considered the therapy of choice for many years, but are now regarded ever more critically^[21,22]. Surgical skill is very important and adherence to principles of hemostasis, gentle tissue handling and complete debridement of diseased tissue are imperative to success. The technique of repair will vary depending on the location of the fistula and extent of local and distant disease activity^[23-25].

Rectal advancement flap surgery is the most frequently used approach. A flap consisting of mucosa, submucosa, and some circular muscle fibers is elevated. The fistula tract is curetted and oversewn with synthetic absorbable suture. The distal end of the flap is then trimmed and sutured distal to the fistula opening. The repair is undertaken from the high-pressure side of a high low-pressure shunt, the primary source of the fistula is excised and a layer of intact healthy tissue is interposed. Rectal advancement flaps are best suited for Crohn's RVF when the fistula is low, the rectum is relatively spared, and there is no significant anal stenosis. However, this technique is contraindicated in patients with extensive ulceration or stricturing of the anal canal and transitional zone^[26,27]. Alternative or additional surgical procedures include a rectal sleeve advancement flap and a vaginal advancement flap. An advancement sleeve flap may be a better option for those with circumferential anal canal or low rectal disease and a normal proximal rectum. This approach removes all diseased tissue in the anal canal and allows "normal"

rectal tissue to be sutured to the neodentate line^[28]. Compared with a rectal flap, a vaginal flap is easier to mobilize. Sher *et al*^[29] treated 14 patients using a transvaginal flap with excellent results, with complete healing in 13 patients. The authors believed that the success of this technique was to be attributed to the fact that they used healthy tissue from the vagina. Unfortunately no other authors have published their experience with this interesting technique and further conclusions are impossible to draw.

Tissue interposition methods were intended to interpose normal healthy tissue between suture lines and bring well-vascularized tissue into the area. The use of a gracilis muscle transplant is one of the various options and has been proposed for failed previous RVF repair. In Lefevre's study, for patients with CD, four of five (80%) presented no recurrent rectovaginal fistula after a median follow-up of 28 mo. They concluded that gracilis muscle transposition was a useful and effective method for the treatment of recurrent RVF, especially in patients with CD, providing a viable tissue flap between the rectum and the vagina. This procedure is associated with minor morbidity and a good success rate, especially in patients with CD^[30]. However, despite healing, postoperative quality of life and sexual activity remained altered. Zmora *et al*^[31] reported that the important technical features of the gracilis transposition procedure were fecal diversion, meticulous hemostasis, tension-free primary repair of the rectum after dissection and mobilization to a level of at least 3 cm above the fistula site, and a viable, tension-free, well-vascularized muscle pedicle. So they recommended it for Crohn's RVF, especially after failed previous repairs.

Advanced improvements in medical treatment and expert surgical management have decreased the need for proctectomy. However, recurrence has a major negative impact on the quality of life. The suboptimal quality of perianal tissues that are affected by CD is probably the origin of the failure to heal. As we know, adult stem cells extracted from certain tissues, such as adipose tissue, can differentiate into different tissues, such as muscle. García-Olmo *et al*^[32] first reported a case of a young patient with CD who had a recurrent rectovaginal fistula that was treated by autologous stem cell transplantation with a lipoaspirate as the source of stem cells. Three months later the RVF still remained closed. The patient has not experienced vaginal flatus or fecal incontinence through her vagina. In any case, cell transplantation to overcome healing problems is a new surgical tool, and careful evaluation of this new modality might provide an opportunity to define a new era in the treatment of surgical challenges associated with healing disorders.

Failure, reoperations and recurrences of rectovaginal fistulas have a major impact on anal continence and quality of life. The technical feasibility of closing rectovaginal fistulas using new bioprosthesis such as SurgisisTM mesh has already been demonstrated. SurgisisTM is a biocompatible mesh generated from lyophilized porcine small intestinal submucosa, which allows host cells to replace and repair damage or defective tissue. The mesh is gradually replaced as the host cells rebuild and remodel

weakened tissue. Its sterile, acellular properties enable it to be used without the complication of rejection. There is a significant reduction in fibrosis during the healing process, because the mesh supports the patient's own connective tissue and smooth muscle growth^[33]. Schwandner *et al*^[34] reported that 21 patients with RVFs were performed with mesh procedures, the majority of them had Crohn's RVFs (nine). After a mean follow-up of 12 months, the success rates in relation to the presence of CD or not were 78% and 83% after primary mesh procedure separately. Besides, the success rates of RVF with only one prior attempt or at least two prior attempts were 88% and 62% respectively. Authors believed that this technique could potentially be an effective alternative or enrichment to "traditional" procedures such as advancement flap repair as the main procedure, or closure with muscle transposition. Further analysis is needed to assess the definitive role of this innovative technique in comparison to traditional surgical technique.

Complex RVFs are uncommon but difficult therapeutic problems. Local repair and flap advancement techniques have a high incidence of recurrence with poor functional outcomes. Transperineal repair with anal sphincter reconstruction, when indicated, and placement of a Martius flap result in improved rates of repair and better functional outcomes. A consecutive series of patients were retrospectively reviewed from a prospective database between 2002 and 2006 in McNevin's study^[26]. They concluded that selected complex RVF can be reliably repaired with good functional outcomes using the Martius flap with anal sphincter reconstruction. Persistent or recurrent fecal incontinence and dyspareunia are common sequela of the underlying perineal injury and repair. However, the number of patients is small. Prospective randomized trials are lacking. Therefore, it remains questionable whether addition of a Martius flap improves outcome after rectovaginal fistula repair^[27].

The protective or definitive stoma (proximal fecal diversion) may be taken into consideration to control the symptoms before proceeding to a more definitive operation. Results are equivocal when a stoma is used in conjunction with repair of a Crohn's perianal fistula. Fecal diversion alone is not successful in healing the fistula despite an improvement of the anorectal Crohn's fistula. Stoma does not ensure success and is probably used in most complicated cases. Creating a stoma remains a controversial subject. Currently the decision to use a stoma is up to the surgeon's judgment at the time of operation with valuable results of physical and laboratory examinations.

RESULTS OF VARIOUS TREATMENTS

A retrospective study reported by Athanasiadis^[35] was concerned on a comparison of different techniques for recovery rates and functional results after repair for RVF in CD. The operations comprised 56 procedures performed in 37 women presenting with RVF. The follow-up period was 7.15 years. Several techniques were performed: transverse transperineal repair ($n = 20$), endoanal direct closure multilayer without flap ($n = 15$), anocutaneous

Table 1 Current reports for different repairs for rectovaginal fistulas with Crohn's disease

Authors	Patients/repairs	Procedure	Primary/overall healing (%)	Recurrence (%)	Follow-up (mo)
Joo <i>et al</i> ^[38] , 1998	20	RAF	-/75		17
Windsor <i>et al</i> ^[39] , 2000	13/21	Local repair	40/77	54	31
Athanasiadis <i>et al</i> ^[35] , 2007	37/56	various	51/73	30	85
	-/20	Transperineal repair	70		
	-/15	Direct closure	73		
	-/14	Anocutaneous flap	86		
	-/7	Advancement flap	29		
Ruffolo <i>et al</i> ^[36] , 2009	52/71	different	56/81	10	109
	-/36	RAF	56		
	-/23	VAF	57		

RAF: Rectal advancement flap; VAF: Vaginal advancement flap.

flap ($n = 14$), and advancement mucosal or full-thickness flap ($n = 7$). The success rates for each of the techniques in the above group were 70, 73, 86, and 29%, respectively. The transperineal repair led to decreased postoperative resting pressures. In the advancement flap technique, the resting and squeezing pressure decreased significantly. The risk of developing a suture line dehiscence leading to a persisting fistula was higher in the advancement flap procedure with 43%. Authors concluded that techniques with a low degree of tissue mobilization such as the direct closure and anocutaneous flap show higher success rates without significant postoperative changes in continence and manometric outcome. Impaired continence was observed only in the advancement flap group, resulting in significant changes in manometric values and recovery rates. The authors preferred to apply the direct multilayer closure technique without flap.

The research studied by Ruffolo evaluated the outcome of surgical repair of RVF in patients with Crohn's disease over a 14-year period and assessed the effect of therapy with antibody against TNF on healing^[36]. The surgical techniques included ileocecal interposition, coloanal anastomosis, perineoproctotomy with sphincter repair, levatorplasty and closure of the fistula, rectal advancement flap and vaginal advancement flap. After initial drainage of anal sepsis and optimization of medical therapy, fistula closure was achieved in 81% of 52 patients. Overall, cumulative closure rates after the first, second, third and fourth attempts were 56, 75, 78 and 81%, respectively. The primary healing rate was similar in patients who received anti-TNF treatment before the first operation (12 of 18 patients) and those who did not (19 of 34), even though the former patients probably presented with more extensive and/or aggressive disease at some stage. This observation supports the notion that anti-TNF- α does not interfere with wound healing^[37]. Furthermore, the current reports for different repairs for rectovaginal fistulas with CD are displayed in Table 1.

Crohn's-related rectovaginal fistulae have significant impact on quality of life including sexual function. El-Gazzaz^[40] and coworkers obtained long-term follow-up of Crohn's-related rectovaginal fistulae to assess variables that influence surgical success and determine its effects on

quality of life and sexual function. Sixty-five women were identified at median follow-up of 44.6 months, of which 30 patients (46.2%) were successfully healed. Methods of repair included advancement flap ($n = 47$), episio-proctotomy ($n = 8$), colo-anal anastomosis ($n = 7$), and fibrin glue or plug ($n = 3$). Twenty-eight women (43.1%) were sexually active at follow-up, and of those, nine complained of dyspareunia, all within the unhealed group of patients. Sexual function and quality of life scores were comparable between healed and unhealed groups. They believed that healing increased when immunomodulators were used within three months before surgery. Smoking and steroids were predictors of repair failure. Regardless of successful healing, quality of life and sexual function were similar. Dyspareunia appears to be higher for women with unhealed fistulas^[41]. Further research and multicenter studies should be performed.

CONCLUSION

Crohn's-related RVF continues to be difficult to treat. Up to now, there has been no ideal treatment option suitable for all patients, and many techniques have been reported with a wide range of success. The reasonable combination of medical and surgical treatments should be recommended. The surgical management of RVF is complex and the best results come in conjunction with individualized care within specialist units. The technique should be carefully chosen depending on the anorectal morphology. Hopefully, ongoing studies will help produce a universally accepted algorithm that may enhance long-term outcomes. Further multicenter studies should be needed to evaluate the long-term effects of various approaches. Besides, the prognosis and the quality of life for affected women should not be ignored.

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S- Editor Tian L L- Editor Cant MR E- Editor Lin YP

Transient micro-elastography: A novel non-invasive approach to measure liver stiffness in mice

Cécile Bastard, Matteo R Bosisio, Michèle Chabert, Athina D Kalopissis, Meriem Mahrouf-Yorgov, Hélène Gilgenkrantz, Sebastian Mueller, Laurent Sandrin

Cécile Bastard, Matteo R Bosisio, Laurent Sandrin, Echosens, Research and Development Department, Paris, F-75013, France

Cécile Bastard, INSERM, U930, Tours, F-37000, France

Cécile Bastard, CNRS, ERL3106, Tours, F-37000, France

Cécile Bastard, Univ François Rabelais, Tours, F-37000, France

Matteo R Bosisio, UPMC Univ Paris 06, UMR 7623, LIP, Paris, F-75005, France

Matteo R Bosisio, CNRS, UMR 7623, Laboratoire d'Imagerie Paramétrique, Paris, F-75005, France

Michèle Chabert, Athina D Kalopissis, INSERM, U872, Centre de Recherche des Cordeliers, Equipe 4, Paris, F-75006, France

Michèle Chabert, Athina D Kalopissis, UPMC Univ Paris 06, UMR S 872, Equipe 4, Paris, F-75006, France

Michèle Chabert, Athina D Kalopissis, Univ Paris Descartes, UMR S 872, Equipe 4, Paris, F-75006, France

Michèle Chabert, EPHE, Laboratoire de Pharmacologie Cellulaire Et Moléculaire, Paris, F-75006, France

Meriem Mahrouf-Yorgov, Hélène Gilgenkrantz, INSERM, U1016, Institut Cochin, Paris, F-75014, France

Meriem Mahrouf-Yorgov, Hélène Gilgenkrantz, CNRS, UMR8104, Paris, F-75014, France

Meriem Mahrouf-Yorgov, Hélène Gilgenkrantz, Univ Paris Descartes, Paris, F-75014, France

Sebastian Mueller, Department of Medicine and Center for Alcohol Research, Liver Disease and Nutrition, Salem Medical Center, University of Heidelberg, 69121 Heidelberg, Germany

Author contributions: Bastard C, Bosisio MR and Sandrin L designed the research; Chabert M, Gilgenkrantz H and Kalopissis AD provided the animal models; Bastard C, Bosisio MR, Chabert M, Gilgenkrantz H and Kalopissis AD performed the study; Chabert M, Kalopissis AD and Mahrouf-Yorgov M analyzed the histological sections; Bastard C, Bosisio MR, Chabert M, Kalopissis AD and Gilgenkrantz H analyzed the data; Bastard C, Bosisio MR, Mueller S and Sandrin L wrote the paper.

Supported by Echosens, Research and Development Department, 153 avenue d'Italie, 75013, Paris, France

Correspondence to: Cécile Bastard, Echosens, Research and Development Department, 153 avenue d'Italie, 75013, Paris, France. cecile.bastard@echosens.com

Telephone: +33-1-44827850 Fax: +33-1-44827860

Received: July 21, 2010 Revised: September 13, 2010

Accepted: September 20, 2010

Published online: February 28, 2011

Abstract

AIM: To develop and validate a transient micro-elastography device to measure liver stiffness (LS) in mice.

METHODS: A novel transient micro-elastography (TME) device, dedicated to LS measurements in mice with a range of measurement from 1-170 kPa, was developed using an optimized vibration frequency of 300 Hz and a 2 mm piston. The novel probe was validated in a classical fibrosis model (CCl₄) and in a transgenic murine model of systemic amyloidosis.

RESULTS: TME could be successfully performed in control mice below the xiphoid cartilage, with a mean LS of 4.4 ± 1.3 kPa, a mean success rate of 88%, and an excellent intra-observer agreement (0.98). Treatment with CCl₄ over seven weeks drastically increased LS as compared to controls (18.2 ± 3.7 kPa vs 3.6 ± 1.2 kPa). Moreover, fibrosis stage was highly correlated with LS (Spearman coefficient = 0.88, $P < 0.01$). In the amyloidosis model, much higher LS values were obtained, reaching maximum values of > 150 kPa. LS significantly correlated with the amyloidosis index (0.93, $P < 0.0001$) and the plasma concentration of mutant hApoA-II (0.62 , $P < 0.005$).

CONCLUSION: Here, we have established the first non-invasive approach to measure LS in mice, and have successfully validated it in two murine models of high LS.

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Key words: Transient elastography; Micro-elastography; Liver stiffness; Liver; Mice; Amyloidosis; Fibrosis; Ultrasound

Peer reviewer: Dr. Joseph J Cullen, MD, Professor, Department of Surgery, University of Iowa Carver College of Medicine, 4605 JCP, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242, United States

Bastard C, Bosisio MR, Chabert M, Kalopissis AD, Mahrouf-Yorgov M, Gilgenkrantz H, Mueller S, Sandrin L. Transient micro-elastography: A novel non-invasive approach to measure liver stiffness in mice. *World J Gastroenterol* 2011; 17(8): 968-975 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/968.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.968>

INTRODUCTION

Transient elastography (TE) (FibroScan[®]) is a novel non-invasive bedside method to assess liver fibrosis *via* liver stiffness (LS)^[1,2]. In various liver diseases, LS was shown to be strongly associated with the degree of liver fibrosis^[2-8]. In these studies, cut-off values have been defined that allow the diagnosis of advanced fibrosis (F3/4). Despite some variability, cut-off values of 8.0 and 12.5 kPa are widely accepted to identify patients with F3 and F4 fibrosis, respectively. Although LS closely correlates with fibrosis stage, it also increases in patients with mild^[9,10] or acute hepatitis^[11], cholestasis^[12], and liver congestion^[13], independently of the degree of fibrosis. Thus, improved diagnostic algorithms require the exclusion of congestion and mechanic cholestasis by ultrasound and measurement of liver function tests prior to LS interpretation^[2].

Despite major efforts worldwide, the molecular mechanisms of liver fibrosis are still poorly understood^[14]. The appreciation of LS as physical parameter has not only improved fibrosis diagnosis, but has also stimulated our understanding of its pathophysiology. Thus, LS seems to directly affect matrix synthesis^[15] and precedes fibrosis progression^[16]. Two recent reports have highlighted the importance of hydrostatic pressure on LS^[12,13], leading to the formulation of the pressure-LS-fibrosis sequence hypothesis^[2]. However, no non-invasive and routinely exploitable methods exist to assess LS in mice, which is the standard animal model for studying fibrosis^[17] and antifibrotic strategies^[18].

Elastographic techniques seem to be the most promising approach to assess tissue stiffness *in vivo*. Although magnetic resonance elastography (MRE)^[19] has been successfully applied to quantify LS in rodents^[20,21], this technique sometimes requires the insertion of a needle to transmit a vibration to the liver^[20]. In addition, MRE techniques remain very expensive, limiting their routine use in animal laboratories. Likewise, alternative techniques, such as ultrasonic static elastography^[22], are restricted to *in vitro* or *in situ* studies in mice^[23], and they are not quantitative^[24]. Recently, acoustic radiation force impulse (ARFI) imaging^[25] has been successfully applied to quantify LS in a rat model of toxic liver fibrosis induced by carbon tetrachloride (CCl₄) injections^[26]. However, the probe had to be coupled to the rat's abdomen through a water-path and a layer of saran wrap. Alternative approaches, such as elasticity and atomic force microscopy^[15,27,28], yield stiffness values at the cellular level; however, how subcellular

stiffness parameters translate into overall organ stiffness and fibrogenesis remains under discussion. Finally, a direct rheological technique only allows the measurement of LS on the explanted organ *ex vivo*^[16].

Here, we introduce a novel miniaturized probe, based on the principle of vibration-controlled transient elastography. Significant changes in the physical parameters, such as vibration frequency, are required because of the small size of the murine liver. We demonstrate that the novel transient micro-elastography (TME) device reproducibly allows the measurement of LS in mice. We successfully validated the technique in two murine models of increased LS: the conventional CCl₄-induced fibrosis model and a transgenic model of systemic amyloidosis.

MATERIALS AND METHODS

Phantoms for stiffness measurements

A homogeneous copolymer-in-oil phantom, comprising Styrene-Ethylene-Butylene-Styrene (4%) and mineral oil, was used to compare the results obtained using TE and TME, as described recently^[29].

Animal models

Carbon tetrachloride fibrosis model: Toxic liver fibrosis was induced by carbon tetrachloride (CCl₄) injections over seven weeks, according to standard protocols (Sigma Aldrich, St. Louis, MO, USA)^[30]. Nine female CD1 mice were divided into three groups (Table 1): the first group ($n = 3$, aged 6 mo) received toxic injections (CCl₄, 5 mL/kg dissolved in paraffin oil, ratio 1:10), the second group ($n = 3$, aged 16 mo) received paraffin oil, and the third group ($n = 3$, aged 4 mo) was not treated. Intraperitoneal injections of CCl₄ or paraffin oil were performed twice a week over seven weeks. For LS measurements, mice were first anaesthetized with isoflurane (0.75%-1% in oxygen).

Mouse model of systemic amyloidosis: Transgenic mice with systemic amyloidosis were generated at the Cordeliers Research Center (UMRS 872, Paris, France) by microinjection of the 3-kilobase genomic clone of the human apolipoprotein A-II (hapoA-II) gene, bearing a stop codon serine mutation. In humans, this mutation results in a longer amyloidogenic protein^[31]. Normal apolipoprotein A-II (apoA-II) is a major protein of high-density lipoproteins, and its plasma concentration is measured by immunonephelometry using a commercial kit (DiaSys, Holzheim, Germany). Three transgenic lines (Y, K and F) with very low, moderate, and high expression levels, respectively, of mutant hapoA-II were obtained (X. Rousset, M. Lacasa, A.D. Kalopissis and M. Chabert, manuscript in preparation). Twenty-seven transgenic mice (9 Y, 8 K, and 10 F), aged between 4 and 12 mo, were included in the study (Table 2). This group comprised 13 males and 14 females (Table 2). Furthermore, four male C57BL/6 mice aged 4 mo were kept as controls (Table 1). The animals were anesthetized by intraperitoneal injection of avertin (tribromoethanol, 2% solution), and LS measurements were performed. Mice were then sacrificed to evaluate the stage of amyloidosis.

Table 1 Characteristics of control mice and CCl₄-induced fibrotic mice

Mouse	Strain	Type	Sex	Liver stiffness (kPa)	Success rate for LSM (%)	% sirius red
1	C57BL/6	Control	M	4.7	75	ND
2	C57BL/6	Control	M	4.0	100	ND
3	C57BL/6	Control	M	4.7	67	ND
4	C57BL/6	Control	M	6.8	86	ND
5	CD1	Control	F	5.0	100	< 0.3
6	CD1	Control	F	3.3	92	0.05
7	CD1	Control	F	2.6	94	0.10
8	CD1	Oil injected	F	5.3	100	0.42
9	CD1	Oil injected	F	9.3	84	0.72
10	CD1	Oil injected	F	5.8	91	0.32
11	CD1	CCl ₄ injected	F	18.8	100	1.84
12	CD1	CCl ₄ injected	F	21.6	100	2.36
14	CD1	CCl ₄ injected	F	14.2	100	2.38

LSM: Liver stiffness measurement ; ND: Not determined.

The livers of 19 mice were removed and weighed. Animal use procedures were in accordance with the recommendations of the European Economic Community (86/609/CEE) and the French National Committee (decree 87/848) for the care and use of laboratory animals.

Amyloidosis staging

To determine the stage of amyloidosis, mice were sacrificed and their livers were subjected to macroscopic examination. Several parameters were considered, including the size of the liver, its apparent rigidity, and modifications of the lobes, such as hypertrophy, and included in an empirically established score from 0 to 4 (Table 3). Representative livers of a normal mouse and an amyloidosis mouse stage 3 are shown in Figure 1. All examinations were performed by M.C. in a blinded fashion, without knowledge of the TME data.

Histological analysis

Immediately after stiffness measurements, transgenic mice with amyloidosis were sacrificed for histological analysis. Their livers were quickly removed after intra-cardiac vascular washing with 0.1 mol/L phosphate buffer and then with 4% paraformaldehyde (PFA). The livers were then cut and immersion-fixed, successively, in 4% PFA and then in sucrose overnight. Small livers pieces were embedded in Tissue-Tek O.C.T. compound 4583 (Sakura Finetek, Torrance, CA, USA), frozen and stored at -80°C until analysis. Tissue sections of 20 µm were specifically stained for amyloidosis with Congo red. Amyloid fibrils consisted solely of hApoA-II; therefore, 5 µm thick liver sections were immunostained with a hApoA-II specific antibody and examined with an LSM-710 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA).

Livers of mice with toxic liver fibrosis were harvested, fixed in 4% neutral-buffered formalin, and embedded in paraffin. 5 µm thick sections were stained with hematoxylin and eosin (HE) or with picosirius red (Sigma Aldrich, St. Louis, MO, USA), as previously described^[32]. For morpho-

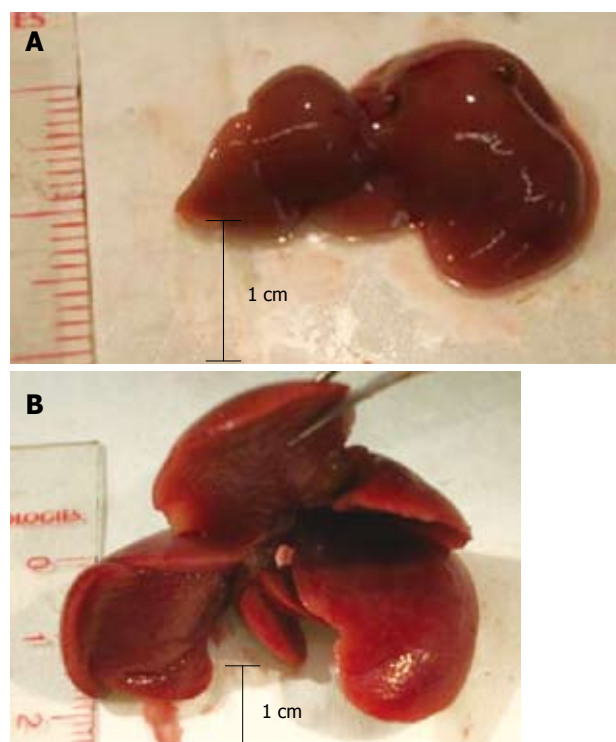


Figure 1 Representative livers from normal control mice (A), and mice with stage 3 amyloidosis (B).

metric analysis, 15 images per animal from at least two different lobes were taken at × 100 magnification. The areas of staining were quantitated using the software Image J 1.37v (National Institutes of Health, Bethesda, MD, USA). The histological results provided by the analysis of these 15 images are more reliable and representative than a mere biopsy.

Transient micro-elastography

TE measures shear wave velocity, and thus determines tissue stiffness using ultrasound to follow the propagation of a low frequency shear wave generated in a tissue by an external vibrator^[33,34]. In humans, TE uses a low frequency vibration of 50 Hz generated by an ultrasonic transducer 9 mm in diameter used as a piston. However, shear stiffness can be overestimated in the near field zone (i.e. under 25 mm for a 50 Hz excitation) because of diffraction effects^[35]. Due to the morphology of mice, measurements have to be performed very close to the vibration source, where diffraction effects are likely to occur. Our modified TME device allows the measurement of elasticity near the probe and is suitable for contact measurements of stiffness at the organ surface. TME comprises a microprobe, a high frequency electronic system (Echosens, Paris, France), and a laptop computer to control the ultrasound system and analyze the data. The microprobe contains an ultrasonic transducer (Imasonic, Besançon, France), used as both the receiver and emitter, which is mounted on a mechanical vibrator to generate a low frequency shear wave. The electronic system is fully programmable, and enables sampling of the radiofrequency data at a frequency up to 200 MHz with a 12-bit precision. Diffraction effects were reduced by increasing the frequency of vibra-

Table 2 Characteristics of the amyloidosis group mice

Mouse	Strain	Sex	Liver weight to body weight ratio (%)	hpaA-II (g/L)	Amyloidosis index	LS (kPa)	Success rate for LSM (%)
1	Y	F	5.1	0.19	0	3.9	90
2	Y	M	ND	ND	0	9.3	83
3	Y	F	ND	ND	0	5.9	100
4	Y	F	4.4	0.22	0	4.1	100
5	Y	F	4.5	0.22	0	4.1	100
6	Y	F	4.6	ND	0	4.7	86
7	Y	F	ND	ND	1	12.6	100
8	Y	M	5.2	0.23	0	3.9	100
9	Y	F	ND	ND	0	11.2	92
10	K	M	ND	0.15	1	23.7	100
11	K	M	16.0	0.55	4	124.0	74
12	K	M	13.8	0.57	2	124.0	57
13	K	F	15.8	0.54	4	168.8	100
14	K	F	22.8	0.80	3	168.8	82
15	K	F	18.2	0.44	3	168.8	100
16	K	F	12.1	0.42	2	94.9	100
17	K	F	5.0	0.28	0	4.4	96
18	F	M	7.6	0.66	1	50.2	100
19	F	M	ND	0.76	1	73.0	100
20	F	M	ND	0.44	1	13.5	92
21	F	F	12.9	ND	2	124.0	95
22	F	M	ND	0.35	2	155.5	100
23	F	F	16.3	0.58	3	168.8	95
24	F	M	15.7	0.53	3	168.8	69
25	F	M	14.9	0.59	3	168.8	100
26	F	M	13.7	0.64	3	124.0	89
27	F	M	10.8	0.59	3	168.8	100

LSM: Liver stiffness measurement; ND: Not determined.

Table 3 Macroscopic liver amyloidosis index obtained by visual assessment and manual palpation

Index	Liver appearance
0	Normal
1	Slightly bigger and/or stiffer
2	Big and stiff (\pm whitish zones)
3	Very big and stiff, changes in the right lobe (\pm whitish zones)
4	Very big and stiff, right lobe transformed and very big (\pm whitish zones)

tion to 300 Hz and reducing the diameter of the piston (2 mm). To improve the performance of TME in mice, we also increased the ultrasound frequency (up to 12 MHz). These parameters allowed the calculation of displacements with better resolution. During the propagation of the low frequency shear wave, the radiofrequency (RF) data were acquired at a repetition frequency of 15000 Hz. The displacements induced in the medium were computed from the RF data using an autocorrelation method and derived *vs* depth to provide a strain image (Figure 2). Analysis of the strain image yields the shear wave velocity and thus elasticity, according to the formula $E = 3\rho V_s^2$ where E , ρ (1 kg/dm³), and V_s are the Young's modulus, the mass density, and the shear wave velocity, respectively. To compute the shear wave velocity, we used a time-of-flight algorithm, as previously described by Sandrin *et al*^[1]. The system enables the measurement of stiffness values (Young's modulus) between 0.5 kPa and 170 kPa. How-

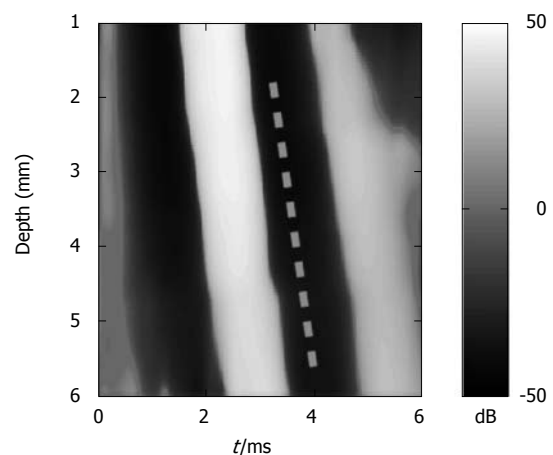


Figure 2 Amplitude of the strains induced in the liver of a mouse with systemic amyloidosis (stage 1), as a function of depth and time. The shear wave velocity (V_s) is the slope of the wave pattern. The steeper the slope, the higher the velocity of the shear wave and the higher the Young's modulus (here $E = 79.3$ kPa).

ever, for high stiffness values (> 100 kPa), the computational step is larger and the measurement less accurate.

Liver stiffness measurement using TME

Anesthetized mice were placed in the spinal position. Abdominal hair was removed and gel was used to ensure the coupling. The best location for LS measurement was below the level of the xiphoid cartilage (Figure 3). We identified some conditions that could limit successful TME measure-

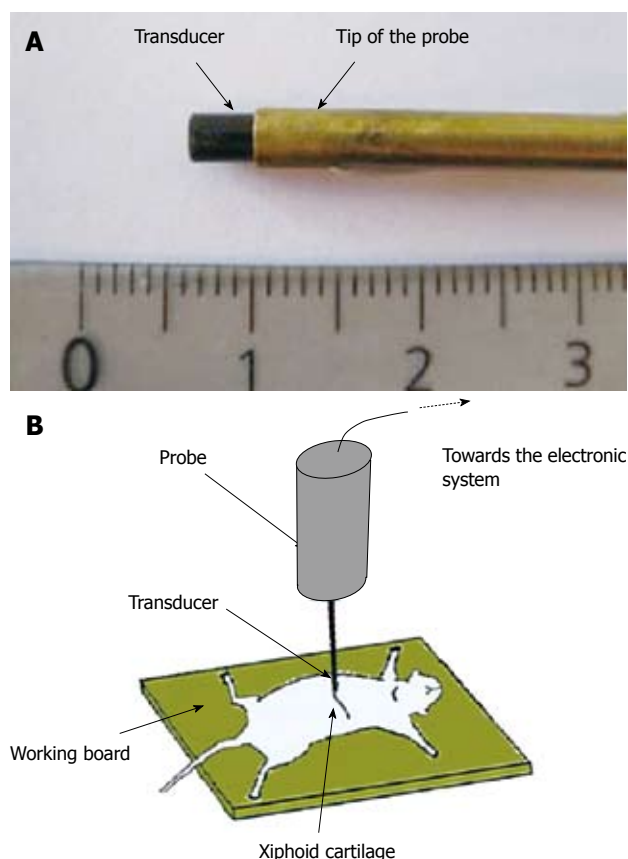


Figure 3 Transducer (A) and experimental setup (B) used in transient micro-elastography.

ments (very small mice, or mice with highly hypertrophic and misshapen livers, as in advanced amyloidosis). Measurements were routinely performed in the median liver lobe, between 2 mm and 5-6 mm below the skin surface, depending on the size of the animal. For each LS value, we performed at least 10 validated measurements, and used the median of the values recorded. The approximate duration of the examination was generally five minutes.

Statistical analysis

The relationship between LS and histology was investigated by computing p-values for the Spearman coefficient, and using Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) and one-sided Mann-Whitney tests. These nonparametric tests are particularly well suited to analyze data from small samples. Correlations with p-values less than 0.05 were considered significant. Bar plots were also used to estimate the stiffness distribution as a function of amyloidosis index. Intra-observer agreement was analyzed using the intraclass correlation coefficient (ICC). All statistical analyses were carried out using Matlab (The MathWorks, Natick, MA, USA).

RESULTS

TME studies on intra-observer agreement and reproducibility

A homogenous copolymer-in-oil phantom with a defined

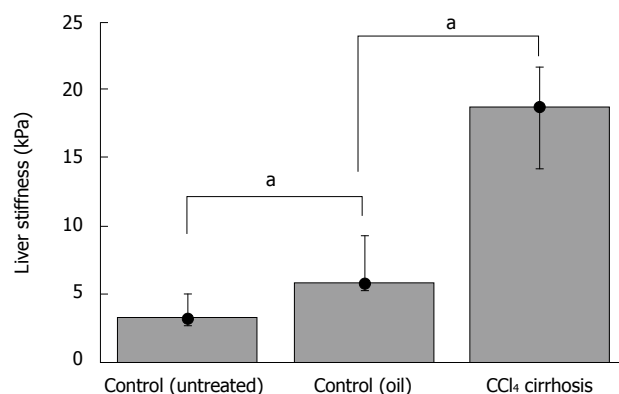


Figure 4 Bar plot showing median liver stiffness in control (untreated vs oil-treated) and CCl₄-induced fibrosis. Liver stiffness is expressed in kPa. Whiskers indicate the extent of the data. The difference between the groups is significant (*P ≤ 0.05, Mann-Whitney).

stiffness was used to establish and compare TME with TE. In TE, the Young's modulus was measured in a region of interest (ROI) located between 25 and 65 mm from the source, whereas with TME, the ROI was located between 2 and 6 mm. TME yielded a slightly higher, but comparable, Young's modulus of 9.9 ± 0.3 kPa as compared to TE (8.2 ± 0.0 kPa). To evaluate the intra-observer agreement, we performed two consecutive series of 10 valid measurements in seven control mice (Table 1) and twelve transgenic mice exhibiting different amyloidosis stages (Table 2). Then, we computed the ICC, which was 0.98. In healthy control mice ($n = 7$), mean LS was comparable to human LS, at 4.4 ± 1.3 kPa. The mean success rate was 88%. Thus, we concluded that, using a vibration frequency of 300 Hz, TME allows for non-invasive and reproducible measurements of LS in mice over a wider stiffness range.

TME in a conventional fibrosis model

We next studied LS in a conventional fibrosis model using CCl₄ injections over seven weeks. Two controls were used (paraffin oil without CCl₄ and untreated controls). Fibrosis was assessed by picrosirius red staining, and stained areas were quantified (Table 1). As expected, fibrotic scars were only detected within the parenchyma of the CCl₄-treated mice, forming bridges between vessels with a very faint inflammatory reaction (not shown). TME showed a significantly higher LS (P -value ≤ 0.05) in the experimental CCl₄-treated group ($E = 18.2 \pm 3.7$ kPa) compared to oil-injected mice ($E = 6.8 \pm 2.2$ kPa) or control mice ($E = 3.6 \pm 1.2$ kPa) (Figure 4). A high correlation was also obtained between LS and CCl₄-induced liver fibrosis, as assessed by picrosirius red staining (Spearman coefficient = 0.88, $P < 0.01$).

TME in a systemic amyloidosis model

Hepatic amyloidosis is known to cause increased LS in humans^[36,37]. We therefore studied LS in a recently established murine amyloidosis model using TME. Amyloid deposits were detected in the livers of 6-mo-old transgenic mice by green birefringence in Congo red stained sections under a polarized microscope (Figure 5A and B).

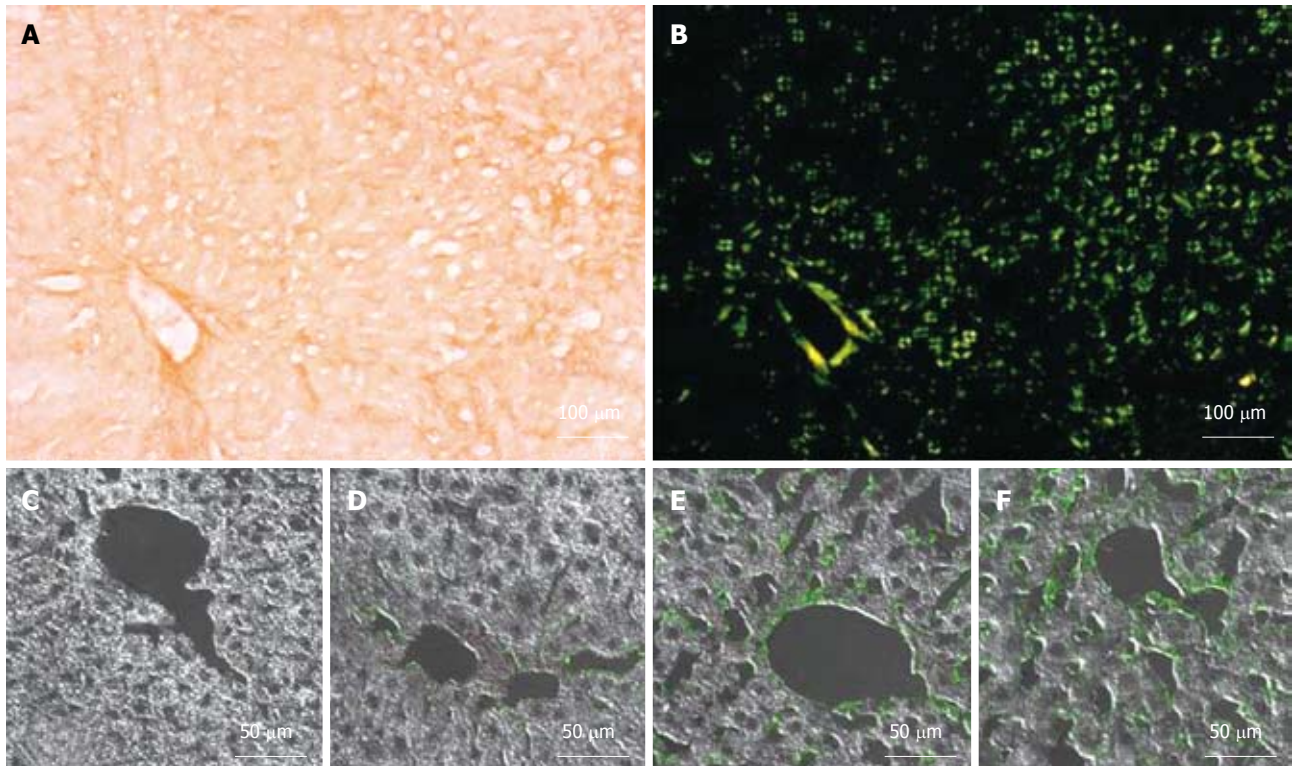


Figure 5 Histological characterization of amyloidosis mice. Light microscopy of a liver section from a 6-mo-old K mouse stained with Congo red (A) and the same section under polarized light showing green birefringence (B); Confocal microscopy of liver sections with immunolocalization of mutant hapoA-II using an anti hapoA-II antibody and CY2 as the secondary antibody (green fluorescence): Control mouse, 8 mo old (C); K mouse, 2 mo old (D); K mouse, 6 mo old (E); F mouse, 8 mo old (F).

Figures 5C-F display liver sections immunostained with a specific anti-hapoA-II antibody (green fluorescence in confocal microscopy) detecting mutant hapoA-II forming amyloid fibrils. Amyloidosis was absent in a control C57BL/6 mouse (Figure 5C), and drastically increased in transgenic mice as a function of age (Figure 5D-F). Figure 6 shows bar plots of LS as a function of amyloidosis index. LS significantly correlated with the amyloidosis index established by macroscopic examination (Spearman coefficient = 0.93, P -value < 0.0001). Interestingly, significant differences in LS were observed between degrees of amyloidosis, and TME was able to clearly discriminate between amyloidosis indexes 0 and 1 (P -value < 0.005). Mice with amyloidosis indexes 3 and 4 exhibited very high stiffness values, very close to the upper detection limit of 170 kPa (Table 2); thus explaining the lack of discrimination between these two indexes by LS measurements. We also found a significant correlation between LS and the ratio of liver weight to body weight (Spearman coefficient = 0.84, P -value < 0.0001), which increases with the progression of the disease because of the progressive deposition of amyloid fibrils in the liver. In addition, the plasma concentration of mutant hapoA-II partly accounts for LS values (Spearman coefficient = 0.62, P -value < 0.005).

DISCUSSION

We here introduce TME for the assessment of LS in mice in a rapid and non-invasive manner. In addition, we successfully studied LS and validated TME in two mouse

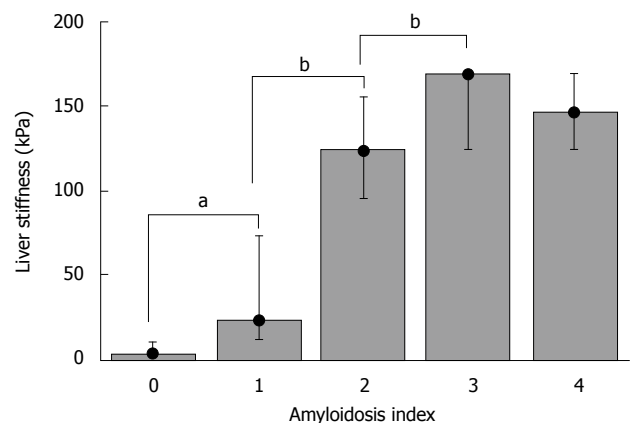


Figure 6 Bar plot showing median liver stiffness as a function of amyloidosis severity. Liver stiffness is expressed in kPa, and amyloidosis severity is given according to the scoring system described in Materials and Methods. Whiskers indicate the extent of the data. The difference between index 0, 1, 2, and 3 is significant (aP < 0.005 and bP < 0.01; Mann-Whitney).

models with increased LS. With a success rate of 88% and an intra-observer variability of 0.98, TME offers the first approach to study LS in small animal models, a prerequisite condition for determining the role of LS in murine fibrosis models.

Interestingly, LS values of control mice (4.4 ± 1.3 kPa) were comparable to those of healthy humans, which should be below 6 kPa^[2]. These LS values are also consistent with those found in rodents using other techniques such as MRE or ARFI^[20,21,26]. These findings emphasize

that normal LS seems to be below 6 kPa, which is independent of liver size.

The investigation performed on mice with CCl₄-induced fibrosis showed the potential of TME for fibrosis quantification in mice. After seven weeks of fibrosis induction, LS had increased to 18.2 ± 3.7 kPa in all livers with a proven cirrhosis stage. Thus, murine LS had clearly passed typical cut-off values for F4 cirrhosis (12.5 kPa). The increase of LS in the CCl₄ model was also in good agreement with the recently reported storage shear modulus determined on explanted non-perfused livers^[16]. Previous MRE and ARFI studies reported much smaller LS values for fibrotic livers, using either CCl₄-induced fibrosis or knock-out models^[20,21,26]. However, the degree of fibrosis is known to vary considerably in the CCl₄ fibrosis model and significantly depends on the species used. Thus, LS of 5.4 and 6.9 kPa have been reported recently in CCl₄ rat models^[21,26], with a very small difference between control and treatment groups of less than 1.5 kPa. We observed a slightly higher LS in the control mice treated with the CCl₄ carrier solvent paraffin oil (6.8 ± 2.2 kPa), as compared to untreated mice (3.6 ± 1.2 kPa). This was most likely due to the older age (12 mo) of the oil-injected group: age has been recognized as factor that independently increases LS in humans^[38,39].

It has been recently demonstrated in humans that hepatic amyloidosis can drastically increase LS up to the detection limit of TE^[36,37]. Here, we reproduced these data in a transgenic murine model of amyloidosis using TME and showed significant correlation between histological and serum markers of amyloidosis. Thus, in contrast to visual assessment and manual palpation, TME allowed the detection of the early stage 1 of the disease. LS also significantly correlated with the amyloidosis index and plasma concentrations of mutant hApoA-II. Comparable to humans, amyloidosis showed the highest LS values reported so far in rodents, significantly exceeding 75 kPa.

In conclusion, TME allows the measurement of LS in mice in a fast, reproducible, and non-invasive manner. TME will be a powerful tool for studying fibrosis in murine models, and in transgenic and knock out mice in longitudinal studies. It will help in the better understanding of the determinants of LS, such as venous pressure^[13] or extrahepatic cholestasis^[12]. Finally, TME will also permit the exploration of anti-fibrotic strategies.

COMMENTS

Background

Transient elastography is a quantitative ultrasound elastography technique, which consists of following with ultrasound the propagation of a low frequency shear wave generated in a tissue by an external vibrator. Based on transient elastography, Fibroscan® (Echosens, Paris, France) is a novel non-invasive bedside method to assess liver fibrosis by measuring liver stiffness. This technique provides an average value of the Young's modulus in a region of interest, comprising an area between 25 and 65 mm below the skin. This device is non-invasive, fully automatic, and generates a result within a few minutes. Its main advantages are its ease of use, good reproducibility, and very good acceptance by patients. Clinical interest in liver stiffness measurement using Fibroscan® has been largely validated for adult patients with chronic liver diseases.

Research frontiers

Over the past decades, the mouse has emerged as one of the best model

organisms for experimental studies of human diseases and drug testing. For example, in liver pathologies, mice have been used in numerous investigations involving antifibrogenic substances. However, no non-invasive and routinely exploitable methods exist to assess liver stiffness in mice.

Innovations and breakthroughs

In the area of small animal experimentation, the use of elastographic techniques has been reported by several groups with magnetic resonance elastography (MRE) and static ultrasound elastography. However, the techniques proposed remain expensive (MRE) and are sometimes invasive and unsuitable for *in vivo* applications. The use of elastographic techniques on small animals is therefore a challenging area of investigation.

Applications

In this study, a novel transient micro-elastography (TME) device dedicated to liver stiffness (LS) measurements in mice was developed. The novel system was validated in both a classical fibrosis model (CCl₄) and a transgenic murine model of systemic amyloidosis. TME could be successfully performed in control mice below the xiphoid cartilage, with a mean LS of 4.4 ± 1.3 kPa, a mean success rate of 88%, and an excellent intra-observer agreement (0.98). Treatment with CCl₄ over seven weeks drastically increased LS as compared to controls (18.2 ± 3.7 kPa vs 3.6 ± 1.2 kPa). Moreover, fibrosis stage highly correlated with LS (Spearman coefficient = 0.88, $P < 0.01$). In the amyloidosis model, much higher LS values were obtained, reaching maximum values of > 150 kPa. LS significantly correlated with the amyloidosis index (0.93, $P < 0.0001$) and the plasma concentration of mutant hApoA-II (0.62, $P < 0.005$). Transient micro-elastography should make it possible to measure the evolution of pathologies such as fibrosis, in the same animal during longitudinal studies. Thus, TME could be a valuable non-invasive tool to assess the evolution of disease as a function of time and the response to treatment of fibrosis in *in vivo* murine models without proceeding to euthanasia.

Terminology

Elastography is a technique used to measure the elasticity of biological tissues. It has been introduced as a novel diagnostic tool in oncology and hepatology. Amyloidoses are a group of β -structure protein deposition diseases. Liver, kidney, spleen, heart, joints, muscles, and gastrointestinal tract are usually involved in the systemic forms of amyloidosis.

Peer review

This study is of interest because it introduces a method for successfully measuring liver stiffness in mice. Unlike previous methods, this method is noninvasive and quantitative. The method is illustrated in a model of CCl₄ induced liver fibrosis and also in an amyloidosis model.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Ma WH

Cinnamon extract suppresses experimental colitis through modulation of antigen-presenting cells

Ho-Keun Kwon, Ji-Sun Hwang, Choong-Gu Lee, Jae-Seon So, Anupama Sahoo, Chang-Rok Im, Won Kyung Jeon, Byoung Seob Ko, Sung Haeng Lee, Zee Yong Park, Sin-Hyeog Im

Ho-Keun Kwon, Ji-Sun Hwang, Choong-Gu Lee, Jae-Seon So, Anupama Sahoo, Zee Yong Park, Sin-Hyeog Im, School of Life Sciences and Immune Synapse Research Center, Gwangju Institute of Science and Technology, Gwangju 500-712, South Korea

Chang-Rok Im, Global leader program, Bugil Academy, Cheonan, Gyeonggi-do 330-941, South Korea

Won Kyung Jeon, Byoung Seob Ko, Korea Institute of Oriental Medicine, Daejeon 305-811, South Korea

Sung Haeng Lee, Chosun University School of Medicine, Gwangju 501-759, South Korea

Author contributions: Kwon HK performed the majority of experiments; Hwang JS, Lee CG and So JS performed TNBS-induced colitis and data analysis; Sahoo A and Im CR gave help with manuscript correction; Jeon WK, Ko BS and Lee SH prepared water soluble fractions of cinnamon extract and performed HPLC and MALDI analysis for quality control; Park ZY and Im SH designed and financed this study.

Supported by Grants from the BioGreen 21 Program, Rural Development Administration (PJ007054), Regional Technology Innovation Program of the MOCIE (RTI05-01-01) and by Korea Healthcare Technology R&D Project, Ministry of Health and Welfare (A080588-20)

Correspondence to: Dr. Sin-Hyeog Im, School of Life Sciences and Immune Synapse Research Center, Gwangju Institute of Science and Technology, Gwangju 500-712, South Korea. imsh@gist.ac.kr

Telephone: +82-62-7152503 Fax: +82-62-7152484

Received: September 20, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate the anti-inflammatory effects of cinnamon extract and elucidate its mechanisms for targeting the function of antigen presenting cells.

METHODS: Cinnamon extract was used to treat murine macrophage cell line (Raw 264.7), mouse primary antigen-presenting cells (APCs, MHCII⁺) and CD11c⁺

dendritic cells to analyze the effects of cinnamon extract on APC function. The mechanisms of action of cinnamon extract on APCs were investigated by analyzing cytokine production, and expression of MHC antigens and co-stimulatory molecules by quantitative real-time PCR and flow cytometry. In addition, the effect of cinnamon extract on antigen presentation capacity and APC-dependent T-cell differentiation were analyzed by [³H]-thymidine incorporation and cytokine analysis, respectively. To confirm the anti-inflammatory effects of cinnamon extract *in vivo*, cinnamon or PBS was orally administered to mice for 20 d followed by induction of experimental colitis with 2,4,6 trinitrobenzenesulfonic acid. The protective effects of cinnamon extract against experimental colitis were measured by checking clinical symptoms, histological analysis and cytokine expression profiles in inflamed tissue.

RESULTS: Treatment with cinnamon extract inhibited maturation of MHCII⁺ APCs or CD11c⁺ dendritic cells (DCs) by suppressing expression of co-stimulatory molecules (B7.1, B7.2, ICOS-L), MHCII and cyclooxygenase (COX)-2. Cinnamon extract induced regulatory DCs (rDCs) that produce low levels of pro-inflammatory cytokines [interleukin (IL)-1 β , IL-6, IL-12, interferon (IFN)- γ and tumor necrosis factor (TNF)- α] while expressing high levels of immunoregulatory cytokines (IL-10 and transforming growth factor- β). In addition, rDCs generated by cinnamon extract inhibited APC-dependent T-cell proliferation, and converted CD4⁺ T cells into IL-10^{high} CD4⁺ T cells. Furthermore, oral administration of cinnamon extract inhibited development and progression of intestinal colitis by inhibiting expression of COX-2 and pro-inflammatory cytokines (IL-1 β , IFN- γ and TNF- α), while enhancing IL-10 levels.

CONCLUSION: Our study suggests the potential of cinnamon extract as an anti-inflammatory agent by targeting the generation of regulatory APCs and IL-10⁺ regulatory T cells.

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Key words: Cinnamon extract; Inflammation; CD4 antigen; Antigen presenting cells; Cyclooxygenase-2; Tumor necrosis factor- α ; Interleukin-10; Inflammatory bowel disease

Peer reviewer: Joan Roselló-Catafau, PhD, Experimental Hepatology, Instituto de investigaciones Biomédicas de Barcelona, Consejo superior de Investigaciones Científicas, Roselló 161, 080036-Barcelona, Spain

Kwon HK, Hwang JS, Lee CG, So JS, Sahoo A, Im CR, Jeon WK, Ko BS, Lee SH, Park ZY, Im SH. Cinnamon extract suppresses experimental colitis through modulation of antigen-presenting cells. *World J Gastroenterol* 2011; 17(8): 976-986 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/976.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.976>

INTRODUCTION

Oriental herbal medicines have been used for the treatment of various types of diseases for thousands of years^[1,2]. Herbal medicines have immunomodulatory effects through diverse mechanisms of action. They can enhance immunity and induce tolerance in specific disease conditions^[3,4]. For example, curcumin derived from *Curcuma longa* has potent anti-inflammatory activities by inhibiting nuclear factor- κ B activation and blocking interleukin (IL)-12 signaling^[5-7]. Andrographolide from *Andrographis paniculata* and herbkinase have potent immunostimulatory activities. They increase lymphocyte proliferation, production of pro-inflammatory cytokines, and the humoral response^[8-10].

Among many herbal medicines, *Cinnamomum cassia* bark is the outer skin of an evergreen tall tree belonging to the family *Lauraceae*. Cinnamon contains various active components including cinnamic aldehyde, cinnamyl aldehyde, tannin, mucus and carbohydrates. Previous studies have shown diverse biological functions of cinnamon extract such as antioxidant, antimicrobial and antidiabetic effects^[11-16]. It also has potent anti-inflammatory properties by inhibiting the production of NO, cyclooxygenase (COX)-2 and prostaglandin (PG)E2 in macrophage cell lines^[17,18]. However, it is still unclear how cinnamon modulates the function of immune cells, especially *in vivo*.

Antigen-presenting cells (APCs) process and present antigens on MHC molecules, and play pivotal roles in regulation of diverse immune responses. They can potentiate active immunity against pathogens while inducing immunotolerance to unharmed or self-antigens^[19]. Recently, many studies have suggested the important role of APCs for induction of immunotolerance by inducing regulatory T cells and desensitization of effector T cells^[20-22]. Regulatory APCs have potent therapeutic possibility to suppress various types of inflammatory immune disorders^[20,23,24]. Among the APCs, regulatory dendritic cells (rDCs) have potent anti-inflammatory activities through

diverse mechanisms of action. These include production of anti-inflammatory cytokines, induction of active cell death of effector T cells, inhibition of T-cell proliferation, and high level expression of indoleamine 2,3-dioxygenase (iDO)^[20]. These immunomodulatory properties of rDCs induce regulatory T cells through cell to cell contact^[23]. The potent anti-inflammatory properties of rDCs have been employed to treat several types of immune disorders including graft rejection, graft-versus-host disease and autoimmune disorders^[24]. Hence, development of immunomodulators to enhance generation of rDCs could be a good strategy for the treatment of diverse inflammatory immune disorders.

In the present study, we evaluated the effects of cinnamon extract on modulation of effector function of APCs *in vitro* and *in vivo*. Treatment with cinnamon extract enhanced generation of rDCs that inhibited APC-dependent T-cell proliferation, and converted CD4⁺ T cells into IL-10^{high} CD4⁺ T cells. Moreover, oral administration of cinnamon extract significantly suppressed experimental colitis by inhibiting expression of pro-inflammatory cytokines while enhancing IL-10 levels.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6-8 wk old) and Do11.10 mice were purchased from SLC (Japan) or Jackson Laboratory (Bar Harbor, ME, USA), respectively. They were maintained under specific pathogen-free conditions in an animal facility at the Gwangju Institute of Science and Technology (GIST). All of the animal experiments were approved by the GIST Animal Care and Use Committee.

Preparation of cinnamon extract

Dried *Cinnamomum cassia* bark (Hwajin Distribution Co., Seoul, Korea) was pulverized and extracted in hot water for 3 h in a hot water extractor. The extract was filtered and the supernatant was concentrated with a rotary evaporator. The extract was then freeze-dried, resulting in a powder extract. The powder extract was suspended in sterilized distilled water at the appropriate concentrations. As we reported in our previous work^[25], HPLC analysis was performed by comparing the levels of trans-cinnamic acid (Sigma, St Louis, MO, USA) and cinnamic aldehyde (kindly provided by Dr. Ehren, Germany) as known standard markers for the quality control of composition of cinnamon extract in each experiment^[25]. Chromatography was carried out using 1% acetic acid (H₂O)/methanol (50:50 v/v) at room temperature on a Phenomenex Luna 5u C₁₈, 10-nm pore size, 250 × 4.60 mm I.D. column. The flow rate of the mobile phase was 2 mL/min. The amount of trans-cinnamic acid and cinnamic aldehyde was about 2.9 mg/g and 7.9 mg/g in each extract, respectively^[25].

Cell lines

Raw 264.7 cells were obtained from the Korean Cell Line

Bank (Seoul National University, Korea) and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 100 U/mL penicillin (Sigma) and 100 U/mL streptomycin (Sigma). Cells were cultured with 0.2 mg/mL cinnamon extract for 24 h and harvested for further analysis.

RNA isolation, cDNA synthesis, quantitative RT-PCR

Total RNA was prepared using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol. For reverse transcription, cDNA was generated using 1 µg total RNA, oligo(dT) primer (Promega, Madison, WI, USA) and Improm-II Reverse Transcriptase (Promega) in a total volume of 20 µL. One microliter of cDNA was amplified using the following RT-PCR primer sets: IL-1β (Forward 5'-GCAACTGTTCTGAACTCAACT-3', Reverse 5'-ATCTTTTGGGGTCCGTCAACT-3'), IL-2 (Forward 5'-CCTGAGCAGGATGGAGAATTACA-3' and Reverse 5'-TCCAGAACATGCCGCAGAG-3'), IL-4 (Forward 5'-ACAGGAGAAGGGACGCCAT-3' and Reverse 5'-GAAGCCCTACAGACGAGCTCA-3'), IL-6 (Forward 5'-GAGGATACCACTCCCAACAGACC-3' and Reverse 5'-AAGTGCATCATCATCGTTGTTC-3'), IL-10 (Forward 5'-ATAACTGCACCCACTTCCCA-3' and Reverse 5'-TCATTTCCGATAAGGCTTGG-3'), IL-12 p40 (Forward 5'-GGAAGCACGGCAGCAGAATA-3' and Reverse 5'-AACTTGAGGGAGAAGTAGGAATGG-3'), IL-17A (Forward 5'-TTCATCTGTGTCTCTGATGCT-3' and Reverse 5'-TTGACCTTCACATCTCTGGAG-3'), interferon (IFN)-γ (Forward 5'-TCAAGTGGCATAGATGTGGAAGAA-3' and Reverse 5'-TGGCTCTGCAGGATTTTCATG-3'), tumor necrosis factor (TNF)-α (Forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and Reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'), transforming growth factor (TGF)-β (Forward 5'-GAAGGCAGAGTTTCAGGGTCTT-3' and Reverse 5'-GGTTCCTGTCTTTGTGGTGAA-3'), HPRT (Forward 5'-TTATGGACAGGACTGAAAGAC-3' and Reverse 5'-GCTTTAATGTAATCCAGCAGGT-3'), B7.1 (Forward 5'-ACCCCAACATAACTGAGTCT-3' and Reverse 5'-TTCCAACCAAGAGAAGCGAGG-3'), B7.2 (Forward 5'-TGTTTCCGTGGAGACGCAAG-3' and Reverse 5'-CAGCTCACTCAGGCTTATGTTTT-3'), B7-DC (Forward 5'-GTGCGATTTTGACCGCAGAG-3', Reverse 5'-CTAGGGATGTGGAACAAAGCC-3'), MHCII (Forward 5'-CACCTCTCGTCTGTTCGGTGAC-3' and Reverse 5'-CCTCTCCCTGATGAGGGGTC-3'), ICOSL (Forward 5'-GACTGAAGTCCGGTGCAATGGT-3' and Reverse 5'-TGGGTTTTTCGATTTGCCAATAGA-3') and PD1L (Forward 5'-ATGCTGCCCTTCAGATCACAG-3' and Reverse 5'-TGTTGATTTTGCGGTATGGG-3').

Immunoblotting

Proteins were resolved by 10% SDS-PAGE, transferred onto PVDF membranes (Bio-Rad) and subjected to Western blotting analysis using anti-Cox-2 (Cayman) and peroxidase-conjugated secondary antibodies (DAKO). Proteins

were visualized with a chemiluminescence kit (Amersham Bioscience). β-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control.

TNBS-induced colitis model

Induction of experimental inflammatory bowel disease (IBD) was carried out as described previously with minor modification^[26]. To induce colitis, 1 mg TNBS in 50% ethanol was injected to slightly anesthetized mice through a catheter inserted into the rectum; 100 µL TNBS-ethanol mixture was carefully poured into the colon. The subsequent course of colitis was evaluated by assessing mortality, body weight decrease, and macroscopic and microscopic observations.

Macroscopic and histological evaluation of colitis

The intestines were removed from mice and evaluated as previously described (Marceau *et al.*, 2004). For histological scoring, intestines were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE) and scored as previously described^[26]. To check the immunological changes, 5 d after IBD induction, mesenteric lymph nodes were obtained, suspended as single cells, and the expression level of cytokines and COX-2 were measured by quantitative real-time PCR or immunoblotting.

Isolation and activation of APCs and CD4⁺ T cells

For isolation of APCs (MHCII⁺ or CD11c⁺ DCs), spleens and lymph nodes isolated from C57BL/6 mice were ground with 30-µm pore meshes at the single cell level. Then, APCs purified with MHCII⁺ Isolation Kit (Miltenyi) or CD11c⁺ DC Isolation Kit (Miltenyi) were washed three times with ice-cold PBS and harvested for further analysis. CD4⁺ T cells from Do11.10 mice (Jackson Laboratory) were purified with CD4⁺ T cells Isolation Kit (Miltenyi).

Cell stimulation and treatment with cinnamon extract

Raw 264.7 cells were stimulated with lipopolysaccharide (LPS) (10 µg/mL) alone or in combination with cinnamon extract (0.2 mg/mL) for 24 h, and then washed three times with PBS. MHCII⁺ APCs or CD11c⁺ DCs were stimulated with LPS (10 µg/mL) alone or in combination with cinnamon extract (0.1, 0.3 and 0.5 mg/mL) for 24 h. After three washes, cells were harvested for further analysis.

Co-culture experiment

APCs (MHCII⁺ or CD11c⁺ DCs) were stimulated with LPS (10 µg/mL) alone or in combination with several doses of cinnamon extract (0.1, 0.3 and 0.5 mg/mL) for 24 h. The cells were incubated in mitomycin C (20 µg/mL) containing medium for 30 min at 37°C, and washed five times with ice-cold PBS. After 24 h stimulation, cells were co-cultured with CD4⁺ T cells obtained from Do11.10 mice in the absence or presence of ovalbumin peptide (Ova; 5, 20, 50 µg/mL) in 96-well plates for 56–72 h. After that, 0.5 µCi [³H]-thymidine (NEN) was added to each well and the cells were incubated for an additional 6 h.

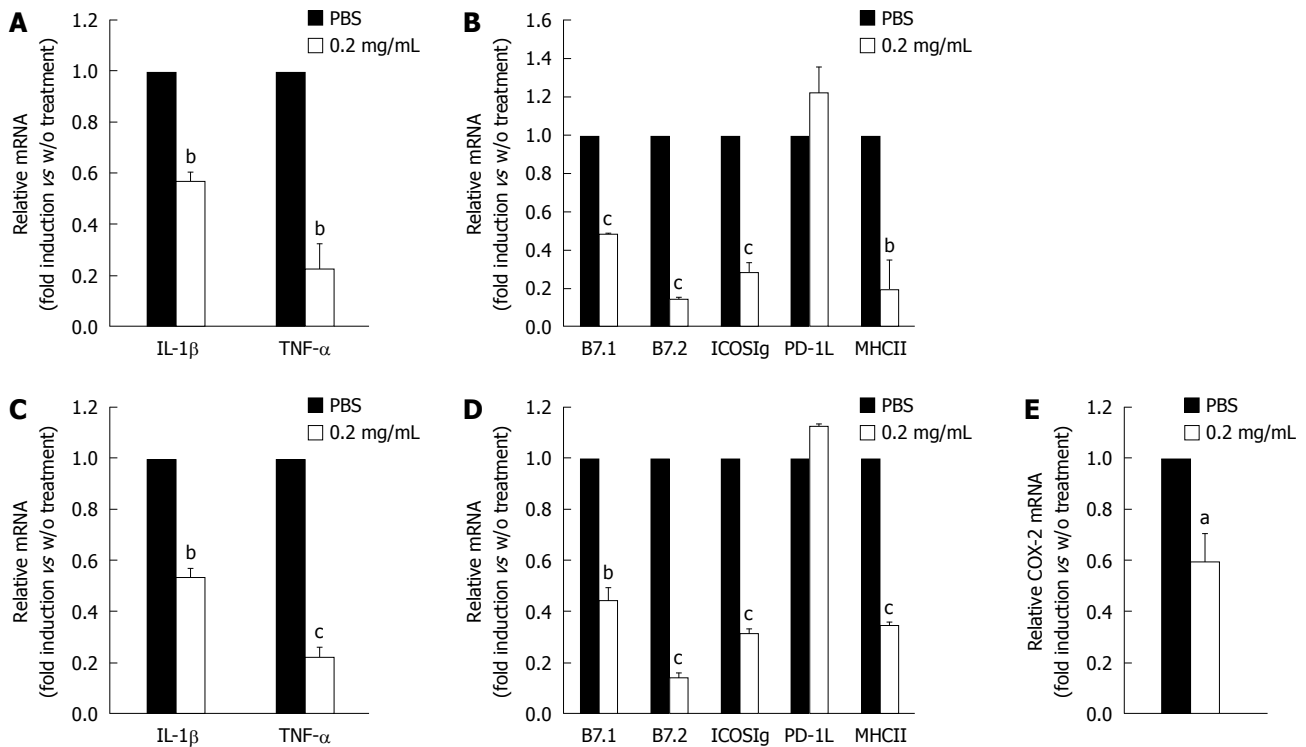


Figure 1 Treatment of cinnamon extract inhibits maturation of macrophage cell line. Murine Raw 264.7 macrophages were stimulated with PMA/ionomycin in the absence or presence of 0.2 mg/mL cinnamon extract. Expression levels of cytokines (A) and co-stimulatory molecules (B) were measured by quantitative real-time PCR. To mimic *in vivo* stimulation, cells were treated with lipopolysaccharide alone or in combination with 0.2 mg/mL cinnamon extract. Expression level of cytokines (C), co-stimulatory molecules (D) and cyclooxygenase (COX)-2 (E) was measured by quantitative real-time PCR. Error bars indicated SD. ^a $P < 0.05$, ^b $P < 0.005$, ^c $P < 0.001$. Data are representative of three individual experiments.

Cells were harvested and [^3H]-thymidine uptake was measured by liquid scintillation counting. For checking functional change of co-cultured CD4^+ T cells, CD4^+ T cells were re-isolated from co-cultured population and mRNAs were obtained to analyze the levels of cytokine expression by quantitative RT-PCR.

Intracellular cytokine staining

To detect intracellular TNF- α and IL-10 positive populations, cells were stimulated with LPS alone or in combination with cinnamon extract for 24 h, fixed with 2% paraformaldehyde (Sigma) for 10 min, permeabilized with permeabilization buffer (0.5% saponin, 1% BSA in PBS) for 30 min, and stained with anti-TNF- α -PE (BD Pharmingen) or anti-IL-10-PE (BD Pharmingen) for 20 min at 4°C. IgG isotypes were used as a control for all flow cytometry analysis. The IgG-positive population was shown to be < 0.2% (data not shown).

Statistical analysis

A two-tailed Student's *t* test was used, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Treatment with cinnamon extract inhibits activation and maturation of APCs

Previous reports have shown that cinnamon components

have a potent anti-inflammatory role by inhibiting NO synthesis^[17,18] without providing any immunological evidence. In this study, we tested the anti-inflammatory efficacy of cinnamon extract and elucidated its working mechanisms by targeting APCs. To test the effect of cinnamon extract on activation and maturation of macrophages we used Raw 264.7 cells (Figure 1). Raw cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24 h in the presence or absence of cinnamon extract at a concentration of 0.2 mg/mL, which did not induce any cell death or morphological change (data not shown). The expression levels of pro-inflammatory cytokines, co-stimulatory molecules and COX-2 were measured by real-time PCR. Raw cells upon activation produced a significant amount of IFN- γ and TNF- α . However, treatment with cinnamon extract significantly decreased expression levels of pro-inflammatory cytokine such as IL-1 β and TNF- α (Figure 1A). Expression level of COX-2, a key inflammatory mediator^[27], was also inhibited by cinnamon extract (Figure 1C). Next, to check the effect of cinnamon extract on maturation of Raw 264.7 cells, we analyzed the expression levels of activation markers for APCs such as MHCII and co-stimulatory molecules [B7.1, B7.2, ICOS ligand (ICOS-L) and PD1 ligand (PD-1L)] (Figure 1B). Consistent with inhibitory effects on pro-inflammatory cytokine expression, all the tested molecules were significantly reduced by treatment with cinnamon extract, except for PD-1L (Figure 1B). Since

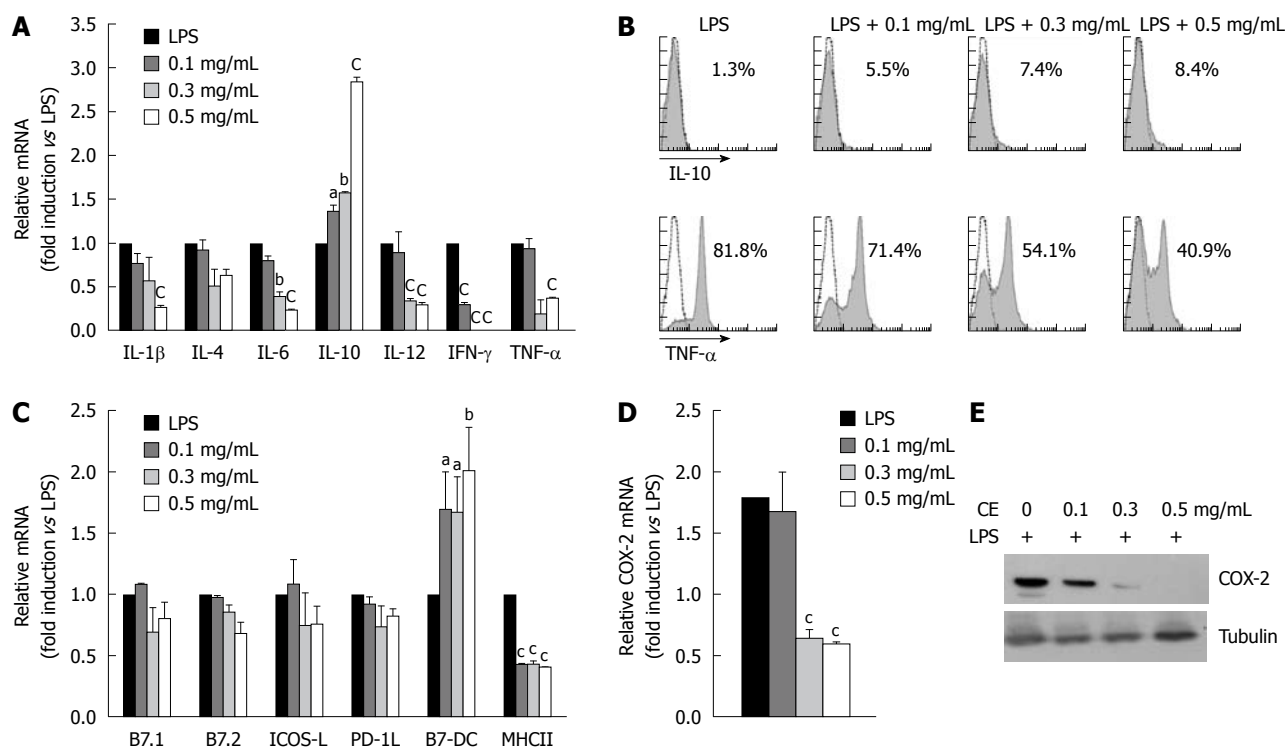


Figure 2 Treatment with cinnamon extract inhibits maturation of MHCII⁺ APCs. MHCII⁺ APCs were stimulated with lipopolysaccharide (LPS) alone or in combination with cinnamon extract (0.1, 0.3 and 0.5 mg/mL). Expression levels of cytokines (A) and co-stimulatory molecules (C) were measured by quantitative real-time PCR. B: Intracellular expression levels of interleukin (IL)-10 and tumor necrosis factor (TNF)-α proteins were analyzed by flow cytometry. Effect of cinnamon extract treatment on cyclooxygenase (COX)-2 expression at the mRNA (D) and protein (E) level was measured by quantitative real-time PCR and immunoblotting, respectively. Error bars indicated SD. ^a*P* < 0.05, ^b*P* < 0.005, ^c*P* < 0.001. Data are representative of three individual experiments. IFN: Interferon.

APCs can be activated by pathogen-associated molecular patterns^[28], to mimic the *in vivo* situation, we stimulated Raw 264.7 cells with LPS, a TLR4 ligand (Figure 1C-E). Cinnamon extract was added during LPS stimulation and its effect on APC maturation and expression of pro-inflammatory molecules was analyzed. LPS treatment significantly increased expression levels of inflammatory cytokines, cell surface molecules and COX-2 compared to non-stimulated cells. However, cinnamon extract strongly inhibited expression of pro-inflammatory cytokines (IL-1β and TNF-α) (Figure 1C), co-stimulatory molecules (B7.1, B7.2, ICOS-L and MHCII) (Figure 1D) and COX-2 (Figure 1E). These results suggest that cinnamon extract has potent anti-inflammatory properties by inhibiting activation and maturation of APCs *in vitro*.

To test further the effect of cinnamon extract treatment on primary APCs, first, we titrated the dose of cinnamon extract in primary MHCII⁺ APCs that did not induce cytotoxic characteristics such as growth inhibition and apoptosis. Up to 0.7 mg/mL cinnamon extract did not show any cytotoxicity (data not shown). Next, primary MHCII⁺ APCs were stimulated with LPS alone or in combination with cinnamon extract (0.1, 0.3 and 0.5 mg/mL) for 24 h, and then the expression levels of cytokines, co-stimulatory molecules and COX-2 were measured by quantitative real-time PCR (Figure 2A-C). Treatment of cinnamon extract significantly decreased LPS-induced expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IFN-γ and TNF-α) in a dose-dependent manner

(Figure 2A). Cinnamon extract significantly upregulated the expression level of IL-10, an immunomodulatory cytokine, in a dose-dependent manner (Figure 2A). To confirm whether cinnamon extract also modulates protein level, we compared IL-10 and TNF-α, as a representative anti- or pro-inflammatory cytokine, respectively, by flow cytometry (Figure 2B). Consistent with mRNA data, treatment with cinnamon extract significantly decreased the TNF-α⁺ while increasing the IL-10⁺ population. LPS-stimulated cells produced high levels of TNF-α⁺ (81.8%), whereas treatment with 0.5 mg/mL cinnamon and LPS significantly reduced its production (40.9%) in a dose-dependent manner (Figure 2B). However, treatment with cinnamon extract significantly increased the IL-10⁺ population from 1.3% (LPS alone) to 8.36% (LPS with 0.5 mg/mL cinnamon extract) in a dose-dependent manner (Figure 2B). In addition, treatment with cinnamon extract significantly decreased COX-2 mRNA (Figure 2D) and protein (Figure 2E) levels in a dose-dependent manner. Next, we tested whether cinnamon treatment also modulated activation stage of primary MHCII⁺ APCs. MHCII⁺ APCs were stimulated with LPS alone or in combination with cinnamon extract, and then expression levels of co-stimulatory molecules were analyzed (Figure 2C). Cinnamon treatment significantly decreased MHCII molecules that play critical roles in T-cell activation. However, cinnamon treatment did not induce significant alteration in expression patterns of co-stimulatory molecules including B7.1, B7.2, ICOS-L and PD-1L (Figure 2D). Cinnamon

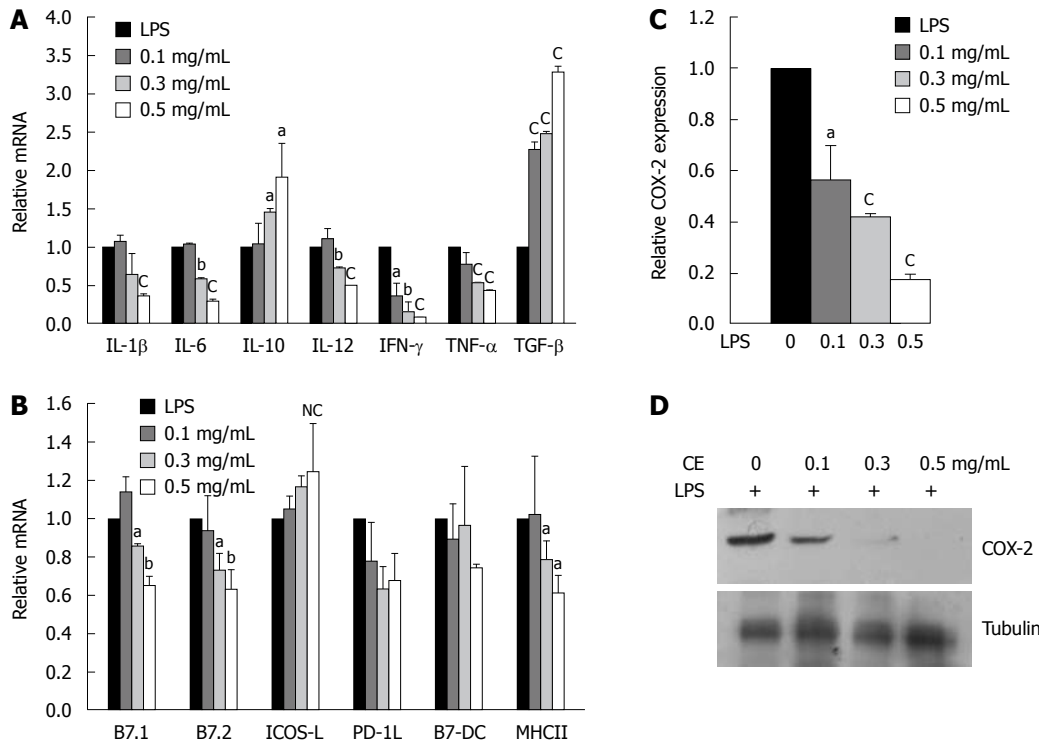


Figure 3 Treatment of cinnamon extract inhibits maturation of CD11⁺ dendritic cells. CD11⁺ dendritic cells (DCs) were stimulated in the absence or presence of cinnamon extract (CE) and expression levels of cytokines [interleukin (IL)-1 β , IL-4, IL-6, IL-10, IL-12, interferon (IFN)- γ , tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β] (A) and co-stimulatory molecules (B7.1, B7.2, ICOS ligand, B7-DC and MHCII) (B) were measured by quantitative real-time PCR. Effect of cinnamon extract (CE) treatment on cyclooxygenase (COX)-2 expression at the RNA (C) and protein (D) level was measured by quantitative real-time PCR and immunoblotting, respectively. Error bars indicated SD. ^a $P < 0.05$, ^b $P < 0.005$, ^c $P < 0.001$. Data are representative of three independent experiments.

treatment significantly increased expression levels of B7 DCs that have regulatory roles in inflammatory immune responses^[29,30]. Hence, these data indicate that primary MHCII⁺ APCs upon treatment with cinnamon extract may gain immunoregulatory properties.

Cinnamon extract treatment inhibits maturation of DCs

Based on the finding that cinnamon extract inhibits activation and maturation of MHCII⁺ APCs, we further tested whether treatment with cinnamon extract exerted a similar effect on DCs. CD11c⁺ DCs were isolated and stimulated with LPS alone or in combination with cinnamon extract for 24 h. Expression levels of cytokines, co-stimulatory molecules and COX-2 were measured by quantitative real-time PCR (Figure 3). CD11c⁺ DCs stimulated with LPS significantly increased expression levels of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-12, IFN- γ and TNF- α . However, expression levels of pro-inflammatory cytokines were markedly reduced in a dose-dependent manner upon cinnamon extract treatment (Figure 3A). Cinnamon extract treatment significantly upregulated the expression levels of immunomodulatory cytokines such as IL-10 and TGF- β ^[31,32] in a dose-dependent manner (Figure 3A). Moreover, treatment with cinnamon extract significantly downregulated COX-2 mRNA (Figure 3C) and protein (Figure 3D) levels in a dose-dependent manner. Cinnamon extract treatment also downregulated the expression levels of B7.1, B7.2 and MHCII (Figure 3B). These data indicate that cinnamon extract inhibited matu-

ration of CD11c⁺ DCs while inducing tolerogenic properties by increasing immunomodulatory cytokines.

Cinnamon extract treatment inhibits APC-dependent T-cell proliferation

One of the major roles of APCs is to process and present antigens in the context of MHCII to T cells, which is a key event for induction of adaptive immune responses. We tested whether cinnamon extract treatment affected APC-dependent T-cell proliferation. Two types of primary APCs were isolated: MHCII⁺ APCs and CD11c⁺ DCs. They were pre-stimulated with LPS alone or in combination with cinnamon extract for 24 h, and then co-cultured with naïve T cells obtained from Do 11.10 mice that specifically expressed Ova-specific T-cell receptors. In the presence of Ova peptide, co-culture was continued for 72 h. T-cell proliferation was mainly dependent on antigen presenting and co-stimulatory capacity of APCs (MHCII⁺ or CD11c⁺). APCs (MHCII⁺ (Figure 4A) and CD11c⁺ DCs (Figure 4B) pre-treated with cinnamon extract significantly lowered APC-dependent T-cell proliferation in a dose-dependent manner (Figure 4A). These results suggest that treatment with cinnamon extract negatively modulated antigen presenting capacity of MHCII⁺ APCs or CD11c⁺ DCs to stimulate T-cell proliferation.

DCs treated with cinnamon extract inhibit Th1 polarization while inducing IL-10^{high} CD4 T cells

DCs play important roles in regulating the activity and dif-

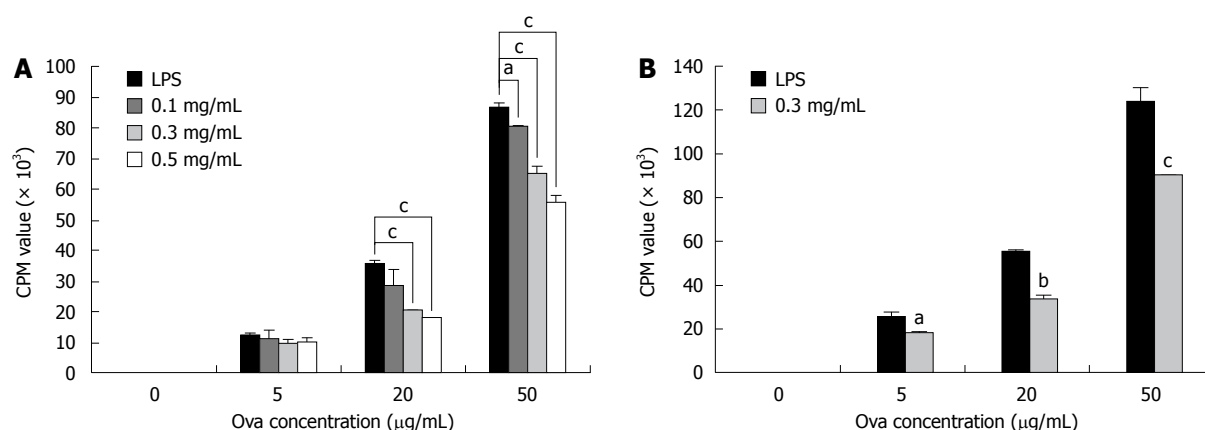


Figure 4 Treatment with cinnamon extract inhibits APC-dependent T-cell proliferation. APC-dependent T-cell proliferation was measured. MHCII⁺ APCs (A) and CD11c⁺ dendritic cells (DCs) (B) were pre-pulsed in the absence or presence of cinnamon extract for 24 h. Then, they were co-cultured with CD4⁺ T cells isolated from Do11.10 mice in the presence of Ova peptide. After 72 h co-culture, T-cell proliferation was measured by [³H]-thymidine incorporation assay. Error bars indicated SD. ^a*P* < 0.05, ^b*P* < 0.005, ^c*P* < 0.001. Data are representative of three independent experiments.

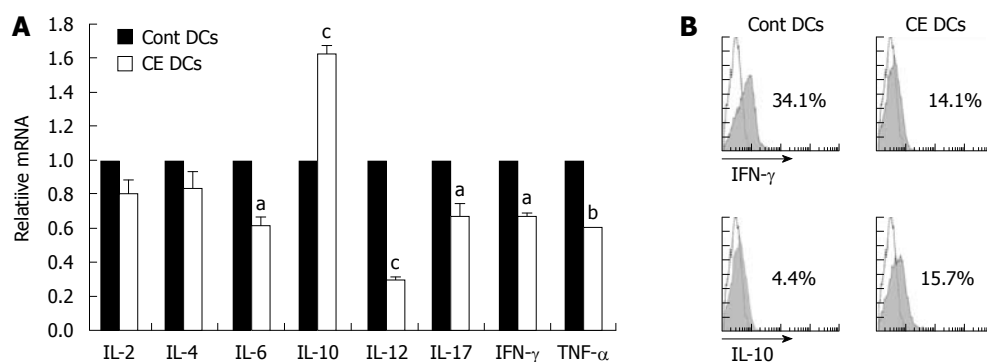


Figure 5 Cinnamon-extract-treated dendritic cells inhibit Th1 polarization. CD11c⁺ dendritic cells (DCs) were pre-pulsed with lipopolysaccharide (LPS) alone or in combination with cinnamon extract (CE) (0.2 mg/mL) for 24 h. Then, they were co-cultured with CD4⁺ T cells isolated from Do11.10 mice. A: After 72 h co-culture, CD4⁺ T cells were re-isolated from each treatment group and relative expression levels of cytokine mRNA were measured by quantitative real-time PCR; B: Intracellular protein levels of interleukin (IL)-10 and interferon (IFN)- γ in CD4⁺ T cells in each treatment group was analyzed by flow cytometry. Error bars indicated SD. ^a*P* < 0.05, ^b*P* < 0.005, ^c*P* < 0.001. Data are representative of three independent experiments.

ferentiation of naïve T cells into diverse T cell lineages such as Th1, Th2 and Th17 types^[33-35]. Since treatment with cinnamon lowered DC-dependent T-cell proliferation (Figure 4), we further tested the effects of cinnamon extract on DC-dependent T-cell polarization. T cells were co-cultured for 72 h with CD11c⁺ DCs pre-pulsed with LPS alone or in combination with 0.3 mg/mL cinnamon extract. Then, T cells were re-isolated and expression levels of T cell lineage-related cytokines were measured (Figure 5). T cells co-cultured with cinnamon-treated CD11c⁺ DCs produced much higher levels of IL-10 expression, while the levels of pro-inflammatory cytokines such as IL-6, IL-12, IL-17, IFN- γ and TNF- α were significantly decreased compared with control DCs (Figure 5A and B). No significant difference was observed in the levels of IL-2 and IL-4 between the treatment groups. These data indicate that CD11c⁺ DCs, upon treatment with cinnamon extract, could induce IL-10^{high} CD4 T cells while inhibiting Th1 polarization.

Oral administration of cinnamon extract ameliorates experimental colitis

To confirm the anti-inflammatory efficacy of cinnamon

extract *in vivo*, we tested whether oral administration of cinnamon extract could prevent progression of TNBS-induced experimental colitis, a typical Th1 type IBD^[36]. Mice were fed orally with PBS alone or in combination with cinnamon extract (50 μ g/g body weight) for 20 d, and experimental colitis was induced by intrarectal injection of TNBS. Clinical symptoms, weight loss and survival rate were monitored for 5 d. Both groups showed significant loss of body weight after 2 d of colitis induction. However, cinnamon-extract-treated mice began to recover weight loss starting from day 3, and almost restored their body weight at day 5, while PBS-treated mice continuously lost body weight during that period (Figure 6A). We also monitored survival rate. Cinnamon-extract-treated mice showed about 95% survival rate, while PBS-treated mice showed 50% survival on day 5 after colitis induction (data not shown). In accordance with weight loss and survival rate, colonic inflammation was significantly reduced in cinnamon-extract-treated mice compared with PBS-treated mice (Figure 6B). Tissue destruction and infiltration of mononuclear cells into the intestine, as shown by HE staining, were also significantly reduced by cinnamon

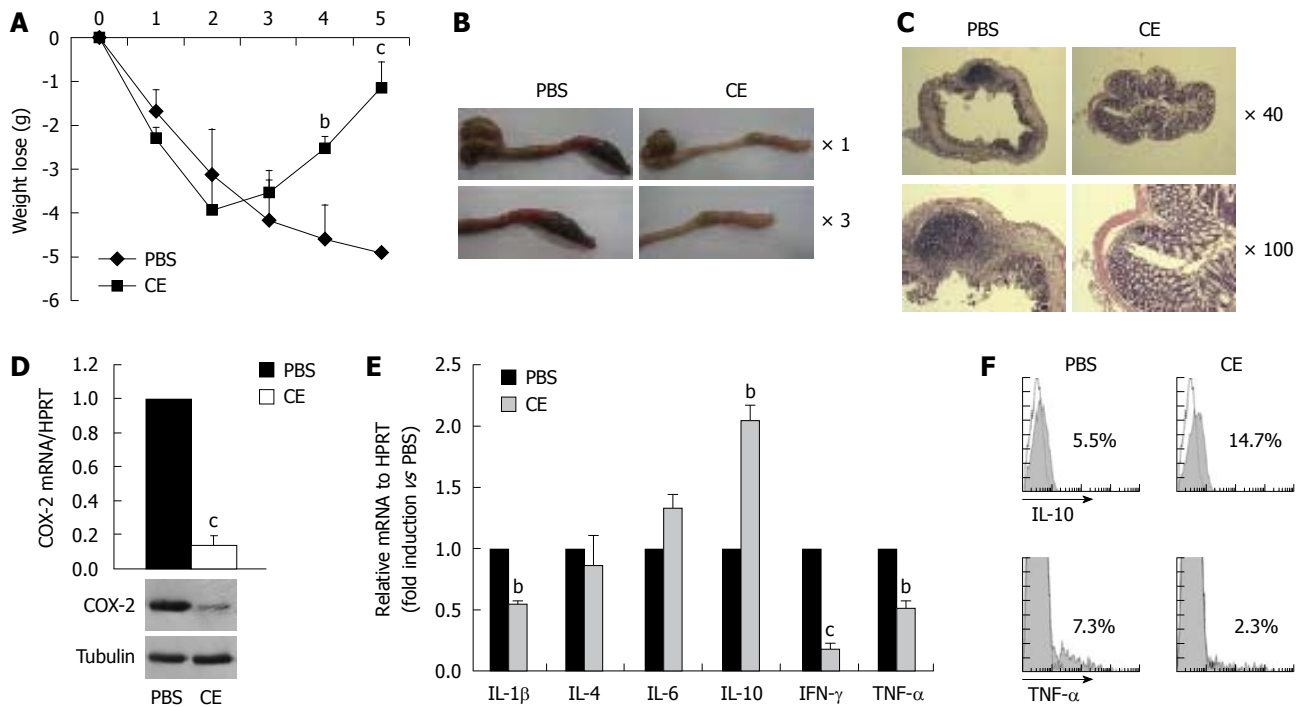


Figure 6 Oral administration of cinnamon extract ameliorates experimental colitis. Mice were orally fed with PBS or cinnamon extract (CE) (50 μ g/g per day) for 20 d, and intestinal colitis was induced by intrarectal injection of TNBS (50 μ g/g in 50% ethanol). A: A change in body weight was monitored for 5 d; B: Gross intestinal changes were analyzed with a magnifying glass 3 d after colitis induction; C: After HE staining, histological analysis was performed by comparing colon sections of treatment groups; D: After induction of inflammatory bowel disease, cyclooxygenase (COX)-2 expression in mesenteric lymph nodes was compared by quantitative real-time PCR and immunoblotting; E: Expression levels of cytokines in mesenteric lymphocytes were measured by quantitative real-time PCR; F: Intracellular protein levels of interleukin (IL)-10 and tumor necrosis factor (TNF)- α were measured by flow cytometry. Error bars indicated SD. $^bP < 0.005$, $^cP < 0.001$. Data are representative of three independent experiments. IFN: Interferon.

extract (Figure 6C). To investigate further the underlying mechanism of cinnamon-extract-mediated IBD protection, we measured the levels of pro- or anti-inflammatory mediators between the treatment groups. First, we measured the mRNA and protein levels of COX-2, an inflammatory mediator that is highly expressed in inflamed tissues and activated immune cells. In accordance with *in vitro* results (Figure 2D and E), oral administration of cinnamon extract significantly reduced the expression levels of COX-2 in mesenteric lymph nodes at both mRNA and protein levels (Figure 6D). We also compared expression level of various pathogenic or protective cytokines such as IL-1 β , IL-4, IL-6, IL-10, IFN- γ and TNF- α in cells isolated from mesenteric lymph nodes of cinnamon-extract-treated or non-treated mice. In agreement with clinical data, cells isolated from mesenteric lymph nodes of cinnamon-extract-treated mice expressed much lower levels of pathological cytokines such as IL-1 β , IFN- γ and TNF- α (Figure 6E). No significant difference in the expression levels of IL-4 and IL-6 was observed between the treatment groups. Oral administration of cinnamon extract significantly upregulated IL-10 expression levels (Figure 6E). To confirm these data at the protein level, IL-10 $^+$ or TNF- α $^+$ populations among total mesenteric lymph node cells were compared by flow cytometry. In accordance with mRNA level, oral administration of cinnamon extract significantly increased the IL-10 $^+$ population (PBS, 5.5% *vs* cinnamon extract, 14.7%) but decreased the TNF- α $^+$ population (PBS, 7.3% *vs* cinnamon extract,

2.3%). These data suggest that the prophylactic effect of cinnamon extract against colitis pathogenesis is mediated by its anti-inflammatory properties.

DISCUSSION

Treatment with cinnamon extract endowed APCs with tolerogenic characteristics resulting in inhibition of T-cell proliferation, T-cell polarization into Th1 type, while leading to IL-10 $^{\text{high}}$ CD4 T cells. Furthermore, oral administration of cinnamon extract significantly suppressed the progression of experimental colitis by increasing IL-10 production while suppressing the levels of pro-inflammatory cytokines.

APCs express high levels of MHC molecules on their surface to present antigen to adaptive immune cells, especially T cells^[19]. APCs are a heterogeneous population constituting various types of cells including DCs, B cells and macrophages^[19]. APCs play important roles in the initiation and enhancement of immune responses, and for induction of immunotolerance as well^[37-39]. DCs are key APCs that determine T-cell response and polarization into Th1 or Th2 cells^[33]. APCs also play pivotal roles in maintenance of immunological homeostasis through various action mechanisms^[20,24,40]. B cells have regulatory properties in modulating diverse inflammatory immune disorders such as IBD, collagen-induced arthritis, and autoimmune encephalomyelitis^[24,41-45]. The regulatory properties of B cells are mainly mediated by production of anti-inflam-

matory cytokines such as IL-10 and TGF- β , which are important to generate regulatory T cells.

rDCs promote immunotolerance rather than immune activation^[20,38,39]. rDCs usually express low levels of MHC molecules^[23], co-stimulatory molecules and pro-inflammatory cytokines (IL-1 β , IL-12 and TNF- α), while expressing high levels of IL-10, TGF- β and iDO. rDCs are resistant to maturation and help generation of regulatory T cells^[23]. Their potent anti-inflammatory functions have been successfully demonstrated in various types of inflammatory immune disorders^[23].

In this study, treatment with cinnamon extract significantly downregulated expression levels of MHCII and co-stimulatory molecules (B7.1 and B7.2) in macrophage cell lines (Figure 1D); primarily MCHII⁺ APCs (Figure 2C) and CD11c⁺ DCs (Figure 3B). Primary MCHII⁺ APCs significantly increased expression levels of B7-DC (PD-L2) upon cinnamon extract treatment in a dose-dependent manner (Figure 2C). B7-DC (PD-L2) is a ligand of PD1 which has potent anti-inflammatory properties to inhibit T-cell activation^[46]. Hence, an increase of B7-DC expression by cinnamon extract may mediate its anti-inflammatory properties.

One of the properties of tolerogenic APCs is to express high levels of immune regulatory cytokines (IL-10 and TGF- β) and iDO, while expressing low levels of pro-inflammatory cytokines^[23]. IL-10 and TGF- β are highly expressed in MCHII⁺ APCs, including B cells, macrophages and DCs, to suppress immune responses^[47,48]. MCHII⁺ APCs upon cinnamon extract treatment produced significantly reduced levels of pro-inflammatory cytokines, including IL-1 β , IL-6, IL-12, IFN- γ and TNF- α , while enhancing IL-10 levels (Figure 2A and B). Treatment with cinnamon extract also reduced NO production, a potent mediator of pro-inflammatory response in cinnamon-pulsed MCHII⁺ APCs (data not shown). In addition, cinnamon-pulsed DCs significantly decreased expression of pro-inflammatory cytokines, while upregulating IL-10 and TGF- β levels (Figure 3A). These results suggest that upregulated IL-10 and TGF- β levels in APCs by cinnamon extract may mediate downregulation of MHCII and co-stimulatory molecules^[47,48].

Tolerogenic APCs inhibit effector T-cell function by suppressing T-cell proliferation and cytokine expression^[23]. Upon LPS treatment, MCHII⁺ APCs and CD11c⁺ DCs strongly induced antigen-specific T-cell proliferation (Figure 4). However, cinnamon-pulsed MCHII⁺ APCs and CD11c⁺ DCs significantly reduced T-cell proliferation in response to antigen stimulation (Figure 4). Upon cinnamon extract treatment, how do APCs reduce their antigen-presenting capacity? An increased production of immunoregulatory cytokines (IL-10 and TGF- β) by cinnamon extract may reduce antigen-presenting capacity because increased levels of IL-10 and TGF- β suppress the expression of MHCII and co-stimulatory molecules on APC surfaces^[47,48]. Another possibility is that cinnamon extract may modulate DC properties. Tolerogenic APCs could generate IL-10^{high} (Tr1) or Foxp3⁺ regulatory T cells^[23]. These latter cells have pivotal roles to suppress ex-

aggerated immune responses in autoimmune, allergic and infectious diseases^[49,50]. Here, we found that T cells cultured with cinnamon-pulsed DCs significantly decreased expression levels of pro-inflammatory cytokines such as IL-6, IL-12, IL-17, IFN- γ and TNF- α , while increasing IL-10 levels (Figure 5), which indicates that cinnamon-pulsed DCs inhibit Th1 polarization of naïve T cells rather than generate IL-10^{high} regulatory T cells. Does cinnamon extract treatment increase Foxp3⁺ regulatory T cells? However, cinnamon extract treatment failed to increase Foxp3 expression within CD4⁺ T cells (data not shown).

COX-2 is a key mediator for PG synthesis. It promotes inflammatory immune response to exaggerate pathogenesis in rheumatoid arthritis, multiple sclerosis and cancer^[51]. In the normal state, expression of COX-2 is almost undetectable. However, many cell types including chondrocytes, macrophages, DCs and epithelial cells express high levels of COX-2 by various stimuli such as IL-1 β , TNF- α , phorbol esters, hypoxia, and LPS^[51,52]. COX-2 plays a pivotal role in inflammation and tumor progression^[53]. In our previous study, we have shown that cinnamon treatment effectively inhibits COX-2 expression in melanoma cell lines and in melanoma models^[25]. In this study, we found that treatment with cinnamon extract also significantly inhibited COX-2 expression in Raw macrophage cells (Figure 1), primary MCHII⁺ APCs (Figure 2), CD11c⁺ DCs (Figure 3), and in cells isolated from mesenteric lymph nodes in experimental colitis (Figure 6). IL-10 and TGF- β downregulate COX-2 expression in the immune system^[54,55], therefore, decreased expression of COX-2 levels (Figures 3, 4 and 6) may contribute to increased expression of IL-10 and TGF- β . However, we still do not know whether COX-2 inhibition by cinnamon is accomplished by intrinsic mechanisms such as active compounds of cinnamon, or extrinsic mechanisms mediated by immunomodulation. Currently, we are trying to identify active compounds from the cinnamon extract that have potent anti-COX-2 activities.

In summary, our study suggests a potent anti-inflammatory function of cinnamon extract that modulates effector function of APCs *in vitro* and *in vivo*. Cinnamon extract strongly inhibits maturation of APCs rather endows them with tolerogenic capacities that produce high levels of anti-inflammatory cytokines. Moreover, oral administration of cinnamon extract strongly ameliorated TNBS-induced experimental IBD by increasing IL-10 levels while downregulating pro-inflammatory cytokines. Further identification of the active components of cinnamon extract could lead to development of potent anti-inflammatory agents that target diverse inflammatory immune disorders.

COMMENTS

Background

Recently, the incidence of inflammatory immune disorders including inflammatory bowel disease, rheumatoid arthritis and allergic diseases has been growing fast. However, any conclusive medication for the treatment of these diseases is not available. Hence, oriental herbal medicines, which have been shown to

have various bioactive components, are regarded as a potent source of medication for the development of immunomodulators.

Research frontiers

Cinnamomum cassia bark contains various bioactive molecules, and has been shown to have diverse biological functions such as antioxidant, antimicrobial and antidiabetic effects. However, although little evidence suggests its immunomodulatory properties such as inhibiting the production of NO, cyclooxygenase-2 and prostaglandin E2 in macrophage cell lines, it is still unclear how cinnamon modulates the function of immune cells, especially *in vivo*. In this study, the authors demonstrated potent anti-inflammatory properties of cinnamon through modulating the properties of antigen-presenting cells (APCs) *in vitro* and *in vivo*.

Innovations and breakthroughs

The work has demonstrated the potent anti-inflammatory properties of cinnamon extract. It modulates the properties of APCs to endow them with immunoregulatory phenotypes. In particular, cinnamon extract treatment of APCs affected their maturation, to have tolerogenic immune function by upregulating expression patterns of tolerogenic marker molecules such as B7-DC and cytokines including interleukin (IL)-10 and transforming growth factor- β . Moreover, cinnamon-extract-treated APCs affected effector functions of T cells by inhibiting their proliferation, while increasing IL-10 production, resulting in potent therapeutic effects in an animal model of intestinal colitis. The data strongly suggest that cinnamon extract is a potent anti-inflammatory herbal medicine that can be used to treat inflammatory bowel disease.

Applications

The authors have provided scientific evidence that cinnamon extract has potent anti-inflammatory properties. Further identification of its active components could lead to development of potent anti-inflammatory agents that target diverse inflammatory immune disorders.

Terminology

Cinnamon is from *Cinnamomum cassia* bark, which is a small evergreen tree belonging to the family Lauraceae. APCs are cells that display foreign antigen complexes with the MHC on their surface, for the recognition of this complex by T-cells, using the T-cell receptors.

Peer review

This is an interesting study that showed the anti-inflammatory effects of cinnamon extract. The authors demonstrated that cinnamon extract acts on APCs and IL-10⁺ regulatory cells. Cinnamon administration ameliorated experimental colitis by inhibiting inflammatory cytokines.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM

Yi-Qi-Zeng-Min-Tang, a Chinese medicine, ameliorates insulin resistance in type 2 diabetic rats

Zeng Zhang, Hong-Li Xue, Yi Liu, Wen-Jian Wang

Zeng Zhang, Hong-Li Xue, Yi Liu, Institute of Chinese Integrative Medicine, Huashan Hospital, Fudan University, Shanghai 200040, China

Wen-Jian Wang, Institute of Chinese Integrative Medicine, Huashan Hospital, Fudan University, Shanghai 200040, China

Wen-Jian Wang, Institute of Integrated Clinical Medicine, Shanghai Academy of Traditional Chinese Medicine, Yueyang Integrative Medicine Hospital, Shanghai Traditional Chinese Medicine University, Shanghai 200437, China

Author contributions: Zhang Z conducted the research, acquisition of data and statistical analysis, and wrote the manuscript; Xue HL designed the research; Liu Y and Wang WJ revised the manuscript.

Supported by The Fok Ying-Tong Education Foundation, China, No. 114036; Leading Academic Discipline Project of Shanghai Municipal Education Commission, No. J50307; and State Administration of Traditional Chinese Medicine

Correspondence to: Wen-Jian Wang, MD, Professor, Institute of Chinese Integrative Medicine, Huashan Hospital, Fudan University, No. 12 Wulumuqi Zhong Road, Shanghai 200040, China. wj6518@163.com

Telephone: +86-21-52888225 Fax: +86-21-62480691

Received: October 13, 2010 Revised: November 17, 2010

Accepted: November 24, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate the effects of the Chinese herbal decoction, Yi-Qi-Zeng-Min-Tang (YQZMT), on insulin resistance in type 2 diabetic rats.

METHODS: Sprague-Dawley rats were divided into two dietary regiments by feeding either normal pellet diet (NPD) or high fat diet (HFD). Four weeks later, the HFD-fed rats were injected intraperitoneally with low-dose streptozotocin (STZ). Rats with non-fasting blood glucose level ≥ 16.67 mmol/L were considered type 2 diabetic and further divided into five subgroups: the type 2 diabetes model group, low-dose, medium-dose

and high-dose YQZMT groups, and rosiglitazone group. Age-matched NPD-fed rats served as controls. YQZMT or rosiglitazone were administered for 8 wk. Intraperitoneal glucose and insulin tolerance tests were performed before and after the treatment to measure the glucose tolerance and insulin sensitivity. Serum levels of biochemical parameters, adipocytokines, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), as well as free fatty acids (FFAs), were also analyzed.

RESULTS: There was significant elevation of insulin resistance and serum levels of fasting glucose (12.82 ± 1.08 mmol/L vs 3.60 ± 0.31 mmol/L, $P < 0.01$), insulin (7197.36 ± 253.89 pg/mL vs 4820.49 ± 326.89 pg/mL, $P < 0.01$), total cholesterol (TC) (8.40 ± 0.49 mmol/L vs 2.14 ± 0.06 mmol/L, $P < 0.01$), triglyceride (2.24 ± 0.12 mmol/L vs 0.78 ± 0.05 mmol/L, $P < 0.01$), low-density lipoprotein cholesterol (LDL-c) (7.84 ± 0.51 mmol/L vs 0.72 ± 0.04 mmol/L, $P < 0.01$) and decrease in high-density lipoprotein cholesterol (HDL-c) (0.57 ± 0.03 mmol/L vs 1.27 ± 0.03 mmol/L, $P < 0.01$) in the low-dose STZ and high-fat diet induced type 2 diabetic group when compared with the control group. Administration of YQZMT induced dose- and time-dependent changes in insulin resistance, glucose and lipid profile, and reduced levels of FFA, TNF- α and IL-6 in the type 2 diabetic rats. After the treatment, compared with the diabetic group, the insulin resistance was ameliorated in the high-dose YQZMT (2.82 g/100 g per day) group, with a significant reduction in serum glucose (12.16 ± 1.00 mmol/L vs 17.65 ± 2.22 mmol/L, $P < 0.01$), homeostasis model assessment of basal insulin resistance (22.68 ± 2.37 vs 38.79 ± 9.02 , $P < 0.05$), triglyceride (0.87 ± 0.15 mmol/L vs 1.99 ± 0.26 mmol/L, $P < 0.01$), TC (3.31 ± 0.52 mmol/L vs 6.50 ± 1.04 mmol/L, $P < 0.01$) and LDL-c (2.47 ± 0.50 mmol/L vs 6.00 ± 1.07 mmol/L, $P < 0.01$), and a significant increase in HDL-c (0.84 ± 0.08 mmol/L vs 0.50 ± 0.03 mmol/L, $P < 0.01$). But the body weight was not changed significantly.

CONCLUSION: YQZMT, which ameliorates insulin resistance and does not cause increase in body weight, may be a suitable therapeutic adjunct for the treatment of type 2 diabetes.

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Key words: Yi-Qi-Zeng-Min-Tang; Insulin resistance; Type 2 diabetes; Lipids; Adipocytokines; Free fatty acids

Peer reviewer: Dr. Juan Carlos Laguna Egea, Catedràtic de Farmacologia/Pharmacology Professor, Unitat de Farmacologia/Pharmacology laboratory, Facultat de Farmàcia/School of Pharmacy, Universitat de Barcelona/University of Barcelona, Avda Diagonal 643, Barcelona 08028, Spain

Zhang Z, Xue HL, Liu Y, Wang WJ. Yi-Qi-Zeng-Min-Tang, a Chinese medicine, ameliorates insulin resistance in type 2 diabetic rats. *World J Gastroenterol* 2011; 17(8): 987-995 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/987.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.987>

INTRODUCTION

The prevalence of type 2 diabetes is dramatically increasing throughout the world. Pathogenesis of this disease involves abnormalities in glucose and lipid metabolism, including inadequate insulin secretion from pancreatic β -cells and insulin resistance^[1,2].

Insulin resistance is a hallmark of type 2 diabetes, characterized by a decreased response of the peripheral tissues to insulin action^[3,4], and it most often precedes the onset of hyperglycemia and predicts development of type 2 diabetes^[5]. Insulin resistance produces elevations in glucose and lipid levels^[6]. It has become increasingly evident that obesity and the concomitant development of inflammation are major components of insulin resistance. Studies have revealed a clear association between pro-inflammatory signaling pathways and decreased insulin sensitivity^[7]. Pro-inflammatory adipokines including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), as well as free fatty acids (FFAs), have been implicated to play important roles in inflammation, insulin resistance, and type 2 diabetes^[8-11]. These inflammatory markers have been proposed to be risk factors for cardiovascular disease (CVD) in type 2 diabetes mellitus patients^[12].

Since insulin resistance both precedes and predicts type 2 diabetes, it is important to develop drugs to reverse insulin resistance^[13]. At present, thiazolidinediones (TZD), the agonists of the peroxisome proliferators-activated receptor (PPAR) γ , are the main agents to improve insulin sensitivity in the liver, adipose tissue, and skeletal muscle, thus improving glycaemic control in patients with type 2 diabetes^[14]. Despite the efficacy, some deleterious side effects of TZDs, including rosiglitazone and pioglitazone, have been noted, such as increasing body weight and aggravating heart failure through fluid retention^[15,16]. Therefore, development of new agents may help treat type 2 diabetic patients with insulin resistance.

Traditional Chinese Medicine (TCM) has a long history in the treatment of type 2 diabetes^[17]. Because of the supposedly less side effects when compared with modern medicine^[18], the use of traditional Chinese medicine and botanicals has been increasing rapidly^[19]. Yi-Qi-Zeng-Min-Tang (YQZMT), a traditional Chinese compound recipe of 10 medicinal herbs, is considered a useful medicine for the amelioration of insulin sensitivity of type 2 diabetic patients. *Radix Astragali* (Huangqi), the chief herb of YQZMT, has been shown to alleviate glucose intolerance and insulin resistance^[20]. It seems that this traditional Chinese herbal compound recipe is valued in the glucose homeostasis, and may be utilized as adjuvant therapy for the control of diabetes and its complications.

The high-fat diet and low-dose streptozotocin (STZ) induced type 2 diabetic rat model mimicking the natural history of the disease events (from insulin resistance to β cell dysfunction) as well as metabolic features of human type 2 diabetes, where the high-fat diet initiated a state of insulin resistance and followed by the addition of low-dose STZ, has been known to induce a mild impairment of insulin secretion characteristic of the later stage of type 2 diabetes mellitus^[21]. For this study, we aimed at investigating the effect of the Chinese herbal decoction YQZMT on insulin resistance in rats with type 2 diabetes.

MATERIALS AND METHODS

Composition and preparation of YQZMT

YQZMT was composed of 10 medicinal herbs, as shown in Table 1. The mixture was decocted for three times by refluxing with water (1:8, w/v) for 2 h, 1 h and 1 h, respectively. The solution obtained was concentrated to give an extract. The yield of YQZMT extract was 36.76% (w/w) compared with the original herbs. The process was manipulated by the National Engineering and Research Center for Traditional Chinese Medicine under internationally certified good manufacturing practice guidelines. Thin-layer chromatography and high-performance liquid chromatography identification was used to authenticate the plants. The YQZMT extract was stored at 4°C, and was diluted to the desired concentrations in distilled water before use.

Animal experiments

Sixty-five Sprague-Dawley rats (male, weight 180-220 g) were obtained from Shanghai Slaccas Laboratory Animal Company Limited, Shanghai, China. Animals were housed in standard polypropylene cages (three rats/cage) in a temperature- and humidity-controlled room with a 12 h light-dark cycle. All the rats were provided with rat normal pellet diet (NPD) (Shanghai Slaccas Laboratory Animal Company Limited, Shanghai, China) and water ad libitum, prior to the dietary manipulation.

After 1 wk of acclimatization, the rats were randomly divided into two dietary regiments consisting of 10 and 55 rats by feeding either NPD or high fat diet (HFD) (18% lard, 8% yolk powder, 2% cholesterol, 0.2% sodium cholate and 71.8% powdered NPD, as a percentage of

Table 1 Composition of Yi-Qi-Zeng-Min-Tang

Component	Part used	Amount used (%)
<i>Radix Astragali</i>	Root	16.0
<i>Mung bean coating</i>	Seed bark	16.0
<i>Folium Perillae</i>	Leaf	10.6
<i>Phellodendron amurense</i> Rupr.	Bark	10.6
<i>Pollen Typhae</i>	Pollen	10.6
<i>Serissa foetida</i>	Whole strain	10.6
<i>Ramulus Cinnamomi</i>	Twig	6.4
<i>Radix Aconiti Lateralis Preparata</i>	Prepared root	6.4
<i>Coptis Chinensis</i> Franch	Root	6.4
<i>Rhizoma Alismatis</i>	Tuberous stem	6.4

total kcal, manufactured by Shanghai Slaccas Laboratory Animal Company Limited, Shanghai, China) ad libitum, respectively, for the initial period of 4 wk.

After the 4 wk of dietary manipulation, the 55 HFD-fed rats were injected intraperitoneally (ip) with low-dose STZ (35 mg/kg, Sigma), while the 10 NPD-fed rats were injected ip with vehicle citrate buffer (pH 4.4) in a dose volume 1 mL/kg 3 d after the STZ or vehicle injection, non-fasting blood glucose (NFBG) was measured in whole blood collected from the tail vein by a portable Glucometer (Accu-Check Active, Roche Diagnostics Limited, Germany). Rats with NFBG level ≥ 16.67 mmol/L were considered diabetic and selected for further studies. Fasting blood was collected from retro-orbital plexus of the rats under light ether anesthesia using capillary tubes at day 7 after the STZ or vehicle injection to measure biochemical parameters [fasting glucose level, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and insulin]. The rats were fed on their respective diets until the end of the study.

Fifty diabetic rats (the diabetic success rate was 90.9%) were further divided into five subgroups: type 2 diabetes model group (MOD, $n = 10$), low-dose YQZMT group (LDY, $n = 10$), medium-dose YQZMT group (MDY, $n = 10$), high-dose YQZMT group (HDY, $n = 10$), and rosiglitazone group (ROS, $n = 10$). The age matched NPD-fed rats served as control group (CON, $n = 10$). Administration of YQZMT or rosiglitazone lasted 8 wk. LDY, MDY and HDY rats were dosed orally with YQZMT 0.47, 1.41, 2.82 g/100 g per day, respectively. ROS rats were given by oral gavage (4 mg/kg per day), this dose was selected since it was found to rapidly induce PPAR γ -dependent genes^[22]. CON and MOD group rats were given with an equal volume of distilled water once a day for 8 wk. Fasting blood was collected from retro-orbital plexus of the rats under light ether anesthesia using capillary tubes at 4 wk and 8 wk after treatment. The experiments were approved by the ethics committee of our institution. All procedures were in accordance to the rules and guidelines of the Experimental Animal Center of Fudan University.

Intraperitoneal glucose and insulin tolerance tests

The intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were performed at day 7 after the STZ or vehicle injection in diabetic ($n = 5$) and con-

trol ($n = 5$) rats, also at the end of the experiment in various groups ($n = 5$ for each group). After an overnight fast (12–16 h), fasting blood was collected from retro-orbital plexus (time 0), and then 50% glucose solution (2 g/kg body weight) or neutral insulin (Novo Nordisk, Denmark) was injected intraperitoneally. Blood samples were collected from retro-orbital plexus at 30, 60, and 120 min for measurement of glucose. The areas under the glucose curves (AUC) were calculated for each parameter by the trapezoidal rule in IPGTT. In ITT, the value was presented as a percentage of initial glucose level.

Blood sampling and analysis

Blood sample of rats were collected from retro-orbital plexus under light ether anesthesia using capillary tubes. Samples were centrifuged at $2500 \times g$ for 10 min at 4°C, blood serum was removed and aliquot for the respective analytical determinations, stored at -80°C until analysis. The serum was analyzed for glucose, TG, TC, HDL-c, LDL-c, and insulin. Serum glucose concentration was measured by glucose oxidase method (Applygen Technologies Inc, Beijing, China). Insulin was analyzed by rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Sweden). Levels of TG, TC, HDL-c and LDL-c were assayed with enzymatic assay kits (Shanghai Kexin Biotechnology Research Institute, Shanghai, China). Serum levels of FFA, TNF- α and IL-6 were measured by commercially available rat ELISA kits. The homeostasis model assessment of basal insulin resistance (HOMA-IR) = fasting glucose (mmol/L) \times fasting insulin (IU/L)/22.5. Lower HOMA-IR values indicated a greater insulin sensitivity, whereas higher HOMA-IR values indicated a lower insulin sensitivity (insulin resistance).

Statistical analysis

Data were presented as mean \pm SE. The unpaired Student's t test was used for analyzing the data between two groups. Statistical differences among more than two groups were determined using one-way analysis of variance. P value < 0.05 was considered statistically significant. The statistical SPSS Version 16.0 software was used for statistical calculations.

RESULTS

General characteristics of NPD-fed rats and HFD-fed/STZ induced diabetic rats

Type 2 diabetic rats induced by STZ (35 mg/kg, ip) after 4 wk of HFD feeding exhibited significant hyperglycemia, dyslipidemia and hyperinsulinemia compared with NPD-fed control rats injected with vehicle citrate buffer (1 mL/kg, ip) (Table 2). In addition, the feeding of HFD for 4 wk resulted in significant increase (data not shown) in body weight as compared with NPD-fed rats, and STZ produced reduction in the body weight of the HFD-fed rats, which was still considerably higher than NPD-fed control rats injected with vehicle citrate buffer (Table 2). The HOMA-IR score in the diabetic rats was 6.2-folds higher than in the control rats (Table 2).

Table 2 General characteristics of normal pellet diet-fed rats and high fat diet-fed/streptozotocin induced diabetic rats (mean \pm SE)

	NPD (<i>n</i> = 10)	HFD+STZ (<i>n</i> = 50)
Body weight (g)	411.30 \pm 2.74	457.90 \pm 2.43 ^b
Glu (mmol/L)	3.60 \pm 0.31	12.82 \pm 1.08 ^b
FINS (pg/mL)	4820.49 \pm 326.89	7197.36 \pm 253.89 ^b
HOMA-IR	18.74 \pm 1.56	97.26 \pm 4.98 ^b
TG (mmol/L)	0.78 \pm 0.05	2.24 \pm 0.12 ^b
TC (mmol/L)	2.14 \pm 0.06	8.40 \pm 0.49 ^b
HDL-c (mmol/L)	1.27 \pm 0.03	0.57 \pm 0.03 ^b
LDL-c (mmol/L)	0.72 \pm 0.04	7.84 \pm 0.51 ^b

Glu: Fasting serum glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment of basal insulin resistance; TG: Triglyceride; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; NPD: Normal pellet diet; HFD: High fat diet; STZ: Streptozotocin. ^b*P* < 0.01 vs NPD group.

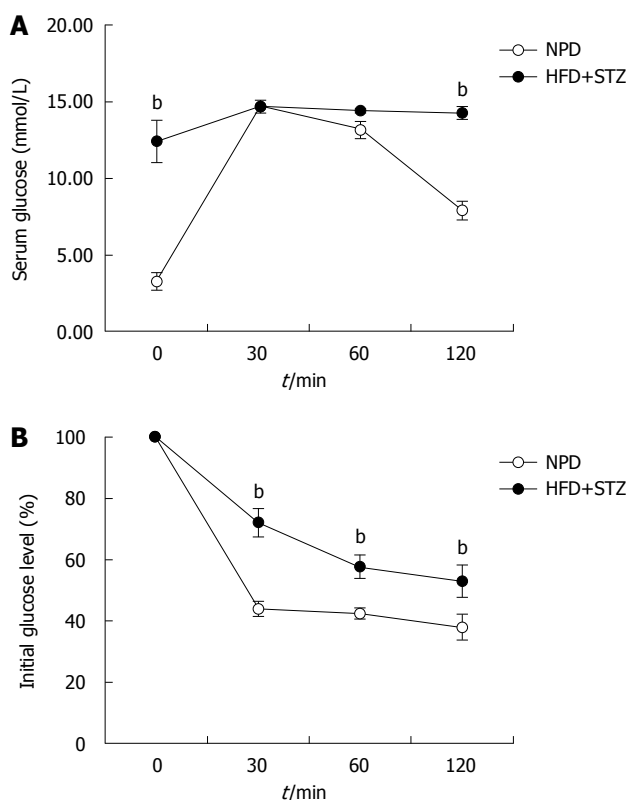


Figure 1 Intra-peritoneal glucose tolerance test and insulin tolerance test in normal pellet diet-fed control rats and high fat diet-fed/streptozotocin induced type 2 diabetic rats. A: Serum glucose during intra-peritoneal glucose tolerance test in normal pellet diet (NPD)-fed rats and high fat diet (HFD)-fed/streptozotocin (STZ) induced diabetic rats (*n* = 5/group); B: Percentage of initial glucose level during insulin tolerance test in NPD-fed rats and HFD-fed/STZ induced diabetic rats (*n* = 5/group). ^b*P* < 0.01 vs NPD group.

IPGTT and ITT in NPD-fed rats and HFD-fed/STZ induced diabetic rats

IPGTT and ITT were carried out in NPD-fed control rats and HFD-fed/STZ induced type 2 diabetic rats to measure glucose tolerance and insulin sensitivity. Figure 1A

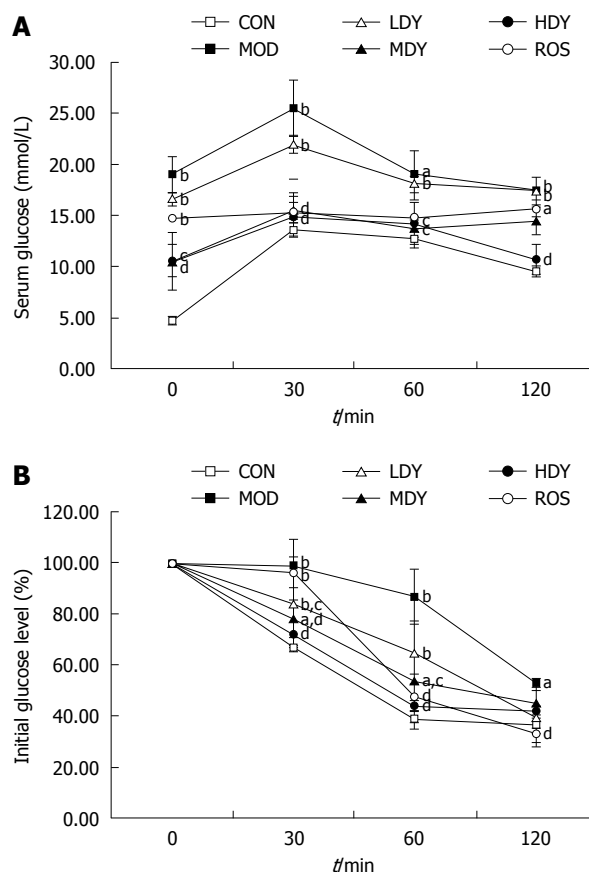


Figure 2 Intra-peritoneal glucose tolerance test and insulin tolerance test in diabetic rats after 8 wk of treatment. A: Serum glucose during intra-peritoneal glucose tolerance test (IPGTT) in diabetic rats (*n* = 5/group); B: Percentage of initial glucose level during insulin tolerance test (ITT) in diabetic rats (*n* = 5/group). YQZMT: Yi-Qi-Zeng-Min-Tang; CON: Control group; MOD: Type 2 diabetes model group; LDY: Low-dose YQZMT group; MDY: Medium-dose YQZMT group; HDY: High-dose YQZMT group; ROS: Rosiglitazone group. ^a*P* < 0.05, ^b*P* < 0.01 vs CON group; ^c*P* < 0.05, ^d*P* < 0.01 vs MOD group.

shows that HFD+STZ, as expected, led to glucose intolerance, since serum glucose concentrations increased from fasting levels of 12.41 ± 1.38 mmol/L to nearly 14.75 ± 0.2 mmol/L by 30 min and were still greatly increased over basal levels 2h after the glucose challenge. The NPD-fed control rats showed a significant elevation in serum glucose concentrations at 30 min but returned nearly to the basal levels within 2h after the glucose administration. Accordingly, the AUC was significantly greater in the HFD+STZ group than in the NPD group (1704 ± 40.05 mmol/L per minute *vs* 1315.2 ± 60.45 mmol/L per minute, *P* < 0.01). To investigate the differences in insulin sensitivity, we performed an ITT at different time points (Figure 1B). Insulin was given intra-peritoneally and blood was collected for the measurement of glucose. After insulin administration, the percentage of initial glucose level in HFD+STZ was shown to be significantly higher than in the NPD group during 120 min. These results indicated that HFD with STZ 35 mg/kg injection developed a diabetic model which was an analogue to type 2 diabetes mellitus with insulin resistance.

Table 3 General characteristics of diabetic rats after 4 wk of treatment (mean \pm SE)

	CON (<i>n</i> = 10)	MOD (<i>n</i> = 10)	LDY (<i>n</i> = 10)	MDY (<i>n</i> = 10)	HDY (<i>n</i> = 10)	ROS (<i>n</i> = 10)
Body weight (g)	496.20 \pm 13.40	418.08 \pm 16.06 ^b	434.80 \pm 13.15 ^b	433.75 \pm 10.99 ^b	443.14 \pm 13.15 ^a	439.30 \pm 15.27 ^b
Glu (mmol/L)	3.95 \pm 0.27	18.40 \pm 1.63 ^b	17.57 \pm 0.86 ^b	14.35 \pm 1.60 ^{b,c}	13.20 \pm 0.64 ^{b,d}	13.00 \pm 1.29 ^{b,d}
FINS (pg/mL)	3866.43 \pm 249.23	9553.83 \pm 899.22 ^b	7170.76 \pm 739.99 ^b	7228.81 \pm 480.47 ^a	7114.41 \pm 762.97 ^a	8363.51 \pm 564.91 ^b
HOMA-IR	16.02 \pm 1.16	191.49 \pm 28.32 ^b	133.39 \pm 13.41 ^b	99.67 \pm 12.97 ^{b,c}	98.99 \pm 12.40 ^{b,c}	107.66 \pm 13.01 ^{b,c}
TG (mmol/L)	1.38 \pm 0.13	2.99 \pm 0.56 ^b	2.91 \pm 0.47 ^b	2.62 \pm 0.44 ^a	2.58 \pm 0.77	2.46 \pm 0.31 ^a
TC (mmol/L)	2.06 \pm 0.11	8.06 \pm 0.69 ^b	8.08 \pm 0.80 ^b	6.49 \pm 1.40 ^b	5.45 \pm 0.64 ^{b,c}	7.79 \pm 0.78 ^b
HDL-c (mmol/L)	1.02 \pm 0.04	0.61 \pm 0.08 ^b	0.71 \pm 0.07 ^a	0.82 \pm 0.07	0.87 \pm 0.11 ^c	0.84 \pm 0.11
LDL-c (mmol/L)	1.04 \pm 0.08	7.46 \pm 0.75 ^b	7.37 \pm 0.82 ^b	5.67 \pm 1.40 ^b	4.58 \pm 0.70 ^{b,d}	6.95 \pm 0.85 ^b

Glu: Fasting serum glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment of basal insulin resistance; TG: Triglyceride; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; CON: Control group; MOD: Type 2 diabetes model group; LDY: Low-dose Yi-Qi-Zeng-Min-Tang (YQZMT) group; MDY: Medium-dose YQZMT group; HDY: High-dose YQZMT group; ROS: Rosiglitazone group. ^a*P* < 0.05, ^b*P* < 0.01 vs CON group; ^c*P* < 0.05, ^d*P* < 0.01 vs MOD group.

Table 4 General characteristics of diabetic rats after 8 wk of treatment (mean \pm SE)

	CON (<i>n</i> = 10)	MOD (<i>n</i> = 10)	LDY (<i>n</i> = 10)	MDY (<i>n</i> = 10)	HDY (<i>n</i> = 10)	ROS (<i>n</i> = 10)
Body weight (g)	562.73 \pm 12.17	424.15 \pm 18.30 ^b	412.60 \pm 12.89 ^b	444.88 \pm 12.02 ^b	445.71 \pm 15.54 ^b	470.80 \pm 19.9 ^{b,c}
Glu (mmol/L)	3.85 \pm 0.33	17.65 \pm 2.22 ^b	14.48 \pm 0.68 ^b	12.76 \pm 0.56 ^{b,d}	12.16 \pm 1.00 ^{b,d}	12.70 \pm 0.55 ^{b,d}
FINS (pg/mL)	3863.25 \pm 316.14	1973.70 \pm 237.35 ^a	3025.17 \pm 296.30	2275.06 \pm 256.45	1815.21 \pm 187.26 ^a	2087.53 \pm 220.78 ^a
HOMA-IR	16.59 \pm 2.65	38.79 \pm 9.02 ^b	48.48 \pm 4.74 ^b	30.37 \pm 3.57	22.68 \pm 2.37 ^c	21.66 \pm 2.13 ^c
TG (mmol/L)	0.83 \pm 0.15	1.99 \pm 0.26 ^b	1.88 \pm 0.31 ^b	1.19 \pm 0.16 ^c	0.87 \pm 0.15 ^d	1.05 \pm 0.16 ^d
TC (mmol/L)	1.56 \pm 0.15	6.50 \pm 1.04 ^b	5.80 \pm 0.66 ^b	4.51 \pm 0.76 ^b	3.31 \pm 0.52 ^{b,d}	4.71 \pm 0.51 ^b
HDL-c (mmol/L)	1.06 \pm 0.14	0.50 \pm 0.03 ^b	0.69 \pm 0.05 ^b	0.78 \pm 0.04 ^d	0.84 \pm 0.08 ^d	0.97 \pm 0.08 ^d
LDL-c (mmol/L)	0.50 \pm 0.05	6.00 \pm 1.07 ^b	5.11 \pm 0.67 ^b	3.73 \pm 0.50 ^b	2.47 \pm 0.50 ^{b,d}	3.74 \pm 0.49 ^b

Glu: Fasting serum glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment of basal insulin resistance; TG: Triglyceride; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; CON: Control group; MOD: Type 2 diabetes model group; LDY: Low-dose Yi-Qi-Zeng-Min-Tang (YQZMT) group; MDY: Medium-dose YQZMT group; HDY: High-dose YQZMT group; ROS: Rosiglitazone group. ^a*P* < 0.05, ^b*P* < 0.01 vs CON group; ^c*P* < 0.05, ^d*P* < 0.01 vs MOD group.

General characteristics of diabetic rats after treatment

After 4 and 8 wk of administration with YQZMT or rosiglitazone to the diabetic rats, body weight, serum glucose, insulin level, HOMA-IR value and lipids are shown in Tables 3 and 4. The levels of serum glucose, HOMA-IR, TG, TC, and LDL-c were significantly higher, while body weight and HDL-c were significantly reduced in the MOD group compared with that in the CON group, which again demonstrated that the disease animal model was established (Tables 3 and 4). At 4 wk, the MOD group had higher serum glucose levels with higher serum insulin levels compared with the CON group (Table 3), suggesting that the HFD+STZ caused insulin resistance. At 8 wk, serum insulin levels decreased in MOD group (Table 4). These results suggest that diabetes in HFD+STZ rats was the result of insulin resistance followed with relative insulin deficiency, as in human type 2 diabetes.

At 4 wk, the high-dose YQZMT (HDY) showed a significant reduction in serum glucose, HOMA-IR, TC and LDL-c, and a significant increase in HDL-c compared with MOD, while rosiglitazone (ROS) only reduced serum glucose and HOMA-IR (Table 3). After 8 wk of treatment, TG in HDY also showed a significant reduction (Table 4). The medium-dose YQZMT (MDY) also significantly reduced serum glucose and TG, and increased HDL-c. YQZMT of all oral dosage did not influence the body weight of diabetic rats. However, diabetic rats receiv-

ing rosiglitazone reduced TC and LDL-c, but without significant differences compared with the MOD group, and gained more body weight than the HDY group (Table 4). The HOMA-IR score in MOD group was 2.3 times higher than in the CON group, which markedly fell to 55.8% of ROS group and 58.5% of HDY group (Table 4).

IPGTT and ITT in diabetic rats after 8 wk treatment

IPGTT and ITT were carried out in diabetic rats after 8 wk of treatment to measure glucose tolerance and insulin sensitivity. Serum glucose levels were significantly elevated during the IPGTT in MOD compared with CON at all time points (Figure 2A). Accordingly, the AUC was roughly two-folds larger in MOD rats than in CON rats (2424.6 ± 372 mmol/L per minute vs 1340.1 ± 126.75 mmol/L per minute, *P* < 0.01). YQZMT and rosiglitazone attenuated the glucose intolerance, as the AUC was significantly smaller during the IPGTT in MDY (1674 ± 205.95 mmol/L per minute, *P* < 0.01), HDY (1567.8 ± 327.75 mmol/L per minute, *P* < 0.01), and ROS (1812.6 ± 134.55 mmol/L per minute, *P* < 0.01) compared with MOD rats. To investigate the differences in insulin sensitivity, we performed an ITT at different time points (Figure 2B). After insulin administration in CON group, glucose concentrations declined rapidly; however, the glucose concentrations declined slowly in MOD group. This demonstrated that MOD group presented insulin resis-

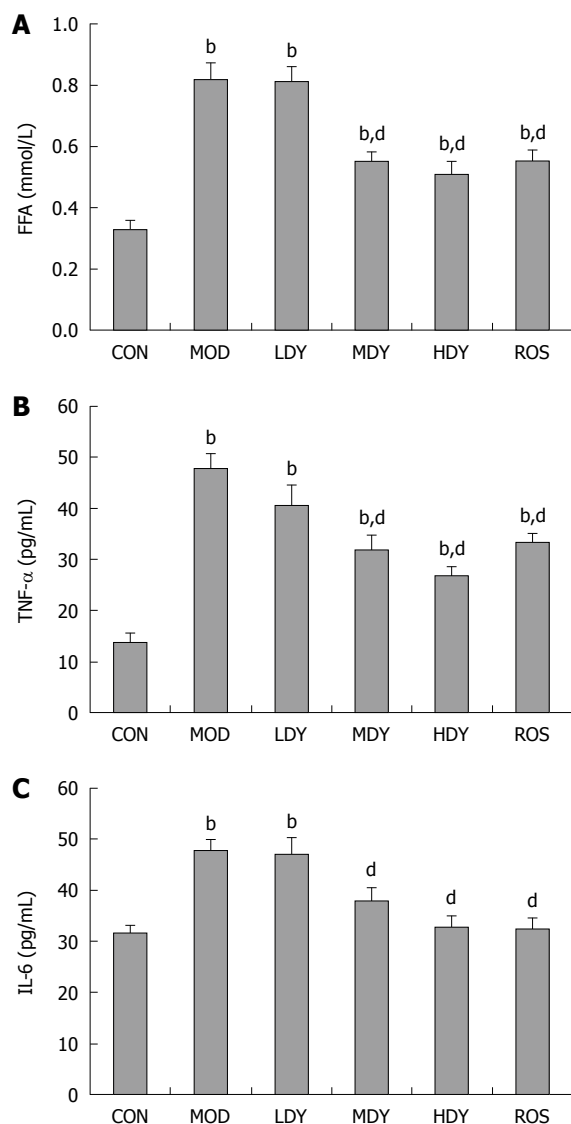


Figure 3 Free fatty acid and adipocytokines in diabetic rats after 8 wk treatment. A: Values of free fatty acid (FFA) in diabetes after 8 wk treatment; B: Values of tumor necrosis factor- α (TNF- α) in diabetes after 8 wk treatment; C: Values of interleukin-6 (IL-6) in diabetes after 8 wk treatment. CON: Control group; MOD: Type 2 diabetes model group; LDY: Low-dose Yi-Qi-Zeng-Min-Tang (YQZMT) group; MDY: Medium-dose YQZMT group; HDY: High-dose YQZMT group; ROS: Rosiglitazone group. Bar graphs indicate mean \pm SE. ^b $P < 0.01$ vs CON group; ^d $P < 0.01$ vs MOD group.

tance. YQZMT and rosiglitazone ameliorated the insulin resistance, since the percentage of initial glucose level in LDY, MDY, HDY and ROS was shown to be significantly lower than that of MOD group during 120 min.

FFA and adipocytokines in diabetic rats after 8 wk of treatment

The serum levels of FFA, TNF- α and IL-6 in diabetic rats after 8 wk of treatment are illustrated in Figure 3. When compared with CON group, the MOD group showed significant increase in serum levels of FFA (0.82 ± 0.05 mmol/L *vs* 0.33 ± 0.03 mmol/L, $P < 0.01$, Figure 3A), TNF- α (47.98 ± 2.75 pg/mL *vs* 13.91 ± 1.74 pg/mL, $P < 0.01$, Figure 3B), and IL-6 (47.77 ± 1.92 pg/mL *vs*

31.60 ± 1.45 pg/mL, $P < 0.01$, Figure 3C).

The diabetic rats treated with medium- and high-dose YQZMT (MDY and HDY group) and rosiglitazone (ROS group) showed significant reduction of the following parameters when compared with the untreated diabetic group (MOD group): serum levels of FFA (MDY *vs* MOD: 0.55 ± 0.03 mmol/L *vs* 0.82 ± 0.05 mmol/L; HDY *vs* MOD: 0.51 ± 0.04 mmol/L *vs* 0.82 ± 0.05 mmol/L; ROS *vs* MOD: 0.55 ± 0.04 mmol/L *vs* 0.82 ± 0.05 mmol/L, $P < 0.01$, respectively, Figure 3A), TNF- α (MDY *vs* MOD: 32.18 ± 2.88 pg/mL *vs* 47.98 ± 2.75 pg/mL; HDY *vs* MOD: 26.76 ± 1.94 pg/mL *vs* 47.98 ± 2.75 pg/mL; ROS *vs* MOD: 33.49 ± 1.75 pg/mL *vs* 47.98 ± 2.75 pg/mL, $P < 0.01$, respectively, Figure 3B), and IL-6 (MDY *vs* MOD: 37.84 ± 2.65 pg/mL *vs* 47.77 ± 1.92 pg/mL; HDY *vs* MOD: 32.74 ± 2.27 pg/mL *vs* 47.77 ± 1.92 pg/mL; ROS *vs* MOD: 32.54 ± 2.08 pg/mL *vs* 47.77 ± 1.92 pg/mL, $P < 0.01$, respectively, Figure 3C).

DISCUSSION

The therapeutic effects for type 2 diabetes are limited due to unavailability of effective medications. TCM has demonstrated a good practice in the treatment of diabetes mellitus and its complications^[17,20,23,24]. The present study was undertaken to investigate the effect of Chinese herbal decoction YQZMT on insulin resistance in high-fat diet and low-dose STZ-induced diabetic rats.

The chronic consumption of a high-fat diet is strongly associated with development of obesity^[25] and can induce insulin resistance in human and animals^[26-28]. The high-fat diet and low-dose STZ induced type 2 diabetic rat model mimicks the natural history of the disease events (from insulin resistance to β cell dysfunction) as well as metabolic features of human type 2 diabetes^[21,29]. In the present work, the model group of type 2 diabetes showed significant increase in serum levels of glucose, HOMA-IR value, TC, TG, LDL-c and decrease in HDL-c, coupled with impaired glucose tolerance and insulin sensitivity when compared with the normal control group. Insulin secretion of the model group rats, which was maintained initially, gradually declined but was not depleted at the end of the study. These results indicated the successful development of type 2 diabetes rat model with insulin resistance.

Administration of YQZMT induced dose- and time-dependent changes in biochemical parameters in the type 2 diabetic rats. In general, high-dose YQZMT exhibited the best effect. Treatment for 8 wk with high-dose YQZMT was found to significantly decrease the high serum glucose concentration, HOMA-IR, TC, TG, LDL-c, FFA, TNF- α , IL-6, and increase HDL-c compared with the model group, with a similar effect of rosiglitazone. However, rosiglitazone group reduced TC and LDL-c without significant differences. And a trend towards a decrease in serum insulin concentration could be found in the high-dose YQZMT group compared with the model group, while no change was seen in rosiglitazone group. IPGTT and ITT also verified that high-dose YQZMT

markedly improved glucose tolerance and insulin resistance. Like other studies^[30], we identified an increase in body weight in the group receiving rosiglitazone, and this was not found in groups administered with YQZMT.

Dyslipidemia contributes directly to development of type 2 diabetes mellitus as lipolytic products may induce gluconeogenesis in the liver, thus contributing to hyperglycemia^[31]. Well-established relationship of LDL-c levels with cardiovascular risk and the availability of proven treatments support LDL-c as the primary target^[32]. Furthermore, multiple epidemiologic studies have established a low level of HDL-c as an independent risk factor for CVD^[33]. While LDL-c-lowering strategies have consistently reduced cardiovascular risk, currently available options to increase low HDL-c levels are only moderately effective and associated with tolerance issues. In our study, reduction of serum TG (56.3%), TC (49.1%), LDL-c (58.8%) and increase of HDL-c (68%) were achieved after 8-wk treatment with high-dose YQZMT compared with the model group. However, diabetic rats receiving rosiglitazone treatment reduced TC and LDL-c, but without significant differences. It demonstrates that YQZMT may be a promising new strategy to address diabetic dyslipidemia and to reduce cardiovascular risk.

In accordance with the significant decrease of serum glucose, TG, TC and LDL-c, increase of HDL-c, amelioration of insulin resistance, and significant decrease of FFA, TNF- α and IL-6 levels were also observed in the high-dose YQZMT group compared with the model group. Chronically elevated FFA may impair insulin secretory function through the “lipotoxicity hypothesis” and can also induce or aggravate insulin resistance and contribute to the development of type 2 diabetes^[34-39]. The fact that TNF- α impairs insulin signaling, has been proven to be due to stimulation of serine phosphorylation of IRS (*via* activity of the serine kinase inhibitor of nuclear factor- κ B kinase), leading to both degradation of IRS and inhibition of tyrosine phosphorylation which is essential to insulin signaling and action^[8,40,41]. IL-6 plays a direct role in insulin resistance at the cellular level by inhibiting insulin receptor signal transduction through induction of suppressor of cytokine signaling-3^[42] and insulin metabolic actions including inhibition of insulin-induced glycogen synthesis^[43]. Our study confirmed again that the circulating FFA, TNF- α and IL-6 are elevated in established type 2 diabetes^[44-47] and rosiglitazone has the property of reducing levels of FFA, TNF- α and IL-6^[30,48,49]. Significant reduction of serum FFA, TNF- α and IL-6 was achieved after 8-wk treatment with medium- and high-dose YQZMT, similarly with rosiglitazone.

YQZMT may mediate glucose and lipid metabolism *via* ameliorating insulin resistance by down-regulating adipocytokines and fatty acids metabolism.

In conclusion, YQZMT has beneficial effects in insulin resistance, glycaemic control, dyslipidemia, FFA and adipocytokines in type 2 diabetes mellitus. Our findings demonstrate that YQZMT displays the insulin sensitization characteristic of rosiglitazone, but unlike rosigli-

tazone, does not cause any increase in body weight. Administration of YQZMT may be a suitable adjunct for the treatment of insulin resistance patients. Further studies will be required to identify the ingredients and chemicals in YQZMT responsible for the beneficial effects observed in the present study.

ACKNOWLEDGMENTS

We thank Teng Zhang, Wei-Hua Chen and Jian Ying for their technical assistance.

COMMENTS

Background

The prevalence of type 2 diabetes is dramatically increasing throughout the world. Insulin resistance is a hallmark of type 2 diabetes, and it most often precedes the onset of hyperglycemia and predicts development of type 2 diabetes. At present, thiazolidinediones (TZD), the agonists of the peroxisome proliferator-activated receptor γ , are the main agents to improve insulin sensitivity in the liver, adipose tissue, and skeletal muscle, thus improving glycaemic control in patients with type 2 diabetes. Despite the efficacy, some deleterious side effects of TZDs, including rosiglitazone and pioglitazone, have been noted, such as increasing body weight and aggravating heart failure through fluid retention. Therefore, development of new agents may be helpful in the treatment of type 2 diabetic patients with insulin resistance. Yi-Qi-Zeng-Min-Tang (YQZMT), a traditional Chinese compound recipe consisting of 10 medicinal herbs, is considered to be a useful medicine for the amelioration of insulin resistance of type 2 diabetes.

Research frontiers

Insulin resistance is a hallmark of type 2 diabetes, characterized by a decreased response of the peripheral tissues to insulin action. Insulin resistance produces elevations in glucose and lipid levels. It has become increasingly evident that obesity and the concomitant development of inflammation are major components of insulin resistance. Studies have revealed a clear association between pro-inflammatory signaling pathways and decreased insulin sensitivity. Pro-inflammatory adipokines including tumor necrosis factor- α , interleukin-6, as well as free fatty acids (FFAs), have been implicated to play important roles in inflammation, insulin resistance, and type 2 diabetes. The results of this study indicate that YQZMT has beneficial effect in insulin resistance, glycaemic control, dyslipidemia, FFA and adipocytokines, and does not cause any increase in body weight in high-fat diet and low-dose streptozotocin induced type 2 diabetic rats.

Innovations and breakthroughs

This study has established a model of type 2 diabetes with insulin resistance, and the beneficial effect of YQZMT was observed on insulin resistance, glycaemic control, dyslipidemia, FFA and adipocytokines in type 2 diabetes mellitus. YQZMT displays the insulin sensitization characteristic of rosiglitazone, but unlike rosiglitazone, does not cause any increase in body weight.

Applications

YQZMT, which ameliorates insulin resistance and does not cause increase in body weight, can be used as an adjunct for the treatment of type 2 diabetes.

Terminology

YQZMT, a traditional Chinese decoction, consisting of *Radix Astragali*, *mung bean coating*, *Folium Perillae*, *Phellodendron amurense Rupr*, *Pollen Typhae*, *Serissa foetida*, *Ramulus Cinnamomi*, *Radix Aconiti Lateralis Preparata*, *Coptis Chinensis Franch*, and *Rhizoma Alismatis*.

Peer review

Work by Zhang *et al.*, described in the present manuscript, generates data indicating a therapeutic effect of an herbal extract of Chinese medicinal plants in a rat model of insulin resistance and diabetes mellitus. The therapeutic effect is shown as changes in plasma markers of metabolic alterations and inflammation related to insulin resistance and diabetes, as well as whole body tests such as intraperitoneal glucose tolerance test and insulin tolerance test.

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S- Editor Tian L L- Editor Ma JY E- Editor Zheng XM

Effects of CPG ODN on biological behavior of PANC-1 and expression of TLR9 in pancreatic cancer

Han-Qing Wu, Bo Wang, Shi-Kai Zhu, Yuan Tian, Jing-Hui Zhang, He-Shui Wu

Han-Qing Wu, Bo Wang, Shi-Kai Zhu, He-Shui Wu, Department of Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Yuan Tian, Jing-Hui Zhang, Laboratory of General Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Author contributions: Wu HS designed the research; Wu HQ, Wang B, Zhu SK, Tian Y and Zhang JH performed the research; Wu HQ, Wang B, Zhu SK analyzed the data; Wu HQ and Wu HS wrote the paper.

Supported by The National Natural Science Foundation of China, No. 30972898

Correspondence to: He-Shui Wu, MD, Department of Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China. heshuiwu@163.com

Telephone: +86-27-85351621 Fax: +86-27-85351673

Received: August 19, 2010 Revised: November 3, 2010

Accepted: November 10, 2010

Published online: February 28, 2011

Abstract

AIM: To determine the expression of toll-like receptor 9 (TLR9) in pancreatic tumor and the effects of cytosine phosphate-guanosine oligodeoxynucleotides 2216 (CPG ODN2216) on biological behavior of pancreatic carcinoma cell line PANC-1 and explore their clinical significance.

METHODS: The immunohistochemistry and Western blot were used to determine the expression of TLR9 protein in pancreatic cancer tissues, and immunofluorescence staining was performed to detect the TLR9 protein expression in pancreatic carcinoma cell line PANC-1. To assess the effects of CPG ODN2216 on the invasive property of Panc-1 cells, *in vitro* cell adhesion, wound-healing scrape, and invasion and cell colony formation were evaluated.

RESULTS: TLR9 was highly expressed in pancreatic

cancer tissues and PANC-1 cells. The percentage of positive cells expressing TLR9 protein in human pancreatic tissues, paracancerous tissues and normal tissues were 73.3%, 33.3% and 20.0%, respectively, and the protein expression level of TLR9 was gradually descending ($P < 0.05$). *In vitro* tests in wound-healing scrape, cell adhesion, colony formation and matrigel invasion showed that the adhesion and motility of PANC-1 cells in CPG ODN 2216 treatment group were significantly lower than in the control group ($P < 0.05$). The cell growth assay showed that the proliferative ability of PANC-1 cells in treatment group was significantly decreased and CPG ODN2216 had an inhibitive effect in the growth of Panc-1 cells in a dose and time-dependent manner ($P < 0.05$).

CONCLUSION: The gene of TLR9 is correlated with the invasive and metastatic potential of human pancreatic carcinoma, and CPG ODN2216 induces the inhibition of migration and invasion of Panc-1 cells.

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Key words: Cytosine phosphate-guanosine oligodeoxynucleotides 2216; Pancreatic cancer; Toll-like receptor 9; Biological behavior

Peer reviewer: Seong Gyu Hwang, Professor, MD, Department of Internal Medicine, CHA Bundang Medical Center, CHA university, #351, Yatap-Dong, Bundang-Gu, Seongnam, Gyeonggi-Do, 463-712, South Korea

Wu HQ, Wang B, Zhu SK, Tian Y, Zhang JH, Wu HS. Effects of CPG ODN on biological behavior of PANC-1 and expression of TLR9 in pancreatic cancer. *World J Gastroenterol* 2011; 17(8): 996-1003 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/996.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.996>

INTRODUCTION

Pancreatic cancer is a highly malignant tumor of digestive

system, with a very poor prognosis^[1,2]. In the developed countries, the pancreatic cancer ranks the fourth in the mortality of malignant tumors^[3]. The five-year survival rate is less than 5% and the median survival time is 5-6 mo. Although 10%-15% of patients have the opportunity for radical surgery, the median survival time is only 10-18 mo and the 5-year survival rate is about 17%-24%^[4,5]. The poor prognosis of pancreatic cancer patients is mainly due to local invasion and early metastasis^[6]. Most patients with pancreatic cancer are diagnosed when they are found to have local invasion or distant metastasis. Therefore, further studies of the molecular biological behavior of pancreatic cancer are necessary to improve the diagnosis of pancreatic cancer, prevention and treatment.

Toll-like receptors (TLRs) are pathogen receptors which have been recently discovered to have the ability of identifying specific types of micro-organisms, namely the pathogen-associated molecular patterns (PAMPs)^[7,8]. Recent studies found that the expression of TLRs occurred not only in immune cells, but also in normal epithelial cells and tumor cells^[9]. The activity of TLRs in tumor cells may delay the anti-tumor function of immune cells, interfere with the role of the immune response, leading ultimately to tumor progression^[10-12].

TLR9 is an important member in this family, with an ability to induce significant secretion of cytokine and chemotactic factors by B cells and dendritic cells, such as interleukin (IL)-12, IL-6, interferon- γ (IFN- γ), monocyte proinflammatory and matrix metalloproteinase^[13]. High-level expression of TLR9 was detected in a wide variety of tumors, however, it remains unknown whether TLR9 is expressed and what role it plays in pancreatic cancer. In order to investigate the role of TLR9 in the development of pancreatic cancer, the immunohistochemistry and Western blotting were used to determine the expression of TLR9 protein in pancreatic cancer tissues and immunofluorescence staining was also performed to detect the TLR9 protein expression in pancreatic carcinoma cell line PANC-1. And we employed TLR9-targeting ligand-synthetic CPG ODN2216 to stimulate pancreatic cancer cell Panc-1 and observe the changes in malignant phenotype of this cell line in terms of proliferation, growth, tumorigenic and invasive ability and the correlation between *TLR9* gene and the pancreatic cancer cells. All these will benefit the future research of the specific mechanism of TLR9 gene.

MATERIALS AND METHODS

PANC-1 cell line and cells culture

The pancreatic cancer cell line Panc-1 was supplied by the laboratory of the Department of General Surgery, Wuhan Union Hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) which contains 10% fetal bovine serum (FBS) and 100 μ mol/mL mycillin under 5% CO₂ at 37°C.

Tissue slices

Fresh human pancreatic cancer tissues ($n = 30$) and ho-

mologous peritumoral tissues (1-2 cm from the edge of cancer) were collected from pancreatic cancer patients. The normal human pancreatic tissues ($n = 30$) were obtained from non-cancer patients who had to be depancreatized for other reasons. All of the specimens were taken from patients with pancreatic cancer who were treated surgically in the Pancreatic Surgery Center of Wuhan Union Hospital and confirmed by post-operational pathology.

Main reagents and antibodies

CpG-ODN2216 (serial number: GGGGGACGATC-GTCGGGGGG) was synthesized, specifically phosphorothioate decorated and polyacrylamide gel electrophoresis (PAGE) purified by Sheng Gong Bio-engineering Company, Shanghai, China; TLR9 monoclonal antibody was provided by the Cell Signaling Company, USA; matrigel and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Company, USA; and immunohistochemistry and immunofluorescence reagent kits were obtained from Boster Bio-engineering Co., Ltd., Wuhan, China.

Western blotting

Conventional methods were used to extract tissue proteins and the consistency of protein was measured by the method of Coomassie brilliant blue. Fifteen μ L protein was taken for 10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter by electrolysis. To block the non-specific antigen, the sections were incubated with 5% skim milk added with 1:750 diluted rabbit anti-human antibody TLR9 monoclonal antibody, followed by overnight incubation at 4°C. Membranes were washed with Tris-Buffered Saline Tween-20 (TBST) containing 0.1% Tween20, added with goat anti-rabbit IgG antibodies (Boster Bio-engineering Co., Ltd., Wuhan, China) tagged with horseradish peroxidase (HRP) and incubated at room temperature for 2 h. After being washed and added with ECL Plus reagent, an enhanced chemiluminescence substrate, the membranes were exposed on X-ray films for 30 s, followed by routine development and fixation. Finally, the images were scanned and analyzed by an image analysis software for gel electrophoresis (Band scan v5.0).

Immunohistochemistry

The streptavidin peroxidase (SP) methods were used for the immunohistochemical studies. The staining step followed the routine process. The paraffin sections were prepared, dewaxed and hydrated by alcohol. After being immersed in 3% hydrogen peroxide for 10 min, the sections underwent antigen retrieval in 0.01 mol/L citrate buffer solution (pH 6.0) and then blocked at room temperature for 30 min by 5% goat serum albumin with subsequent removal of sealing solution. The appropriately diluted primary antibody was added, and the control antibody was replaced with phosphate buffer solution (PBS) and kept at 4°C overnight. They were rinsed with PBS for 3 times, 5 min each time; biotinylated secondary antibodies were incubated at room temperature for 30 min and washed with PBS for 3 times; and horseradish peroxidase (HRP)

labeled avidin was added and incubated at room temperature for 30 min. Finally, after PBS rinsing, DAB-staining, hematoxylin staining, dehydration, and septum pellucidum mounting, results were observed under microscope.

Immunofluorescence method

Pancreatin-digested pancreatic cancer cells were used to prepare single cell suspension and the cell suspension was dropped onto glass slides. Creeping plates were taken out after 24 h or longer according to the cell growth state and rinsed with PBS for 3 times (5 min each time), followed by 15 min fixation with 4% paraformaldehyde, PBS rinse and 20 min perforation by 0.5% Triton.

After rinsed with PBS for 3 times, the PANC-1 cell slides were blocked for 30 min by 5% bovine serum albumin (BSA). The first antibody dilution was added directly after dryness while PBS was used in the control group. Following overnight incubation at 4°C and rinsing with PBS for 3 times, the diluted second antibody was added and incubated for 1 h at 37°C. Finally, the slides were observed under a fluorescence microscope after PBS rinse for 3 times, and added with diluted Strept Avidin Biotin Complex - fluoresceine isothiocyanate (SABC-FITC) and incubated for 30 min at 37°C. PBS rinse was repeated for 3 times and sealed by water-soluble sheet.

Cell growth assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Beyotime Bio., Shanghai, China) according to the method described previously^[14]. Pancreatic cancer cell suspension was transferred into 96-well tissue culture polystyrene (TCP) plates with 1×10^3 cells or 100 μ L per well. The cells were cultured at 37°C under 5% CO₂ and saturated humidity. After cell attachment at time point of 24 h, CPG ODN2216 of various concentrations (0.1, 0.5, 1, 5 and 10 mg/L) was added to act on Panc-1 cells, followed by addition of 20 μ L CCK8 of 5 mg/mL at time points of 24, 48 and 72 h, respectively and 4 h cell culture. Finally, after thorough shaking and mixing, absorbance values were determined by enzyme-linked immunosorbent assay (ELISA) reader. Inhibition rate (IC) = (A control group - A drug group)/A control group \times 100%. The curve of inhibited proliferation was plotted and IC50 was obtained.

Scratch adhesion test

Panc-1 cellular suspension at a concentration of 2×10^5 /mL was inoculated into a 6-well culture plate at 3 mL per well, and cultured for 24 h. In the next step, culture medium was removed and replaced by CPG ODN2216 culture medium at concentrations of 1 and 10 mg/L, respectively after the cell growth rate reached 80%. Supernatant was abandoned and scratches were carried out in the center of wells using a scraping cutter. Four marks were used as test sites at an equal distance along the scratch edge and the average value was obtained.

Cell adhesion test

Collagen gel overlays were prepared on 96-well TCP plates, followed by air dryness, BSA blocking and 60min

incubation. The cell concentrations of treatment group (interfered by CPG ODN at 1 and 10 mg/L for 24 h) and control group was adjusted to 5×10^5 /mL, and both cellular suspensions were inoculated in transwells, leading to 200 μ L/well. With 1h incubation, PBS rinsing, addition of 5 mg/ml methyl thiazolyl tetrazolium (MTT) and 4 h culture, the culture medium was abandoned and the remainder was dissolved in DMSO for 20 min. Lastly, absorbance values were measured at 490 nm wavelength. Cell adhesion inhibition rate = (A control group-A drug group)/A control group \times 100%. Each group contained 3 repeated wells and the average values were obtained.

In vitro invasion assay

Cell invasion *in vitro* was performed in Matrigel Invasion Chambers (Becton Dickinson Co., Franklin Lakes, NJ) in 24-well culture plates, as described previously^[15]. Thirty μ L synthetic basal membrane was added into transwell upper chamber and underwent air dryness. Then, 200 μ L of 24 h starvation-cultured cells was added respectively and the concentration of CPG ODN was adjusted to 1 and 10 mg/L. DMEM culture medium (500 μ L) containing 10% FBS as chemokine was added into the lower chamber of invasive cabinet. It was taken out of cabinet after 24 h culture at 37°C under 5% CO₂ and then cotton swab was used to rub away unigrated cells and superfluous liquid in upper chamber. After that, the cabinet was fixed for 10 min in 4% polyoxymethylene and dyed for 30 min with 1% crystal violet, followed by thorough membrane washing with deionized water. Finally, cells were observed and counted under microscope. Each group contained 3 repeated wells and the average values were obtained.

Cell cloning test

Panc-1 cells were digested with pancreatin and single cell suspension was prepared by blowing method, and then inoculated into a 6-well plate at about 200/well. Three groups were set up, one group without CPG ODN2216, the other two containing 1 and 10 mg/L CPG ODN2216, respectively. Each group contained two repeated wells. Then, the inoculated cells were cultured in the incubator till d 14. Cell culture was suspended when visible clone appeared and then culture medium was removed, rinsed by PBS, dyed for 30 min with hematoxylin and PBS rinse was repeated. Finally, the formation rate of cell clone was calculated and photographed by digital camera. The test was repeated 3 times.

Statistical analysis

Experimental data was analyzed by Statistical Package for Social Sciences(SPSS) software(SPSS, Inc., Version 16.0,USA), All of the data were represented by mean \pm SD and *T* test was performed for statistical analysis, and *P* values < 0.05 were considered statistically significant.

RESULTS

Western blotting test

As shown in Figure 1, the expression of glyceraldehyde-

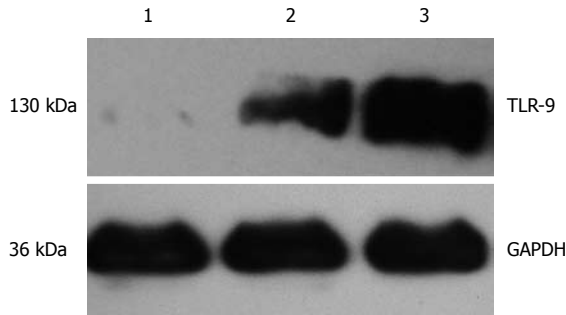


Figure 1 Western blotting test for toll-like receptor 9 protein. 1: Normal human pancreatic tissue; 2: Human peritumoral tissue; 3: Human pancreatic cancer tissue. TLR-9: Toll-like receptor 9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

3-phosphate dehydrogenase (GAPDH) protein was detected in cancer tissues, homologous peritumoral tissues and normal pancreatic tissues and protein band was located at 36 kDa. TLR-9 was expressed at 130 kDa. The expression level of TLR9 in cancer tissues was significantly higher than in both peritumoral tissues and normal pancreatic tissues.

Expression of TLR9 protein in pancreatic cancer tissues

Immunohistochemistry showed that TLR9 protein was mainly expressed in the cytoplasm of cancer cells, exhibiting yellowish and reddish brownness in an even manner. The expression of TLR9 protein was detected in both peritumoral tissues and normal pancreatic tissues, showing light yellowness and the expression level was lower than in the pancreatic cancer tissues. The total positive rates of TLR9 protein were 73.3% in human pancreatic cancer (22/30) and 33.3% (10/30) and 20% (2/10) in pancreatic peritumoral tissues and normal pancreatic tissues, respectively. There were statistical differences among the three groups ($\chi^2 = 13.99$, $P < 0.01$), (Figure 2).

Expression of TLR9 protein in Panc-1

The high expression of TLR9 in Panc-1 cells was found by immune fluorescence method. Upon excitation by ultraviolet, cytoplasm displayed green fluorescence whereas control group did not show the fluorescence (Figure 3).

Effects of CPG ODN2216 on proliferation of pancreatic cancer cell line Panc-1

CPG ODN2216 of different concentrations (0.1, 0.5, 5 and 10 mg/mL) showed inhibitory effects on Panc-1 cell line, and the growth inhibition rate increased markedly with concentration and time. Growth inhibition curve was plotted and the IC₅₀ at 24, 48 and 72 h was 65.1, 16.43 and 4.47 mg/mL, respectively. The inhibitory effect was time and dose dependant (Figure 4).

Cell scratch test

The results of scratch test showed that in contrast with control group, migration ability of Panc-1 cell was decreased with addition of CPG ODN2216 and higher CPG ODN2216 concentration led to significantly lower

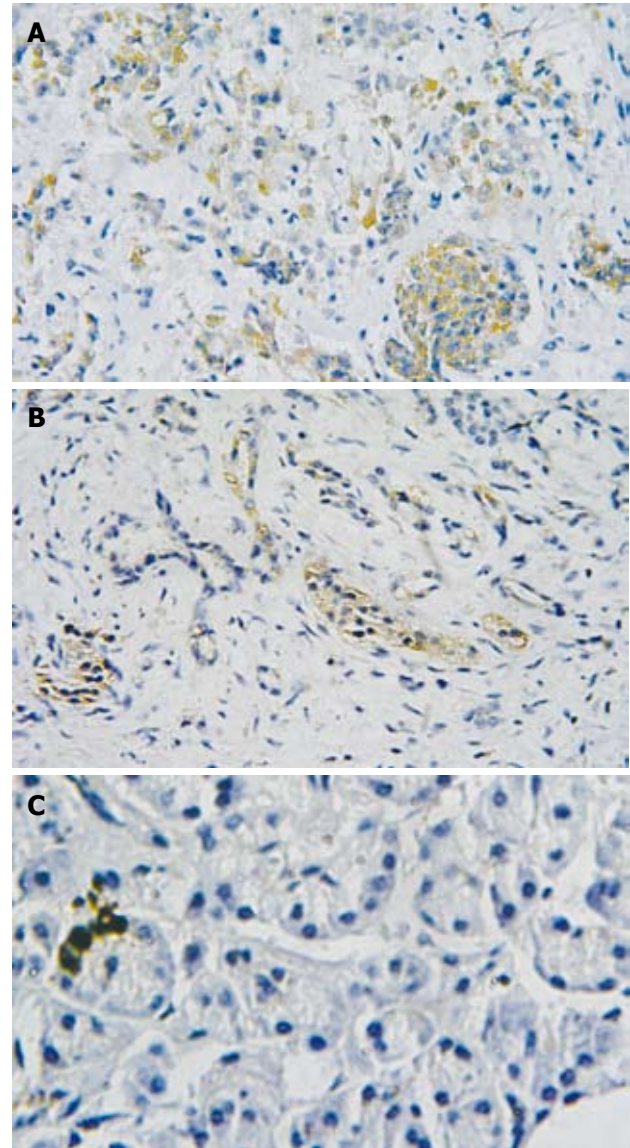


Figure 2 Expression of toll-like receptor 9 protein. Brown color displays the positive expression. A: Human pancreatic cancer tissue ($\times 200$); B: Peritumoral human pancreatic cancer tissue ($\times 200$); C: Normal pancreas tissue ($\times 400$).

migration ability. Migration distance after 48 h was 20.20 ± 1.06 mm at 10 mg/L and 17.37 ± 0.70 mm at 1 mg/L and 13.27 ± 1.11 mm in the control group, the difference being statistically significant ($P < 0.05$), (Table 1, Figure 5).

Effects of CPG ODN2216 on the adhesion of pancreatic cancer cell line Panc-1 and its in vitro invasive ability

The adhesive inhibition rates were 12.5% and 2.5% respectively in 1 mg/mL CPG ODN treated group and 10 mg/L CPG ODN group, respectively. Cell adhesion strength was significantly lower than control group ($t = 25.4$, 14.9 $P < 0.01$) and the inhibitory effect appeared dose-dependent ($t = 6.32$, $P < 0.01$). The control group had higher invasive ability, and the higher the concentration of CPG ODN2216, the fewer cells crossed the membrane. Invasion inhibitory rates were 21.6% and 59.5%, respectively ($t = 6.59$, 13.79 $P < 0.01$), showing a dose-effect relationship ($t = 18.29$ $P < 0.01$), (Table 2, Figure 6).

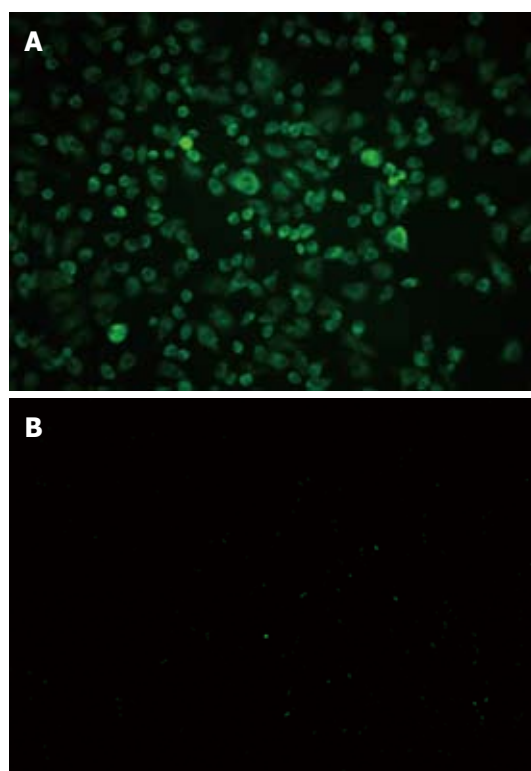


Figure 3 Immunofluorescence staining test. The expression of toll-like receptor 9 (TLR9) was mainly detected in Panc-1 cell cytoplasm ($\times 200$). A: TLR9 was highly expressed in Panc-1 cells; B: TLR9 was negatively expressed in control group.

Cell colony formation

Hematoxylin stained cells in 10 mg/mL CPG ODN2216 treated group showed significantly fewer colonies per square centimeter ($0.27 \pm 0.13/\text{cm}^2$) than in group 1 mg/L (1.08 ± 0.11) and control group (1.17 ± 0.12), with statistical difference between the latter two groups ($t = 8.29$, 8.67 $P < 0.01$) but without significant difference between treatment group and control group ($P > 0.05$), (Figure 7).

DISCUSSION

Pancreatic cancer has a high degree of malignancy and develops stealthily, seriously threatening the human health. Therapeutic effects have been low by the available treatment methods and prognosis is poor. Epidemiological studies showed that both the incidence and mortality of pancreatic cancer are hiking in recent years^[16]. Currently, in China pancreatic cancer jumped from 16th to 6th in the death rate caused by a variety of cancer. Therefore, search for target of gene therapy for pancreatic cancer has always been the focus in this field. Substantial findings demonstrate that signaling pathway induced by TLRs and TLR9 may play an important role in the development of tumors and the up-regulation of TLRs expression may be closely connected with the stomach, lung, bowel cancers and so on^[17]. The expression of TLR9 in tumor is expected to provide new regimens for tumor chemotherapy and immunotherapy. In this study, we used Western blotting and immunohistochemical analysis to detect the expression

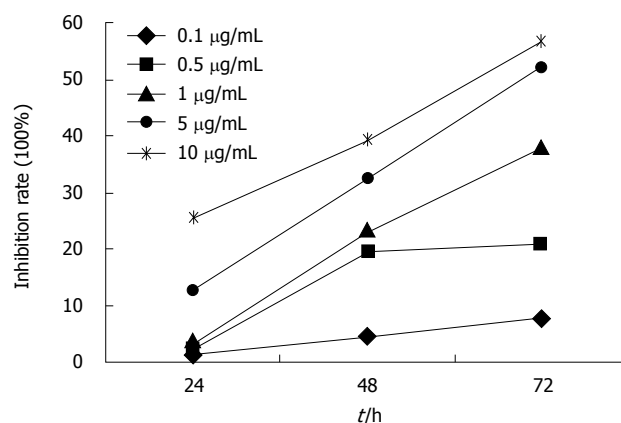


Figure 4 Inhibitory effect of cytosine phosphate-guanosine oligodeoxynucleotides 2216 of different concentrations on Panc-1 cell line with growth inhibition rate increasing remarkably with concentration and time.

Table 1 Migration width in different groups (mean \pm SE)

Time	Control group (mm)	1 mg/L group (mm)	10 mg/L group (mm)
0 h	23.98 \pm 1.71	24.02 \pm 1.67	22.52 \pm 1.43
48 h	13.27 \pm 1.11 ^b	17.37 \pm 0.70 ^b	20.20 \pm 1.06 ^b

Compared with control group, ^b $P < 0.01$.

Table 2 Mobility, adhesion and invasion ability of different groups ($n = 3$, mean \pm SE)

Group	Cell adhesion rate (%)	Cell number of invasion
Control group	0	198 \pm 13
1 mg/L	12.5 \pm 0.85 ^b	144 \pm 5 ^b
10 mg/L	22.5 \pm 2.62 ^a	80 \pm 6 ^a

Control vs 1 mg/L group, ^a $P < 0.05$. Control vs 10 mg/L group, ^b $P < 0.01$.

of TLR9 gene in pancreatic cancer tissues and found that the expression of TLR9 gene in pancreatic cancer tissues was significantly higher than in pancreatic peritumoral tissue and normal pancreatic tissues. This is in agreement with Droemann's^[18] findings in lung cancer study. We also determined the expression of TLR9 gene in pancreatic cancer cells using immunofluorescence method and found high level expression of TLR9 protein in pancreatic cancer cells, mainly in endochylema. In order to investigate the significance of high expression of TLR9 gene in pancreatic cancer cells and clarify its effects, we applied specific ligands of TLR9, namely CPG ODN2216 and pancreatic cancer cell Panc-1, to study the function of CPG ODN and its influence on TLR9 gene in human pancreatic cancer cells.

In recent years it has been verified that CPG ODN has a strong immunoregulatory effect in Th1 direction, mainly through its combination with TLR9 and induction of secretion of Th1 polarizing cell factors such as IFN- γ , IL-12 and so on, promoting the differentiation of Th0 to Th1^[19,20]. For example, CPG DNA penetrates into dendritic cells (DCs) by means of sequence indepen-

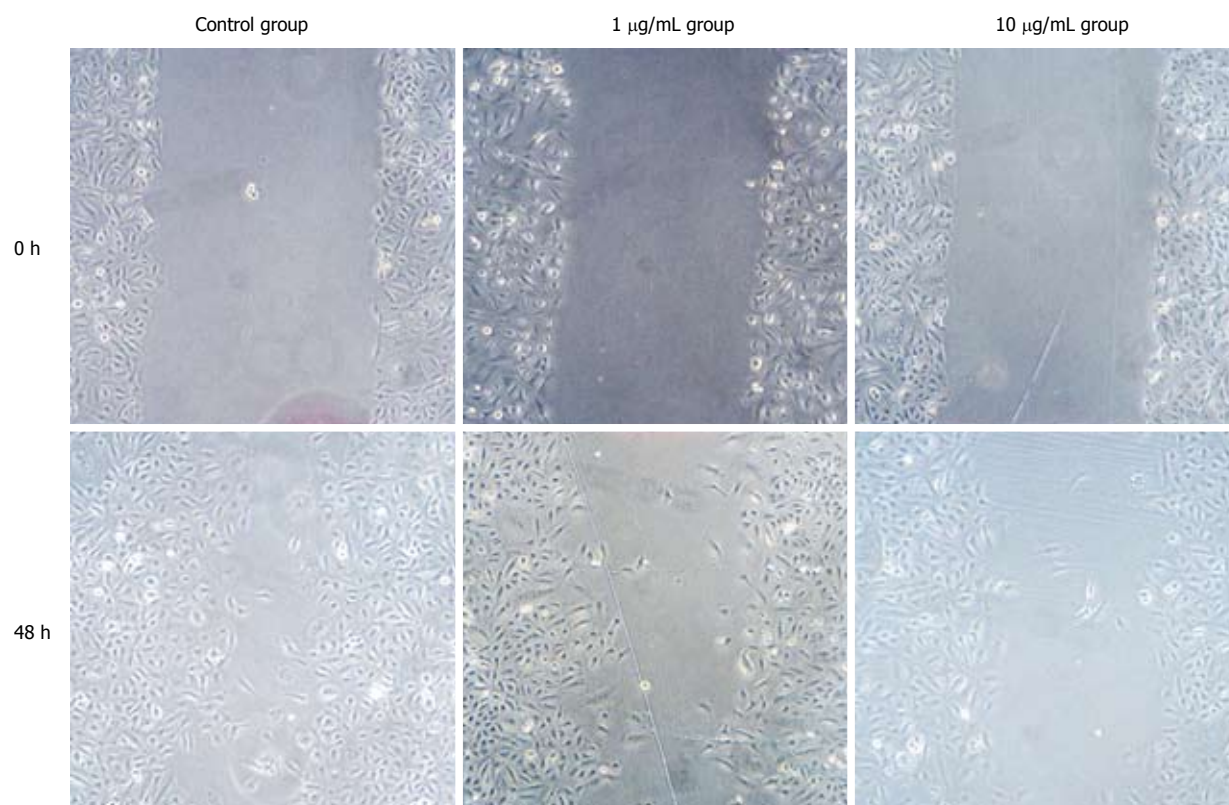


Figure 5 Panc-1 cell scratch test at various time points ($\times 400$). Compared with control group, a higher cytosine phosphate-guanosine oligodeoxynucleotides 2216 concentration led to significantly lower migration ability ($P < 0.01$).

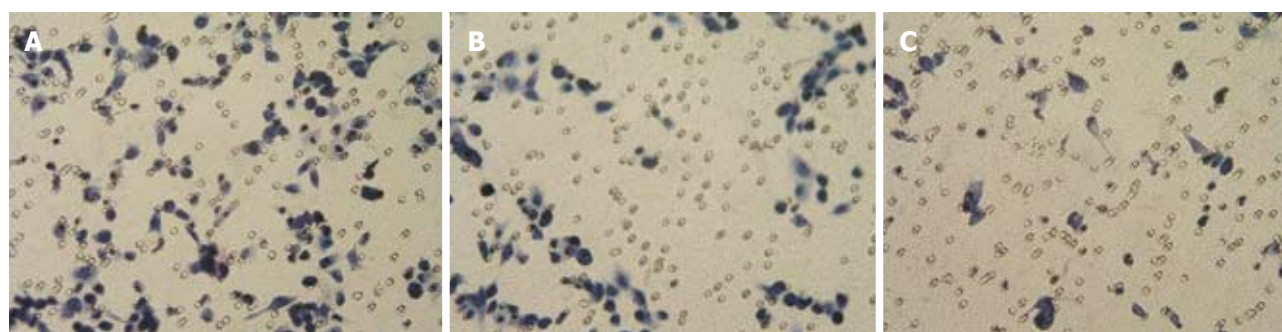


Figure 6 Invasion ability of Panc-1 in cabinet ($\times 200$). A: In control group, there are more Panc-1 cells penetrating through filter membrane; B: The cell number of 1 mg/mL cytosine phosphate-guanosine oligodeoxynucleotides (CPG ODN) treated group penetrating through filter membrane decreased significantly (A vs C, $P < 0.01$); C: The Panc-1 cell number of 10 mg/mL CPG ODN treated group penetrating through filter membrane further decreased (A vs B, $P < 0.01$).

dent, receptor-mediated endocytosis, then acts on TLR9 specifically in lysosomal area and finally activates signal transduction through interleukin-1 receptor associated kinase (IRAK1), interferon regulatory factor 7 (IRF7), TNF receptor-associated factor 6 (TRAF6), mitogen-activated protein kinases (MAPK) and nuclear factor κ B (NF- κ B) pathway and adaptor myeloid differentiation primary response gene (88) (MyD88)^[21-24]. However, at present, CpG ODN, which is used as an immunoadjuvant to induce Th1 immune response on human lung adenocarcinoma A549 cell by means of TLR9, may be adopted for monotherapy or supplement to immunization therapy to treat cancers^[25]. As for the direct effect of CPG ODN on pancreatic cancer, there has been no report in the literature so

far. We combined CPG ODN2216 and pancreatic cancer cell and found by CCK-8 assay that the growth and proliferation of pancreatic cancer cells were inhibited *in vitro* and the inhibitory effect was time-dose dependent.

One of the important biological characteristics of malignant tumor is its invasion into adjacent tissues and subsequent distant metastasis. Invasion is recognized as the foundation and precondition of distant metastasis and distant metastasis is the continuation and further development of invasion. Factually, they are two stages of the same process, and pancreatic cancer invasion and metastasis into adjacent tissues occur in the early stage. Only by preventing the two biological behaviors at proper time can we defeat pancreatic cancer. Like other tumors, invasion

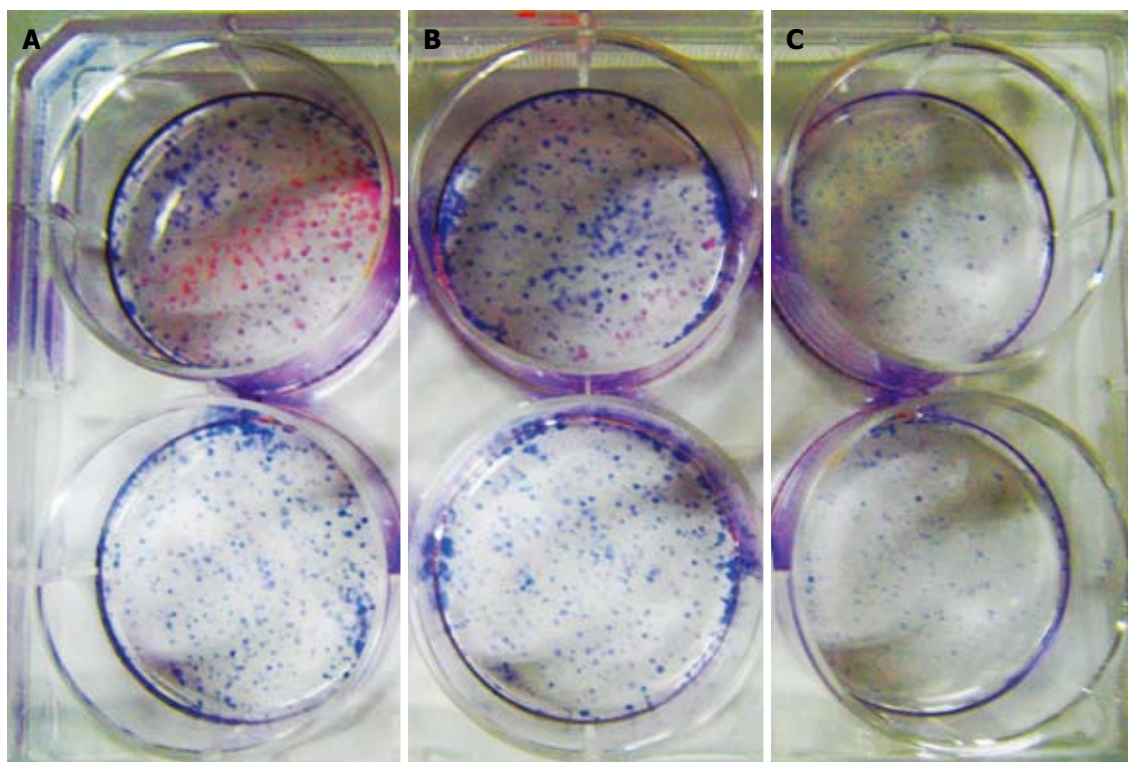


Figure 7 Cell cloning test. A: More colonies are formed in the control group; B: The colony formed in the 1 mg/L cytosine phosphate-guanosine oligodeoxynucleotides (CPG ODN) treated group is the same as in the control group ($P > 0.05$); C: There are fewer colonies formed in the 10 mg/L CPG ODN treated group (A vs B, $P < 0.01$).

and metastasis of pancreatic cancer is a process characterized by polygenic participation, multi-steps and multi-stages^[26]. At the same time, the adhesion and invasion process increases the malignant tumor survival and metastasis ability, that is a committed step in the tumor transition process^[27]. To further investigate the role of TLR9 gene in the process of tumor invasion and metastasis, TLR9 specifically ligand-CPG ODN2216 and pancreatic cancer cell line Panc-1 were applied in the subsequent tests such as scratch adhesion test, adhesion test, Transwells *in vitro* invasion assay, cell clone test and so on. Scratch adhesion test is considered as one of the classic ways to determine cell immigration ability and adhesion and invasion test can relatively ideally simulate and reflect the invasion behavior and ability of tumor cells. Cell clone test can also be used to detect the proliferation ability of cancer cells. CPG ODN2216 had weakened *in vitro* migration, membrane anchor ability and clone proliferation ability of Panc-1 cells compared with the cells in the control group. Therefore, the expression of TLR9 can influence the degree of malignancy of human pancreatic cancer cell line.

In conclusion, there is high expression of TLR9 in both human pancreatic tissues and Panc-1 cells. TLR9 may increase the cell abilities of invasion, metastasis and adhesion to promote the occurrence and development of pancreatic neoplasm, and play an important role in invasion and metastasis of pancreatic cancer. However, the questions of how the gene regulates cell migration and invasion, and what transduction system ligand CPG ODN relies on to influence TLR9, remain to be answered and the specific mechanism need to be further studied.

COMMENTS

Background

Pancreatic cancer is a highly malignant digestive tumor with a very poor prognosis. Recently, an increasing number of studies reported that toll-like receptors (TLRs) were related to malignancies and involved in tumor progression, but whether TLRs, such as TLR9, is expressed in pancreatic cells remains unknown. Regulation of TLR9 signaling pathway in pancreatic cancer cells, and the relationship between the biological impact of behavior change are also unclear.

Research frontiers

Activating TLRs pathway in tumor cells could promote the proliferation and inhibit the apoptosis, leading to migration, invasion and angiogenesis of tumor, but it remains unknown whether TLR9 is expressed and what role it plays in PANC-1. There is no report about the direct effect of cytosine phosphate-guanosine oligodeoxynucleotides (CPG ODN) on pancreatic cancer in the literature so far. The authors demonstrate that the high expression of TLR9 exists in both human pancreatic tissues and Panc-1 cells. TLR9 may increase the cell abilities of invasion, metastasis and adhesion to promote the occurrence and development of pancreatic neoplasm, and play an important role in invasion and metastasis of pancreatic cancer.

Innovations and breakthroughs

This is the first study to report that TLR9 is expressed in both human pancreatic tissues and Panc-1 cells, and first demonstrate that TLR9 may increase the cell abilities of invasion, metastasis and adhesion to promote the occurrence and development of pancreatic neoplasm, and play an important role in invasion and metastasis of pancreatic cancer.

Applications

This study demonstrated that TLR9 may be a novel marker for the progression and prognosis of pancreatic cancer, and may provide a new strategy for the treatment of patients with pancreatic cancer.

Terminology

CPG ODN 2216 (cytosine phosphate-guanosine oligodeoxynucleotides 2216): the TLR9-targeting ligand-synthetic. It has a strong immunoregulatory effect in Th1 direction, mainly through its combination with TLR9 and induction of secretion of Th1 polarizing cell factors such as IFN-7 and IL-12, promoting the differentiation of Th0 to Th1.

Peer review

The authors examined the expression of TLR9 in both human pancreatic tissue and Panc-1 cells, and demonstrated a role for TLR9 in adhesion, migration and invasion of human Panc-1 cells. This is an interesting article in which the experimental design is well thought out and the results are intriguing.

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S- Editor Sun H L- Editor Ma JY E- Editor Ma WH

Prophylactic PEG placement in head and neck cancer: How many feeding tubes are unused (and unnecessary)?

Mohammad F Madhoun, Matt M Blankenship, Derek M Blankenship, Greg A Kreml, William M Tierney

Mohammad F Madhoun, Matt M Blankenship, William M Tierney, Section of Gastroenterology, Department of Internal Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States

Derek M Blankenship, Department of Biostatistics, Institute for Health Care Research and Improvement, Baylor Health care System, Dallas, TX 75206, United States

Greg A Kreml, Department of Otorhinolaryngology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States

Author contributions: Madhoun MF and Blankenship MM were involved in data acquisition, design, analysis and writing the manuscript; Tierney WM and Kreml GA were involved in design, analysis, revision and final approval of the manuscript; Blankenship DM was involved in statistical analysis, writing and final approval of the manuscript.

Correspondence to: Mohammad F Madhoun, MD, Section of Gastroenterology, Department of Internal Medicine, University of Oklahoma Health Sciences Center, 920 SL Young Blvd; WP1360, Oklahoma City, OK 73104, United States. mohammad-madhoun@ouhsc.edu

Telephone: +1-405-2715428 Fax: +1-405-2717186

Received: February 15, 2010 Revised: April 15, 2010

Accepted: April 22, 2010

Published online: February 28, 2011

Abstract

AIM: To determine the rate of use and non-use of prophylactic percutaneous endoscopic gastrostomy (PEG) tubes among patients with head and neck cancer (HNC) patients.

METHODS: All patients with HNC undergoing PEG between January 01, 2004 and June 30, 2006 were identified. Patients (or their next-of-kin) were surveyed by phone and all available medical records and cancer registry data were reviewed. Prophylactic PEG was defined as placement in the absence of dysphagia and prior to radiation or chemoradiation. Each patient with a prophylactic PEG was assessed for cancer diagnosis, type of therapy, PEG use, and complications related to PEG.

RESULTS: One hundred and three patients had PEG tubes placed for HNC. Thirty four patients (33%) could not be contacted for follow-up. Of the 23 (22.3%) patients with prophylactic PEG tubes, 11/23 (47.8%) either never used the PEG or used it for less than 2 wk. No association with PEG use *vs* non-use was observed for cancer diagnosis, stage, or specific cancer treatment. Non-use or limited use was observed in 3/6 (50%) treated with radiation alone *vs* 8/17 (47.1%) treated with chemoradiation ($P = 1.0$), and 3 of 10 (30%) treated with surgery *vs* 8 of 13 (62%) not treated with surgery ($P = 0.21$). Minor complications were reported in 5/23 (21.7%). One (4.3%) major complication was reported.

CONCLUSION: There is a high rate of unnecessary PEG placement when done prophylactically in patients with head and neck cancer.

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Key words: Head and neck cancer; Percutaneous gastrostomy tube; Prophylactic

Peer reviewer: Misha Luyer, MD, PhD, Department of Surgery, Orbis Medical Centre, Postbus 5500, Sittard, 6130 MB, The Netherlands

Madhoun MF, Blankenship MM, Blankenship DM, Kreml GA, Tierney WM. Prophylactic PEG placement in head and neck cancer: How many feeding tubes are unused (and unnecessary)? *World J Gastroenterol* 2011; 17(8): 1004-1008 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1004.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1004>

INTRODUCTION

Significant weight loss and resultant malnutrition in patients undergoing radiation and or chemotherapy for head and neck cancer (HNC) are recognized clinical concerns^[1].

Impaired swallowing function may be further compromised by odynophagia resulting from therapy induced mucosal injury of the pharynx and esophagus. Morbidity related to poor nutritional intake during treatment may include dehydration, hospitalization, compromised treatment compliance, reduced quality of life and the potential for a negative impact on survival^[2]. Multiple interventions have been implemented to help ameliorate the impact of cytoreductive therapy on weight loss and nutritional status, including the use of percutaneous endoscopic gastrostomy (PEG) tube. Published studies suggest that HNC patients undergoing radiation and/or chemotherapy may benefit from prophylactic placement of PEG tubes^[3-9]. In our institution, many patients with newly diagnosed HNC scheduled to receive radiation and/or chemotherapy are offered a PEG tube before the start of treatment regardless of whether they have significant dysphagia. This practice is primarily in patients who are treated with combination chemotherapy and radiation based on the impression that there is added pharyngeal toxicity with this regimen.

We have observed anecdotally that a number of our HNC patients who received a prophylactic PEG tube in fact never used them. We performed a retrospective database study of all patients in whom PEG tube was placed for HNC to determine the prevalence of unused prophylactically placed PEG tubes. Data were also analyzed for possible factors predictive of unused PEGs or PEGs used for less than 2 wk.

MATERIALS AND METHODS

We performed a retrospective review of a prospectively maintained endoscopic database to identify all patients with HNC undergoing PEG between January 1, 2004 and June 30, 2006 at both the Oklahoma City Veteran Affairs and Oklahoma University Medical Center hospitals. Patients were surveyed by phone and all available medical records and cancer registry data were reviewed. If the patient was deceased, his or her next of kin was interviewed. Prophylactic PEG was defined as placement in the absence of dysphagia and prior to radiation or chemoradiation. A PEG was deemed definitely prophylactic if the patient's medical record was suggestive of prophylactic PEG placement and the patient confirmed a lack of pre-procedure symptoms by telephone survey. Possible prophylactic was defined as being when the patient's medical record indicated prophylactic PEG tube but the patient could not be contacted to confirm. Patients with definitely prophylactic PEG were assessed for: cancer diagnosis, type of therapy (radiation alone, chemoradiation, with or without surgery), whether the PEG was ever used, duration and extent of PEG use, and complications related to PEG. Patient demographics, including age, sex, and body mass index (BMI), were also recorded. Specific diagnoses and treatment modalities were examined for a correlation with PEG use *vs* non-use or limited use. For statistical purposes patients were grouped as early (Stage I or II) or late (Stage III or IV) HNC according to the American

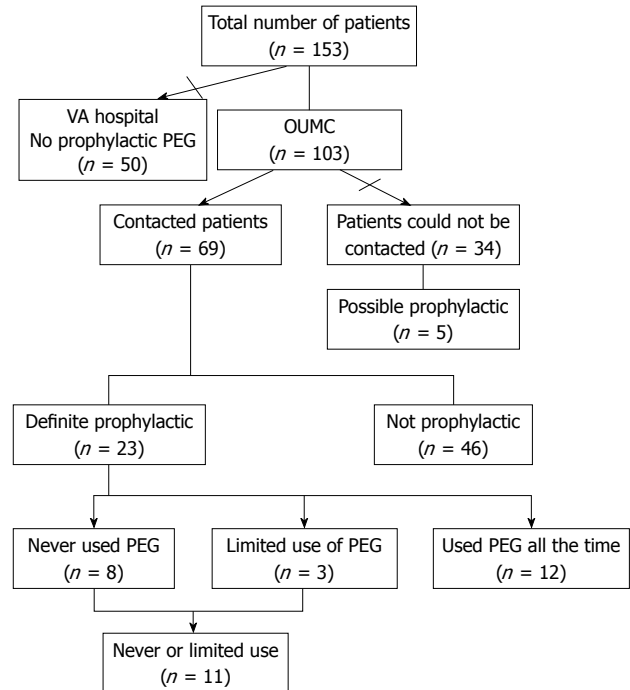


Figure 1 Patient selection. PEG: Percutaneous endoscopic gastrostomy; VA: Veteran Affairs; OUMC: Oklahoma University Medical Center.

Joint Committee on Cancer stage grouping for HNC^[10]. HNC was defined as any malignant tumor involving the lips, any region of the oral cavity, tongue, soft palate, pharyngeal wall, pyriform sinuses, supraglottic larynx, glottic larynx (true vocal cords and the mucosa of the anterior and posterior commissures) and subglottic larynx (extends to the inferior border of the cricoid cartilage).

Statistical analyses

Frequency and percentage of use (none or limited) by clinical parameters (tumor site, stage) or treatment modality (radiation, chemoradiation, surgery) are reported and graphically represented using bar charts. Fisher's exact test was employed to test for an association between nonuse of PEG and each variable.

RESULTS

One hundred and fifty three patients underwent PEG placement for HNC during the pre-specified period of time; of those, 50 patients were excluded because initial review of medical records revealed they did not meet the criteria for prophylactic PEG tube (Figure 1). Of the remaining 103 patients, 34 patients (33%) could not be contacted for follow-up. Chart review revealed that 5/34 patients not contacted likely had undergone prophylactic PEG. Of the 69 patients with available survey data it was established that 23 (22.3%) patients (19 males; 4 females; mean age 58 years) had definitely undergone prophylactic PEG. Ten patients (43.5%) had stage III HNC; seven patients (40.4%) had stage IV HNC. Fifty two percent (12/23) were classified as oropharyngeal; 39.1% (9/23)

Table 1 Patients with prophylactic percutaneous endoscopic gastrostomy tube; demographic, stage, type of treatment, complications and use of percutaneous endoscopic gastrostomy tube

Age (yr)	Sex	Site	Stage	Treatment	Complications	PEG use
58	M	Base of tongue	III	XRT		Never
64	M	Supraglottis	IV	CTX/XRT--Surg		Never
48	M	HNC, NOS	N/A	XRT/CTX--Surg		Never
66	M	Hypopharynx	III	XRT/CTX	Leaking	Never
31	F	Glottis	III	CTX/XRT	Perforation	Never
40	M	Tonsil	IVa	XRT	Leaking	Never
76	M	Supraglottis	II	XRT--Surg		Never
51	M	Larynx, NOS	III	CTX/XRT	Leaking	Never
41	M	Larynx, NOS	IVa	CTX/XRT	Pain and leaking	1 wk
47	M	Tonsil	III	CTX/XRT	Pain	2 wk
79	F	Tonsil	N/A	CTX/XRT	Pain	2 wk
54	M	Base of tongue	III	XRT/CTX--Surg		Months
65	M	Supraglottis	IVa	CTX XRT--Surg		Months
24	F	NPC	IIb	CTX XRT--Surg		Months
66	M	Larynx, NOS	N/A	XRT		Months
70	M	Base of tongue	II	XRT		Continuous
57	F	Supraglottis	III	CTX/XRT	Pain	Continuous
71	M	Base of tongue	IVa	CTX/XRT--Surg		Continuous
78	M	Base of tongue	IVa	Surg-CTX/XRT		Continuous
54	M	Supraglottis	III	XRT/CTX	Pain	Continuous
56	M	Tonsil	IVa	CTX/XRT--Surg		Continuous
71	M	Base of tongue	III	CTX/XRT		Continuous
58	M	Base of tongue	III	XRT--Surg		Continuous

PEG: Percutaneous endoscopic gastrostomy; XRT: Radiation therapy; CTX: Chemotherapy; N/A: Not available; NOS: No origin specified; HNC: Head and neck cancer. Staging is reported according to American Joint Committee on Cancer stage grouping for HNC.

were classified as laryngeal cancer, and one case was nasopharyngeal cancer. The treatment regimens of all patients are outlined in Table 1. All patients received various combinations of radiation, chemotherapy, and surgery. Four patients (17.4%) received radiation as the only treatment. Ten patients (43.5%) underwent surgical resection. Of these, 9 (90%) received preoperative radiotherapy and or chemoradiotherapy and one received chemoradiotherapy post operatively. Patients not using the PEG relied entirely on oral intake but the use of nutritional supplements could not be assessed (Table 1).

Of the 23 patients who underwent prophylactic PEG, 8 (34.7%) never used their PEG for feeding, and 3/23 (13%) used it for less than 2 wk. No association with PEG use *vs* non-use was observed for cancer diagnosis, stage, or specific cancer treatment (Figure 2).

Overall, complications occurred in 13/69 (18.8%). In the prophylactic PEG group, minor complications (pain and leaking at the PEG site) were reported in 5/23 (21.7%). One (4.3%) major complication (gastric perforation due to PEG tract disruption at time of tube removal) was reported. There was no difference in complication rates among patients who used the PEG for more than 2 wk *vs* those who never used it or used it for less than 2 wk; 17.4% (8/46) *vs* 21.7% (5/23), $P = 0.748$.

DISCUSSION

Not all cancer patients benefit from aggressive nutritional support as the pathophysiology behind weight loss and malnutrition is complex. HNC cancer patients are gener-

ally identified as a group of patients that may be helped by caloric supplementation^[3]. Many centers advocate prophylactic PEG tube placement in HNC cancer patients to prevent the rapid weight loss associated with aggressive cytoreductive treatments. In order to establish the practice of prophylactic PEG tube as an appropriate plan of care for these patients, certain end points need to be addressed, including: (1) effect on weight loss; (2) complication rates; (3) effect on quality of life; and (4) overall survival. With regard to weight loss prevention, multiple studies (retrospective and prospective) have demonstrated a benefit of prophylactic tube placement over on demand PEG tube placement. A retrospective study on 88 patients, found that the use of gastrostomy significantly reduced weight loss and the rate of hospitalization^[11]. Nguyen *et al*^[8] and Wiggeraad *et al*^[9] retrospectively evaluated the efficacy of prophylactic PEG tube. They found that the mean weight loss during treatment for all patients with prophylactic PEG was 8.5 kg and 2.3 kg, respectively, although no control group was available for comparison. Piquet *et al*^[6] prospectively compared patients selected for prophylactic PEG (i.e. age greater than 70, BMI less than 20, or recent weight loss greater than 10%) against comparable historical controls. Based on the criteria, 74% of patients qualified for prophylactic PEG. Patients prospectively evaluated for prophylactic intervention experienced significantly less weight loss and fewer hospitalizations for dehydration compared with a group managed with on demand PEG^[6].

PEG tube placement is considered relatively safe and has a low rate of significant associated complications but it is not an entirely benign procedure. Common complica-

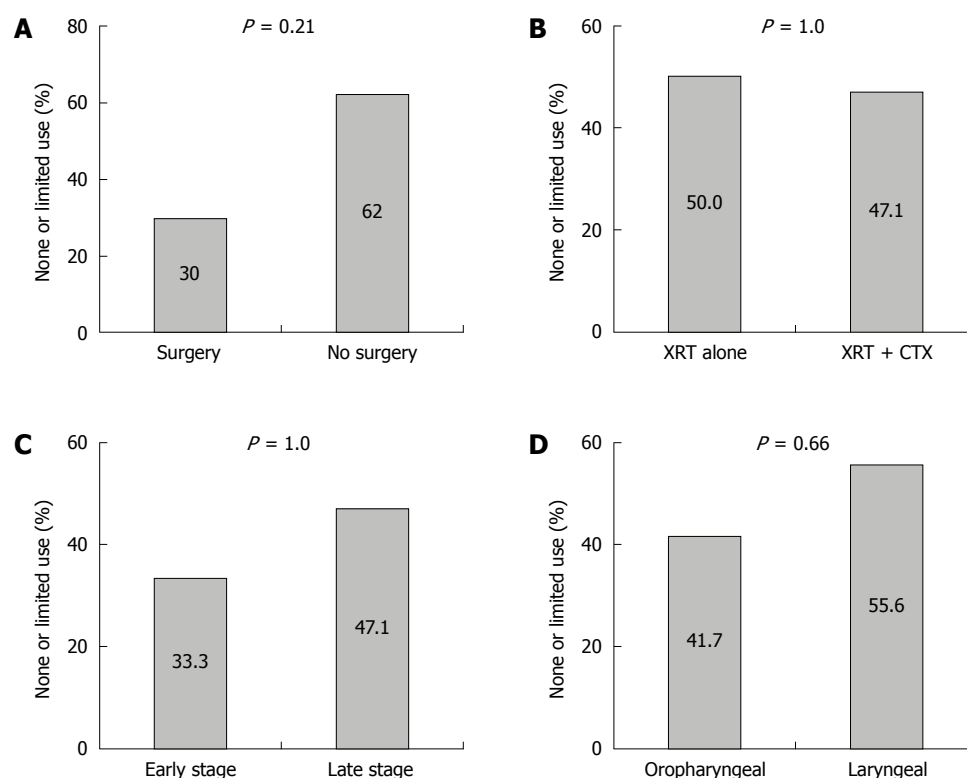


Figure 2 Percutaneous endoscopic gastrostomy use vs non-use observed for specific cancer treatment (A, B), stage (C), or cancer diagnosis (D). XRT: Radiation therapy; CTX: Chemotherapy.

tions associated with PEG tube placement include local site infection, tube blockage, and migration or dislodgement. Serious complications such as peritonitis, abscess, or fistula development are relatively uncommon^[12]. A recent systematic review of 2379 HNC patients found a PEG associated fatality rate of 2.2% and a pooled major complications rate of 7.4%^[13]. When reviewed in a meta-analysis of PEG placement in general mixed patient populations, PEG placement in HNC was associated with higher fatality rate (2.2% *vs* 0.33%) but similar overall major complication rate (7.4% *vs* 9.4%)^[14]. The frequency of complications observed following PEG tubes placement has varied in the literature depending upon the definition used and the population under study. In one study of 314 patients, 13% had minor complications and 3% had major complications^[15]. In our study, the overall complications rate observed (18.8%) is consistent with previously published rates.

Another rare but potential complication specific to this indication for PEG is metastasis of the primary tumor to the gastrostomy site, which has been described in multiple case reports^[16]. The balance between these risks and the potential benefits must be weighed. Overall quality of life should be considered. One study demonstrated that PEG placement prevented deterioration of the quality of life index during radiation therapy^[17]. On the other hand, a recent study was done to evaluate the impact of clinical predictors and other influences on long-term quality of life in patient with HNC, and found that patients with gastrostomy tubes had lower quality of life scores relative

to patients without a PEG ($P < 0.001$)^[18]. There has been no study done to evaluate the effect of prophylactic PEG tube in overall survival. Likewise the cost-effectiveness of this approach is undefined.

Limitations of our study include the inherent bias of retrospective studies in general, and the fact that one third of our patients were not available for phone survey yielding a small sample size. The study is a retrospective observational analysis on the use rates in patients with prophylactic tubes; hence temporally accurate nutritional parameters were not reliably available. There were a small number of patients with a possibly prophylactic PEG, but we choose to focus the analysis on patients that were definitely prophylactic. Inclusion of those five additional patients did not significantly alter the use rate or the overall conclusion.

Currently, there are no practice guidelines for patient selection regarding prophylactic PEG tube placement exist. Clinical judgment, in addition to patient and family preference, most commonly guides the decision on an individual basis and may vary greatly based on the practice setting.

In our study we determined that a significant number of patients (47%) never used their PEG or used it for less than 2 wk. However, those patients who used it for more than 2 wk uniformly reported that PEG tube had critical impact on their nutritional status and “they would not have survived without it”. Prophylactic PEG placement may be unwarranted in some patients but the selection of patients needs to be better defined to prevent unnecessary

risk exposure. In our study there were no clear disease or treatment parameters predictive of PEG use *vs* non-use. The high rate of non-use or limited use and the inherent risk of complications related to PEG call this practice into question and require careful consideration by the referring physician. It also prompts the need for a large prospective study to better define the patients group most likely to require use of enteral feeds during their therapy.

In summary, our study suggests prophylactic PEG placement prior to HNC therapy is associated with a high rate of non use or limited use. Further prospective studies evaluating specific selection criteria for prophylactic PEG in this setting are needed. Similarly, additional studies are needed to assess the impact of prophylactic PEG tube placement on the cost-effectiveness of cancer care, quality of life, hospital admission rate, and, most importantly, survival.

COMMENTS

Background

Impairment of oral intake occurs in the majority of patients with head and neck cancer (HNC) receiving chemoradiotherapy. Placement of prophylactic percutaneous endoscopic gastrostomy (PEG) tube in asymptomatic newly diagnosed HNC before chemoradiation is a common practice in some centers. In some studies PEG has been associated with a decrease in treatment related weight loss in patients with HNC, but no studies have examined the utilization rate. PEG placement is an invasive procedure, with possible complications. The authors anecdotally noticed a finite rate of non use of prophylactic PEG tubes among those patients.

Research frontiers

This study aimed to determine the prevalence of non use or limited use of prophylactically placed PEG tubes in HNC patients and to evaluate any possible factors that might predict the non use or limited use of prophylactic PEGs.

Innovations and breakthroughs

This is the first study that addressed the issue of use of prophylactic PEG in HNC patients. The result of this study showed that a significant number of patients (47%) with prophylactic PEG tubes never used their PEG or used it for less than 2 wk. No association with PEG use *vs* non-use was observed for cancer diagnosis, stage, or specific cancer treatment.

Applications

Prophylactic PEG placement may be unwarranted in some patients but the selection of patients needs to be better defined to prevent unnecessary risk exposure.

Peer review

This is a well-written retrospective study, and it is important to realize that prophylactic PEG placement has its side effects.

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S- Editor Wang JL L- Editor O'Neill M E- Editor Zheng XM

Demographic determinants of risk, colon distribution and density scores of diverticular disease

Mark Golder, Irina Chis Ster, Pratusha Babu, Amita Sharma, Muhammad Bayat, Abdulkadir Farah

Mark Golder, Centre for Academic Surgery, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, Queen Mary University, London, E1 1BB, United Kingdom

Irina Chis Ster, MRC centre for Paediatric Epidemiology and Biostatistics, University College, London, WC1N 1EH, United Kingdom

Pratusha Babu, Amita Sharma, Muhammad Bayat, Abdulkadir Farah, Department of Radiology, University Hospital Lewisham, London, SE13 6LH, United Kingdom

Author contributions: Golder M was responsible for overall coordination of the study, for data analysis and for drafting the paper; Chis Ster I was responsible for the statistical analysis and its interpretation and contributed to the drafting of the paper; Babu P was responsible for collection of data and for its organisation prior to analysis; Farah A, Bayat M and Sharma A were responsible for analysis of barium enema examinations; all investigators revised the final report and agreed to its final form.

Supported by The University Hospital Lewisham

Correspondence to: Mark Golder, PhD, FRCS (Gen), Centre for Academic Surgery, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, Queen Mary University, London, E1 1BB, United Kingdom. email@msgolder.co.uk

Telephone: +44-20-73777079 Fax: +44-20-73777283

Received: September 17, 2010 Revised: November 30, 2010

Accepted: December 7, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate associations between ethnicity, age and sex and the risk, colon distribution and density scores of diverticular disease (DD).

METHODS: Barium enemas were examined in 1000 patients: 410 male, 590 female; 760 whites, 62 Asians, 44 black africans (BAs), and 134 other blacks (OBs). Risks and diverticula density of left-sided DD (LSDD) and right-sided-component DD (RSCDD = right-sided DD + right and left DD + Pan-DD) were compared using logistic regression.

RESULTS: Four hundred and forty-seven patients had DD (322 LSDD and 125 RSCDD). Adjusted risks: (1) LSDD: each year increase in age increased the odds by 6% (95% CI: 5-8, SE: 0.8%, $P < 0.001$); Asians: odds ratio (OR): 0.23 (95% CI: 0.10-0.53, SE: 0.1, $P \leq 0.001$) and OBs: OR: 0.25 (95% CI: 0.14-0.43, SE: 0.07, $P \leq 0.001$) appeared protected vs Whites; (2) RSCDD: each year increase in age increased the odds by 4% (95% CI: 2-6, SE: 1%, $P < 0.001$); females were 0.60 times (95% CI: 0.40-0.90, SE: 0.12, $P = 0.01$) less likely than males to have RSCDD; BAs were 3.51 times (95% CI: 1.70-7.24, SE: 1.30, $P < 0.001$) more likely than Whites to have RSCDD; and (3) DD density scores: each year increase in age increased the odds of high-density scores by 4% (95% CI: 1-6, SE: 1%, $P < 0.001$); RSCDD was 2.77 times (95% CI: 1.39-3.32, SE: 0.67, $P < 0.001$) more likely to be of high density than LSDD. No further significant differences were found in the adjusted models.

CONCLUSION: Right colonic DD might be more common and has higher diverticula density in the west than previously reported. BAs appear predisposed to DD, whereas other ethnic differences appear conserved following migration.

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Key words: Diverticular disease; Migration; Barium enema; Ethnicity; Colon distribution; Risk

Peer reviewer: Patrick O'Dwyer, MB, BCh, BAO, FRCS (1), MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

Golder M, Chis Ster I, Babu P, Sharma A, Bayat M, Farah A. Demographic determinants of risk, colon distribution and density scores of diverticular disease. *World J Gastroenterol* 2011; 17(8): 1009-1017 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1009.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1009>

INTRODUCTION

Diverticular disease (DD) is a common disease in western societies^[1]. It causes considerable acute and chronic suffering and is a financial burden to health care systems^[2]. However, primary and secondary preventative treatment of DD is not possible because its cause and many aspects of its pathogenesis remain unknown. In particular, it is not known whether differences exist in the risk, colon distribution and density scores of DD between ethnic groups living in the west. Such information could aid diagnosis and point to areas of potential research into the etiology of the disease.

Such differences exist between ethnic groups living in their native countries. In Whites in the west, DD has an overall barium enema frequency of 15%-35%, affects only the left colon in 90%-99% of cases, has no sex predilection, and increases in incidence with age^[3-5]. In Southeast Asia, DD has a barium enema frequency of 8%-22%^[6,7], affects the right side of the colon in 70%-98% of cases^[6,8], has a slight female predilection, and a peak incidence in patients aged 50-60 years^[8,9]. In Sub-Saharan Africa, DD is thought to be uncommon, affects the right colon in 62%-94% of cases, affects males more often than females, and is found in patients aged 45-60 years^[10-12].

Patients with left-sided DD typically present with left-sided abdominal manifestations of acute or chronic inflammation or bleeding, and the diagnosis is usually made simply on history alone, or is confirmed by the combination of endoscopic and/or radiological investigations. In contrast, complications of right colon DD may be difficult to diagnose, because of overlap between associated symptoms and signs and those of other right-sided abdominal conditions, particularly in hospitals where the disease is considered uncommon^[7]. This can lead to misdiagnoses, to inappropriate operations, or to repeated and unnecessary investigations^[7,13], and to an increase in suffering for the patient and cost to the hospital.

This study aimed to investigate whether associations exist between ethnicity, age and sex and the risk, colon distribution and density scores of DD, in patients undergoing barium enema examination for non-emergency gastrointestinal symptoms in London, UK.

MATERIALS AND METHODS

Data collection

The Ethics Committee of University Hospital Lewisham approved the study. One thousand consecutive double contrast barium enema radiographs were analyzed from 1000 patients (410 male, 590 female, mean age: 58.73 years), who presented with non-emergency gastrointestinal symptoms, over 18 mo between 2004 and 2006, at the University Hospital Lewisham, London, UK. Non-emergency cases included those with changes in bowel habits, abdominal pain, and non-massive per-rectal bleeding. Emergency cases were excluded and included those with bowel obstruction, massive colon bleeding, colon perforation, inflammatory mass, or known colon or rectal cancer. These were investigated instead by a combination

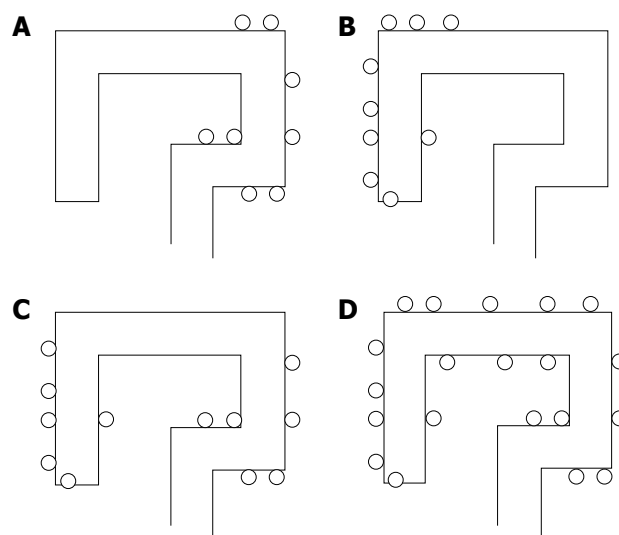


Figure 1 Classification of the pattern of distribution of diverticula throughout the colon. A: Left-sided diverticular disease; B: Right-sided diverticular disease; C: Right and left diverticular disease; D: Pan-diverticular disease.

of computed tomography and colonoscopy.

The London Borough of Lewisham has a population of approximately 258 000, comprising whites (65%), Black Africans (BAs) (10%), Black Caribbean and other blacks (OBs) (15%), Asians (7.5%) and other ethnic groups (2.5%). The proportions of BAs and OBs in the Lewisham population are twice the London average.

Patients were divided into four groups according to self-reported ethnicity: (1) whites ($n = 760$) included White British ($n = 732$) and other white ethnic groups ($n = 29$); (2) Asians ($n = 62$) included Indian ($n = 10$), Chinese ($n = 4$), Bangladeshi ($n = 3$), Pakistani ($n = 4$) and other Asian background ($n = 41$); (3) BA ($n = 44$); and (4) OB ($n = 134$) included Black Caribbean ($n = 80$) and other black ethnicities ($n = 54$). An additional 20 patients of other ethnic background were excluded from the study, because this group did not have epidemiological relevance.

Double contrast barium enema is the most accurate method for the detection of diverticula^[14], therefore, the period of investigation was chosen to coincide with that of a previous temporary reconfiguration of endoscopy services at the hospital, during which time the combination of barium enema and flexible sigmoidoscopy, rather than colonoscopy, was the chosen investigation for non-emergency cases. Three consultant radiologists, AF, MB and AS, assessed the density of diverticula in the sigmoid, descending, transverse, ascending and cecum segments of the colon, according to the following ranking system: no diverticula = 0; 1-5 diverticula = 1; 6-10 = 2; 11-15 = 3; 16-20 = 4; 21-25 = 5; 25+ = 6. The highest score across these segments for each patient was then ranked to either lower density (scores 1-3) or higher density (scores 4-6) for the purpose of statistical analysis.

The pattern of colon distribution of diverticula was first assigned to one of four conventional categories of the disease: left-sided DD (LSDD), right-sided DD (RSDD), right and left DD (R&LDD) and Pan-DD (Figure 1). RSDD, R&LDD and Pan-DD were then grouped together

Table 1 Study sample partition by disease type, ethnicity and sex

Ethnicity sex DD site	Whites		Asians		OBs		BAs		Sub-totals by sex		Totals
	M	F	M	F	M	F	M	F	M	F	
A. LSDD	114	176	3	4	5	12	2	6	126	196	322
B. RSDD	6	4	4	1	2	4	4	1	16	10	26
C. R&LDD	13	10	1	0	1	1	4	1	19	12	31
D. Pan-DD	20	26	3	2	7	5	2	3	32	36	68
No disease	150	241	24	20	34	63	11	10	219	334	553
Sub-totals by sex (all)	303	457	35	27	49	85	23	21	410	590	1000
Sub-totals by ethnicity (all)	760		62		134		44		410	590	1000
Sub-totals by sex (DD overall)	153	216	11	7	15	22	12	11	191	256	447
Sub-totals by ethnicity (DD overall)	369		18		27		23		191	256	447
Right-sided component (B+C+D) (RSCDD)	39	40	8	3	10	10	10	5	67	58	125
	79		11		20		15		67	58	125

OBs: Other blacks; BAs: Black africans; DD: Diverticular disease; LSDD: Left-sided DD; RSDD: Right-sided DD; R&LDD: Right and left DD; RSCDD: Right-sided component DD.

as right-sided component DD (RSCDD), which included all those patients in whom DD affected the right side of the colon.

Statistical analysis

STATA 10.1 statistical package (Stata Corporation, College Station, TX, USA) was used for descriptive statistics, statistical inference and graphs. Groups, defined by sex, ethnicity and disease were compared using ANOVA or non-parametric tests. Logistic regression analysis was used to assess crude and adjusted associations between the odds of DD, DD subtypes (LSDD or RSCDD) and diverticula density, ethnicity, age and sex. Models of the risk of the disease/disease subtype/diverticula density were produced, and the Hosmer-Lemeshow test^[15] was used to assess goodness of fit. Post-estimation analysis was performed to detect possible differences between non-white ethnic groups. The predefined level of type 1 error was adjusted using Bonferroni correction. For the “DD overall” analyses, this was reduced to 0.0083 (0.05 divided by 6, representing the number of all possible comparisons between the four ethnic groups), and for the disease subtype analyses to 0.004 (0.05 divided by 2×6). This was the first investigation of its kind, therefore, no data existed prior to the study, regarding standard deviations for the above parameters. It was therefore not possible to make sample size calculations before the study began. Budgetary constraints limited our sample size to 1000 patients.

RESULTS

Data summary

Data related to diagnosis, ethnicity and sex are summarized in Table 1. Of 1000 patients, 447 (44.7%) had DD, 322 (72%) had LSDD, and 125 (28%) had RSCDD. One hundred and ninety-one (42.7%) were male and 256 (57.3%) were female. The mean age of patients with DD was 62.28 years, which was higher than that of those without DD (55.87 years); difference 6.41 (95% CI: 5.07-7.76, $P < 0.001$). The mean age of patients with LSDD was

2.74 years (95% CI: 0.52-4.95) greater than that of patients with RSCDD, $P = 0.016$ (Bonferroni correction, $P = 0.05/3 = 0.017$). The age distribution in the diseased population did not exhibit normality (Figure 2). The mean ages of BAs (57.6 years) and Asians (56.8 years) were lower than that for whites (62.9 years) and OBs (61.2 years) with the disease. Nevertheless, in BA there was a bimodal age distribution, centered in younger patients (12 less than 60 years old) with a mean age of 47.83 years, and older patients (11 more than 60 years old) with a mean age of 68.18 years.

Diverticular disease (overall)

Age and ethnicity, but not sex, were associated with the risk of DD (Table 2). A 1-year increase in the age of an individual increased the odds of having the disease by 6% (4%-7%), ($P < 0.001$), irrespective of ethnicity. Univariate analysis showed that Asians were 0.43 times (95% CI: 0.25-0.76, $P = 0.004$), and OBs were 0.40 times (95% CI: 0.27-0.61 $P < 0.001$) less likely than whites to have DD. BAs were 2.87-fold (95% CI: 1.42-5.77, $P = 0.003$) more likely to acquire the disease compared with OBs. There was a trend towards BA being more likely than Asians to develop the disease [odds ratio (OR): 2.68, 95% CI: 1.2-5.99, $P = 0.017$], but this was not significant when the pre-defined level dropped to 0.004. No significant differences were found between BAs and whites or between OBs and Asians at the studied sample size. In the adjusted models, sex was dropped because it had no confounding effect. Statistical significance was preserved for OR between OBs and whites ($P < 0.001$) and between BAs and OBs ($P = 0.002$), but the odds in Asians increased to 0.49 of that in whites ($P = 0.018$).

LSDD

Age, but not sex, was a predictor for LSDD, with the percentage increase in odds per year the same as for DD overall (Table 2). Asians and OBs appeared protected against left-sided disease compared with whites, even after adjusting for age, with the odds of LSDD being 23%

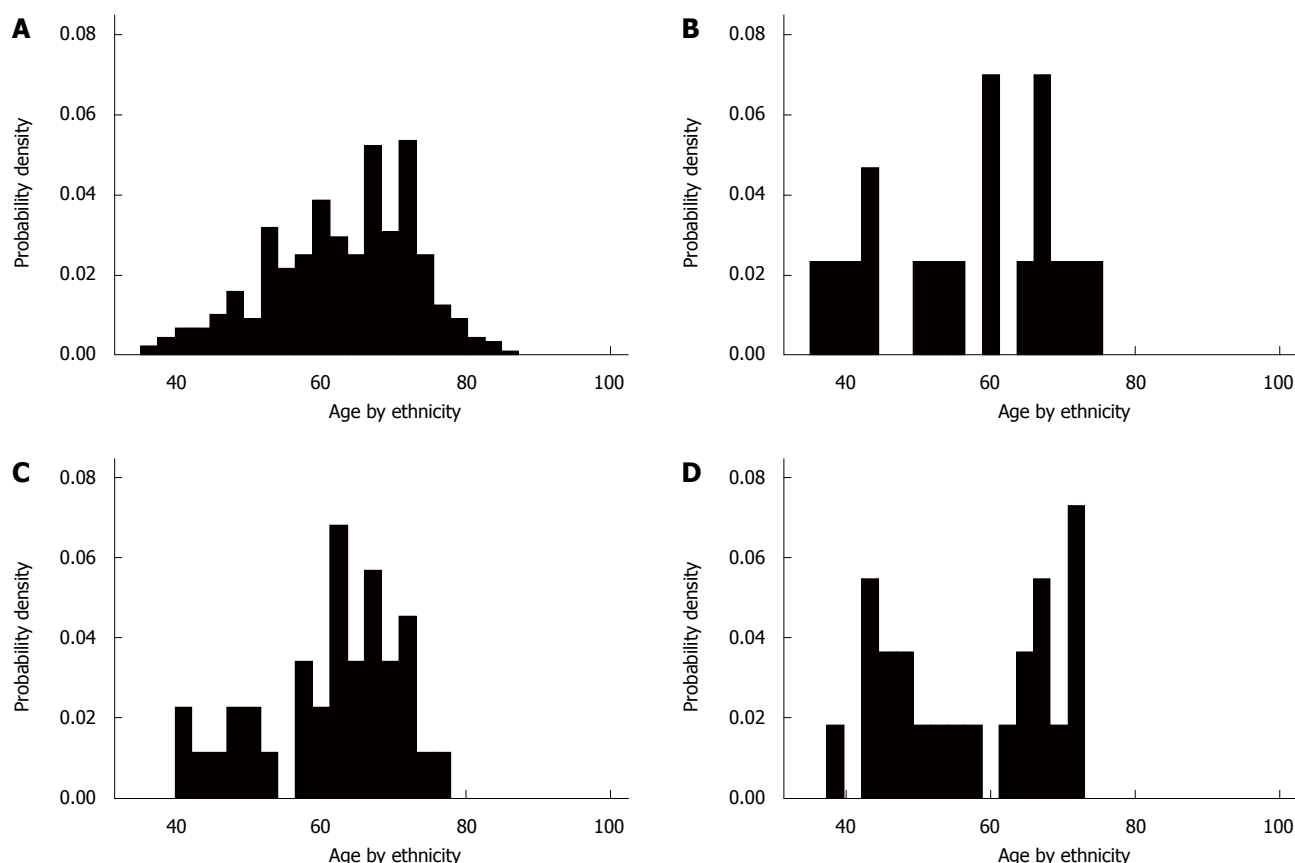


Figure 2 Age and probability density by ethnicity in patients diagnosed with diverticular disease. A: White; B: Asian; C: Other black; D: Black African.

(95% CI: 10-53) and 25% (95% CI: 14-43, $P \leq 0.001$) of that in whites, respectively, (Table 2). The differences between other groups were not statistically significant, at the studied sample size.

RSCDD

Sex was a predictor of RSCDD (Table 2). The odds of women having RSCDD was about 40% less than that in men; an effect that was conserved after adjusting for age and ethnicity (OR: 0.60, 95% CI: 0.40-0.90, $P = 0.01$). Age was a strong predictor, with the adjusted analysis (unchanged from the crude estimate) finding a 4% (95% CI: 2-6, $P < 0.001$) increase in the odds of RSCDD, per year of age. Univariate analysis showed that BAs were 3.54 times (95% CI: 1.75-7.16, $P < 0.001$) more likely than whites to have RSCDD (Table 2); a ratio that was relatively unchanged after adjusting for age and sex (OR: 3.51, 95% CI: 1.70-7.24, $P = 0.001$). BAs were 3.46-fold (95% CI: 1.53-7.86, $P < 0.003$) more likely to be diagnosed with RSCDD than OBs in the crude analysis, but the difference became non-significant following adjustments for age and sex. BAs were more likely to develop RSCDD than Asians, but this trend was not significant at the studied sample size.

Diverticula density

In the analysis of colon segments, Pan-DD had comparatively higher density scores [median (range)]: Pan-DD (sigmoid) 5 (0-6), (descending) 3 (0-6), (transverse) 3 (0-6), (ascending) 2 (0-6), (cecum) 0 (0-6); RSDD (transverse)

0 (0-2), (ascending) 1 (0-2), (cecum) 1 (0-3); R+LDD (sigmoid) 2 (0-6), (descending) 1 (0-3), (ascending) 2 (0-4), (cecum) 1 (0-2); LSDD (sigmoid) 3 (0-6), (descending) 0 (0-6), (transverse) 0 (0-4).

Disease subtype (LSDD and RSCDD) and age were strong predictors of density. The odds of a higher score in RSCDD was 1.91 times that found in LSDD (95% CI: 1.25-2.91 $P = 0.003$), and after adjustment for age, this increased to 2.77 (95% CI: 1.39-3.32, $P < 0.001$) (Table 3). In the final model, each year increase in age yielded a 4% (95% CI: 1-6, $P < 0.001$) increase in the odds of having a higher density score. There was not enough evidence to support an association between diverticula density and sex or ethnicity, in either the crude or final models, at the sample size studied.

Predictions

The tests for Hosmer-Lemeshow statistics were consistent with a good fit of the models to the data; with $P = 0.66$ for DD overall, and $P = 0.65$, $P = 0.71$ and $P = 0.55$ for LSDD, RSCDD and for diverticula density, respectively. Using the adjusted estimates from Tables 2 and 3, the predicted risks for disease overall, disease subtype and higher density (HD), ($R_{DD}/R_{LSDD}/R_{RSCDD}/R_{HD}$, respectively) were computed by:

$$\log [R_{DD}/(1-R_{DD})] = -0.10 - 0.71 \times I_{AS} - 0.84 \times I_{OB} + 0.33 \times I_{BA} + 0.05 \times (\text{age} - 58.73)$$

$$\log [R_{LSDD}/(1-R_{LSDD})] = -0.36 - 1.48 \times I_{AS} - 1.39 \times I_{OB} - 0.48 \times I_{BA} + 0.06 \times (\text{age} - 58.73)$$

Table 2 Crude and adjusted associations between the odds of disease, disease subtypes and demographic parameters

	Univariate analysis		Adjusted analysis-final models	
	OR/proportional odds (SE) (95% CI)	P value	OR/proportional odds (SE) (95% CI)	P value
Disease <i>vs</i> no disease				
Asian (<i>vs</i> white)	0.43 (0.13) (0.25, 0.76)	0.004	0.49 (0.15) (0.27, 0.87)	0.018
OB (<i>vs</i> white)	0.40 (0.08) (0.27, 0.61)	< 0.001	0.43 (0.09) (0.28, 0.65)	< 0.001
BA (<i>vs</i> white)	1.16 (0.36) (0.63, 2.13)	0.63	1.39 (0.45) (0.74, 2.62)	0.31
Sex (F <i>vs</i> M)	0.88 (0.11) (0.68, 1.13)	0.32	-	-
Age (yr) (proportional odds)	1.06 (0.007) (1.04, 1.07)	< 0.001	1.06 (0.007) (1.04, 1.07)	< 0.001
	Post estimation			
BA (<i>vs</i> Asian)	2.68 (1.10) (1.20, 5.99)	0.017	2.83 (1.21) (1.22, 6.56)	0.015
BA (<i>vs</i> OB)	2.87 (1.03) (1.42, 5.77)	0.003	3.22 (1.21) (1.55, 6.71)	0.002
OB (<i>vs</i> asian)	0.93 (0.32) (0.44, 1.74)	0.84	0.87 (0.31) (0.45, 1.79)	0.70
LSDD <i>vs</i> no disease				
Asian (<i>vs</i> white)	0.21 (0.09) (0.1, 0.48)	< 0.001	0.23 (0.1) (0.10, 0.53)	0.001
OB (<i>vs</i> white)	0.24 (0.06) (0.14, 0.40)	< 0.001	0.25 (0.07) (0.14, 0.43)	< 0.001
BA (<i>vs</i> white)	0.51 (0.22) (0.22, 1.18)	0.12	0.62 (0.27) (0.26, 1.46)	0.27
Sex (F <i>vs</i> M)	1.05 (0.15) (0.79, 1.39)	0.75	-	-
Age (yr) (proportional odds)	1.06 (0.008) (1.05, 1.08)	< 0.001	1.06 (0.008) (1.05, 1.08)	< 0.001
	Post estimation			
BA (<i>vs</i> asian)	2.39 (1.39) (0.77, 7.49)	0.13	2.71 (1.62) (0.83, 8.71)	0.10
BA (<i>vs</i> OB)	1.10 (0.53) (0.43, 2.85)	0.84	2.48 (1.26) (0.91, 6.72)	0.075
OB (<i>vs</i> Asian)	2.17 (1.07) (0.83, 5.70)	0.11	1.09 (0.54) (0.41, 2.89)	0.88
RSCDD <i>vs</i> no disease				
Asian (<i>vs</i> white)	1.24 (0.44) (0.61, 2.50)	0.55	1.23 (0.45) (0.60, 2.54)	0.57
OB (<i>vs</i> white)	1.02 (0.28) (0.60, 1.75)	0.94	1.07 (0.30) (0.62, 1.85)	0.82
BA (<i>vs</i> white)	3.54 (1.27) (1.75, 7.16)	< 0.001	3.51 (1.30) (1.70, 7.24)	0.001
Sex (F <i>vs</i> M)	0.57 (0.11) (0.38, 0.84)	0.004	0.60 (0.12) (0.40, 0.90)	0.01
Age (yr) (proportional odds)	1.04 (0.01) (1.02, 1.06)	< 0.001	1.04 (0.01) (1.02, 1.06)	< 0.001
	Post estimation			
BA (<i>vs</i> asian)	2.86 (1.36) (1.12, 7.28)	0.028	2.85 (1.39) (1.10, 7.40)	0.03
BA (<i>vs</i> OB)	3.46 (1.45) (1.53, 7.86)	0.003	3.30 (1.41) (1.42, 7.62)	0.005
OtB (<i>vs</i> asian)	0.82 (0.34) (0.36, 1.87)	0.64	0.87 (0.37) (0.36, 1.87)	0.73

OR: Odds ratio; OB: Other black; BA: Black african; LSDD: Left-sided diverticular disease; RSCDD: Right-sided component diverticular disease.

Table 3 Odds of having a higher density score in relation to disease subtype and demographic parameters

Diverticula density scores	Predictors	Univariate analysis		Adjusted analysis-final model	
		OR (SE) (95% CI)	P value	OR (SE) (95% CI)	P value
Higher <i>vs</i> lower	Disease type: RSCDD <i>vs</i> LSDD	1.91 (0.41) (1.25, 2.91)	0.003	2.77 (0.67) (1.39, 3.32)	0.001
	Asian (<i>vs</i> white)	0.59 (0.32) (0.21, 1.70)	0.33	0.47(0.27) (0.16, 1.43)	0.18
	OB (<i>vs</i> white)	0.57 (0.22) (0.27, 1.22)	0.15	0.41(0.17) (0.18, 0.92)	0.03
	BA (<i>vs</i> white)	0.43 (0.22) (0.16, 1.18)	0.1	0.30(0.17) (0.10, 0.88)	0.03
	Sex (F <i>vs</i> M)	0.98 (0.19) (0.67, 1.45)	0.92	-	-
	Age (proportional odds)	1.032 (0.01) (1.01, 1.05)	0.002	1.04 (0.01) (1.01, 1.06)	< 0.001
		Post-estimation			
	BA (<i>vs</i> Asian)	0.72 (0.52) (0.17, 3.02)	0.66	0.73(0.47) (0.21, 2.60)	0.63
	BA (<i>vs</i> OB)	0.96 (0.62) (0.27, 3.40)	0.95	0.64 (0.48) (0.15, 2.79)	0.55
	OB (<i>vs</i> Asian)	0.75 (0.47) (0.22, 2.56)	0.65	0.87(0.58) (0.24, 3.21)	0.84

OR: Odds ratio; OB: Other black; BA: Black african; RSCDD: Right-sided component diverticular disease; LSDD: Left-sided diverticular disease.

$$\log [R_{RSCDD}/(1-R_{RSCDD})] = -0.31 + 0.21 \times I_{AS} - 0.06 \times I_{OB} + 1.26 \times I_{BA} + 0.04 \times (\text{age} - 58.73) - 0.51 \times I_{FEMALE}$$

$$\log [R_{HD}/(1-R_{HD})] = -0.70 - 0.75 \times I_{AS} - 0.89 \times I_{OB} + 1.20 \times I_{BA} + 0.034 \times (\text{age} - 62.27) + 1.02 \times I_{RSCDD}$$

The symbols $I_{AS}/I_{OB}/I_{BA}/I_{FEMALE}$ were indicators that took the value 1 if an individual belonged to the AS/OB/BA ethnic group or was female, and 0 if otherwise. The linear combination on the right side of each expres-

sion was the linear predictor.

The free terms for disease risk R_{DD} (e.g. -0.10) represented the log odds of having that condition, where age was set to the average of 58.73 years of the population in the study and the ethnic group was set to white. For R_{RSCDD} , the free term had a similar connotation but was in addition set to males. The free term for higher density R_{HD} (e.g. -0.70) represented the log odds of acquiring a

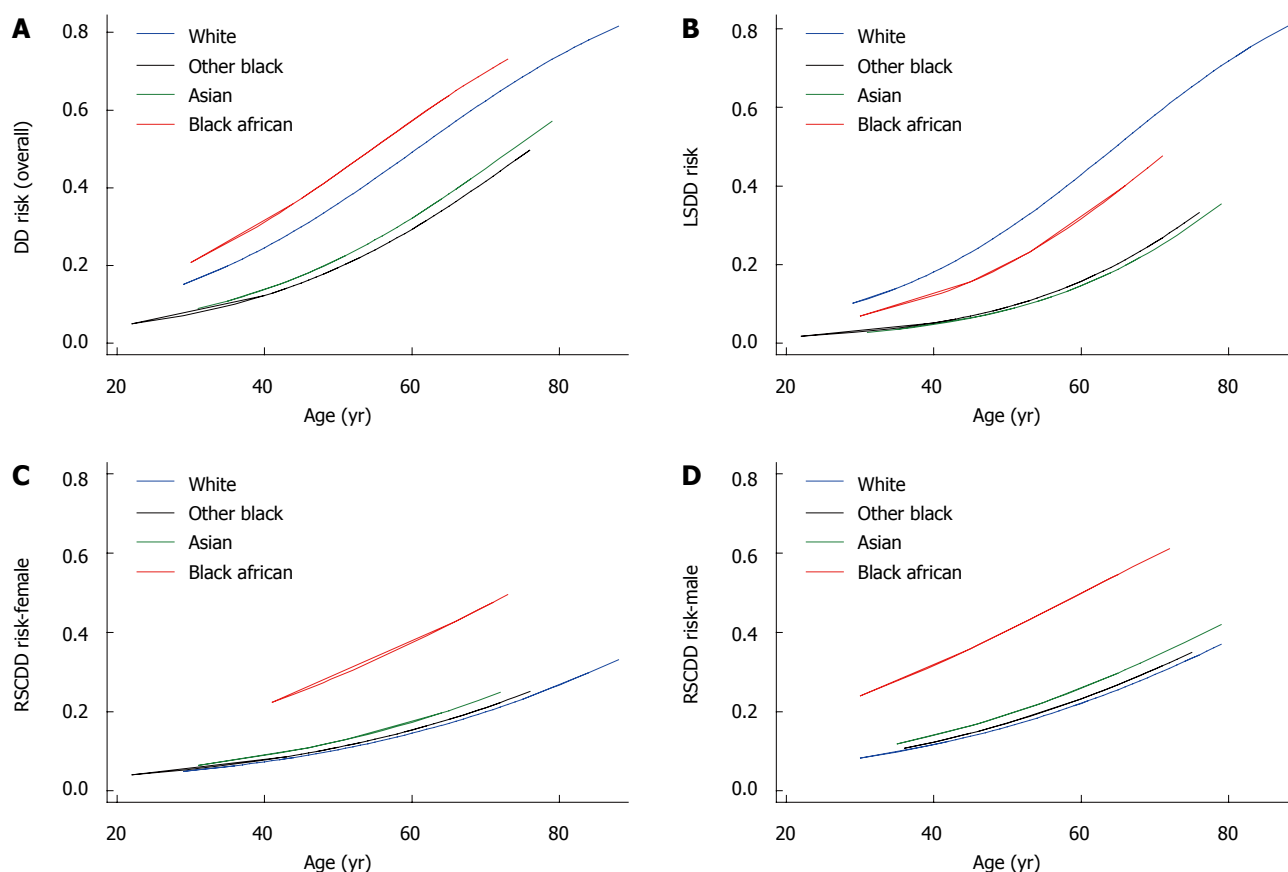


Figure 3 Predictive risk of being diagnosed with diverticular disease by age, ethnicity and sex using the corresponding final models in Table 2. A: Diverticular disease (DD) overall; B: Left-sided DD (LSDD); C: Right-sided component DD (RSCDD)-female; D: RSCDD-male.

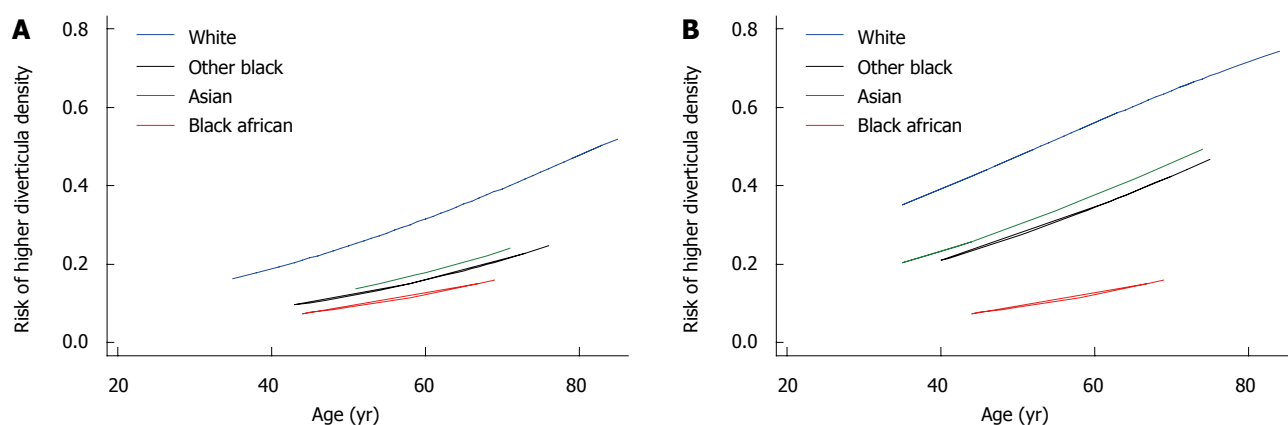


Figure 4 Predictive risks of having a higher diverticula density form, by age and ethnicity. A: Left-sided diverticular disease; B: Right-sided component diverticular disease.

higher density amongst white individuals of 62.27 years of age (mean age in *LSDD*/*RSCDD* patients), with the *LSDD* disease type. The generic risks of disease and higher density were calculated by: $R = [\exp(\text{linear predictor}) / 1 + (\text{linear predictor})]$.

The predicted curves for risk of DD overall, DD subtypes and higher density scores are given in Figures 3 and 4.

DISCUSSION

We investigated demographic factors associated with the

risk, colon distribution and density scores of DD in patients who underwent a barium enema examination for non-emergency gastrointestinal symptoms in London, UK. There were significant differences in the risk and colon distribution of DD between certain ethnic groups, and diverticula density was predicted by colon distribution and age. The results suggest that RSCDD, which comprises Pan-DD (54%), RSDD (21%) and R&LDD (25%), is significantly more common than previous studies in the west, and is most frequent in BAs. The prevalence of DD overall (47%) was high compared with previous studies in

the west (15%-35%)^[3-5] and supports previous reports that the prevalence of DD may be increasing with time^[16,17]. Age, but not sex, was found to be a strong predictor of DD, with the mean age of patients with the disease comparable to that found in previous studies^[5,18], and the chance of having the disease increased by 6% per year, irrespective of sex or ethnicity. Whites and BAs had a similar high predisposition to DD, while Asians and OBs appeared relatively protected.

These results suggest the risk of DD in BAs in London is higher than that of their indigenous counterparts, even if one accounts for an increase in prevalence of DD following urbanization in Africa^[10,12,19]. Our results appear to contradict two previous studies of African migrants in Europe, which found significantly lower rates of admissions with complications of the disease, compared with native whites^[16,20]. They reported that the rate was highest in younger migrants^[16], and that it increased with time following settlement, possibly due to acculturation to a western diet^[20]. However, the low rates of admission in those studies could have been related to low rates of acute complications that required admission amongst Africans with DD, compared with other ethnic groups, and not to an actual low prevalence of the disease. Furthermore, the reported relative high number of admissions in younger blacks could have been due to a higher proportion of blacks in the younger, compared with the older local populations. Such an explanation is supported by the current study, which found that, although there was a peak frequency of DD in young BAs, the predictive risk of the disease increased in a uniform manner with age.

It is possible that our study also suffered bias towards symptomatic patients, because it was an observational study, rather than a population study. It is therefore not possible to draw definitive conclusions from our results on actual population incidence of the disease. However, our study did have an advantage over previous studies, in that it determined with accuracy the proportion of the cases that had the disease. Furthermore, our study design did allow comparisons to be made with previous studies of DD, which all studied patients with symptomatic gastrointestinal disease, and which all included patients with symptomatic and asymptomatic DD. It also had the advantage of providing information on a cohort of patients that was typical of that seen in a standard gastrointestinal outpatient clinic. Any study that includes only asymptomatic patients or those with specific symptoms would appear artificial and could introduce bias towards certain ethnic groups and/or subtypes of the disease.

Our results suggest that the comparatively low risk of DD in Asians^[6,7] is conserved in those who live outside Asia, even though more recent reports have suggested that DD is increasing in frequency in Asia with time^[8,9]. Our results support those of a previous study that showed the admission rate in migrant Asians to be half that of native Europeans^[20]. There have been no previous reports on the prevalence of DD in OB groups, and it remains unclear why they should be protected against the disease.

LSDD was more frequent than RSCDD, but it ac-

counted for a lower proportion of cases compared with previous studies in the west^[3,5]. Age, but not sex, was a strong predictor, with the odds of LSDD increasing by 6% per annum. Whites appeared predisposed, with Asians and OBs significantly protected. The finding that Asians are relatively protected against LSDD suggests that the typical colon distribution of the disease in Asians is conserved following population migration. There were no significant differences in predisposition between BAs and other ethnic groups, at the studied sample size.

RSCDD accounted for 28% of all cases of DD, a far higher proportion than reported previously in the west (10%-17%)^[3,5], even though the sex and age mixes were comparable. This trend was probably due to predominance of Pan-DD in the largest ethnic group, whites. BA ethnicity was a strong predictor of RSCDD. BAs were significantly more likely than whites and OBs to develop RSCDD, and appeared equally predisposed to each of the three subtypes of RSCDD. There was a trend towards BAs being more likely than Asians to develop RSCDD, but this was not statistically significant, at the sample size studied.

Our findings suggest that the BA predisposition to right colon involvement^[10-12] is conserved following population migration. This, together with the fact that this distribution of DD in Africa is conserved even in those regions where the incidence of the disease has increased to western levels^[19] suggests that there could be specific genetic and/or environmental factors involved in the pathogenesis of DD in BAs, and that such environmental factors could be conserved following population migration. Important information on possible genetic and environmental causes of the disease may come from follow-up investigations into whether the patterns of DD found in this study are exhibited by specific generations of migrants.

Evidence suggests that dietary fiber may be protective against DD^[21] but exposure to red meat^[4,22] and other unidentified toxins^[10,12], inflammation^[23], as well as genetic factors^[24] may also be involved. It is probable that an imbalance between protective and promoting factors leads to abnormalities in colonic nerve innervation and connective tissue turnover found in DD^[25] and to the abnormalities in colon motility^[26] and subsequent diverticula formation^[27].

RSCDD and age were found to be strong independent predictors of the higher density form of the disease in the final models, whereas sex and ethnic background were not. It may be argued that because patients with RSCDD were on average twice as likely to have a higher density score compared with those with LSDD, this contradicts previous anecdotal reports that the density of RSDD is less than that of LSDD. However, our results suggest that the relative contribution made by Pan-DD, which accounted for 54% of the total RSCDD, and which had significantly higher median density scores compared with the other disease subtypes, was the reason for this apparent discrepancy.

However, it is not known whether differences in the distribution or density of diverticula affect the risk of acute or chronic complications of the disease. One study

has estimated that acute complications occur in 15%-20% for predominantly LSDD in the west^[28] and another cited 1.0%-4.5% for predominantly RSDD in Southeast Asia, and that patients with only one or two diverticula are least likely to experience acute symptoms^[8]. The proportion of patients diagnosed with DD on barium enema, who then go on to experience a chronic bleed or symptoms of irritable colon, is also unknown, although one uncontrolled study has suggested the latter may be as high as 55%^[29]. Nevertheless, our results suggest that one should maintain a high index of suspicion of RSCDD as a potential diagnosis, both in outpatient and emergency settings.

However, such a diagnosis and its management may not be straight forward. There is considerable overlap between symptoms and signs of RSDD and those of other abdominal conditions including acute appendicitis^[30], irritable bowel syndrome^[26] and colon cancer^[30]. Bleeding from RSDD can also be difficult to manage, because it may mimic upper gastrointestinal bleeding due to the presence of melena, and is less likely to respond to non-surgical treatment, compared with left-sided disease^[31].

Overall, our results suggest that right-colonic DD is more common and has higher density scores in the west than previously reported. BAs appear predisposed to DD, while other ethnic differences appear conserved following migration. Within clinical practice, such knowledge is likely to aid diagnosis and management, and within research, to point to areas of potential future investigation.

COMMENTS

Background

Diverticular disease (DD) is common in western societies, causes considerable suffering and is a financial burden to hospitals. Differences exist in the incidence and colon distribution of DD between indigenous populations worldwide, but it is unknown if these are conserved following ethnic migration.

Research frontiers

Indigenous Asians and Africans appear protected against DD compared with whites in the west. Right-colonic DD is more frequent in Asia and Africa than in the west. The etiology of DD and the reasons for variations in its colon distribution and severity remain unknown.

Innovations and breakthroughs

Right-colonic DD is more common in the west than previously reported and is most frequent in black africans (BAs). BAs appear predisposed to DD, while other ethnic differences appear conserved following migration. The severity of DD is determined by its colon distribution and patient age.

Applications

Within clinical practice, our findings are likely to aid diagnosis and management, and within research, to point to areas of potential future investigation.

Terminology

The density scores were used as an indication of the numbers of diverticula present within the respective segments of the colon, and were calculated by diverticula counts on barium enema examinations.

Peer review

This manuscript presents data on the incidence of diverticulosis in 1000 consecutive barium enema evaluations performed in London, UK. The development of diverticulosis as a result of immigration to the west is an interesting topic and one that has been reported little in contemporary literature.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Comparison of high-resolution ultrasound and MR-enterography in patients with inflammatory bowel disease

Andreas G Schreyer, Cynthia Menzel, Chris Friedrich, Florian Poschenrieder, Lukas Egger, Christian Dornia, Gabriela Schill, Lena M Dendl, Doris Schacherer, Christl Girlich, Ernst-Michael Jung

Andreas G Schreyer, Cynthia Menzel, Chris Friedrich, Florian Poschenrieder, Lukas Egger, Christian Dornia, Gabriela Schill, Lena M Dendl, Ernst-Michael Jung, Institute of Radiology, University Medical Center, 93053 Regensburg, Germany
 Doris Schacherer, Christl Girlich, Ernst-Michael Jung, Department of Interdisciplinary Ultrasound, University Medical Center, 93053 Regensburg, Germany

Author contributions: Schreyer AG, Girlich C and Jung EM designed the research; Schreyer AG, Menzel C, Dendl LM and Schacherer D performed the research; Schreyer AG, Friedrich C, Poschenrieder F, Egger L and Schill G analyzed the data; Schreyer AG, Dornia C and Jung EM wrote the paper.

Correspondence to: Andreas G Schreyer, MD, MBA, Associate Professor, Institute of Radiology, University Medical Center, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany. andreas.schreyer@klinik.uni-regensburg.de
 Telephone: +49-941-9447442 Fax: +49-941-9447402

Received: August 27, 2010 Revised: November 25, 2010

Accepted: December 2, 2010

Published online: February 28, 2011

RESULTS: Two hundred and fifty examinations representing 207 different patients were evaluated. Both modalities assessed similar bowel wall changes in 65% of the examinations, with more US findings in 11% and more MRE findings in 15%. When the reports were analyzed with regard to "bowel wall inflammation", US reported more findings in 2%, while MRE reported more findings in 53%. Stenoses were assessed to be identical in 8%, while US found more in 3% and MRE in 29% ($P < 0.01$). For abscess detection, US showed more findings in 2% ($n = 4$) while MRE detected more in 6% ($n = 16$). US detected more fistulas in 1% ($n = 2$), while MRE detected more in 13% ($n = 32$) ($P < 0.001$). The most common reason for no detected pathology by US was a difficult to assess anatomical region (lesser pelvis, $n = 72$).

CONCLUSION: US can miss clinically relevant pathological changes in patients with IBD mostly due to difficulty in assessing certain anatomical regions.

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Abstract

AIM: To compare the results of high-resolution ultrasound (HR-US) and magnetic resonance enterography (MRE) examinations in patients with inflammatory bowel disease (IBD).

METHODS: The reports of 250 consecutive cases with known IBD, who had an MRE and HR-US examination, were retrospectively analyzed. Using a patient-based approach we evaluated morphological disease features such as affected bowel wall, stenosis, abscess and fistula. The comparison between the two modalities was based on the hypothesis, that any pathological change described in any imaging modality was a true finding, as no further standard of reference was available for complete assessment.

Key words: Crohn's disease; Diagnosis; Inflammatory bowel disease; Magnetic resonance imaging; Ultrasound

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Kórányi S 2A, Budapest H1083, Hungary

Schreyer AG, Menzel C, Friedrich C, Poschenrieder F, Egger L, Dornia C, Schill G, Dendl LM, Schacherer D, Girlich C, Jung EM. Comparison of high-resolution ultrasound and MR-enterography in patients with inflammatory bowel disease. *World J Gastroenterol* 2011; 17(8): 1018-1025 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1018.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1018>

INTRODUCTION

Patients suffering from inflammatory bowel disease (IBD) need a complete bowel assessment for their primary diagnosis. In addition, follow-up examinations are necessary during deterioration of clinical symptoms or for therapy monitoring.

According to most recent national or international guidelines, high-resolution ultrasound (HR-US) should be the first-line diagnostic modality for disease assessment or follow up examinations^[1,2]. If an IBD is assumed, several guidelines recommend a complete sectional imaging of the small bowel. Because of the lack of any ionizing radiation, a dedicated magnetic resonance imaging (MRI) examination of the small bowel is therefore recommended. Currently magnetic resonance (MR) enteroclysis with contrast application *via* a naso-jejunal tube as well as MR enterography (MRE), where the contrast is applied orally, are the methods of choice. Because of the expensive radiological equipment and the need for intravenously applied contrast media, MRE is an examination which is less available and more cost intensive compared to HR-US. On the other hand, US is more subjective and highly dependent on the experience of the examiner, but requires less expensive equipment.

The current literature shows excellent sensitivity of between 78% and 96% with specificity between 67% and 100% for HR-US in IBD^[3]. On the other hand, MRE shows sensitivity of between 82% and 100% with specificity between 71% and 100%. Most studies were performed in an academic environment by specialists in their field. In our experience a total small bowel assessment by HR-US during daily routine is difficult to perform. Several segments of the bowel cannot be visualized by US because of residual air in the bowel, anatomical obstacles in the pelvic region or superposition of other bowel loops.

To assess the diagnostic and therapeutic impact of HR-US and MRE in patients with IBD in a day-to-day clinical setting, we decided to perform a retrospective study. By analyzing and comparing the written reports retrospectively, we intended to avoid the study bias sometimes caused by extremely motivated study examiners, and depict the report quality for both modalities under everyday circumstances.

In this study we compared and evaluated the results of HR-US and MRE examinations in patients suffering from IBD, with regard to clinically relevant disease features such as bowel wall affection, stenosis, fistula, abscess and indirect inflammation indicators such as local lymphadenopathy, mesenteric fat injection or enlarged local vessels.

MATERIALS AND METHODS

Patient selection

Using our radiological information system, we retrospec-

tively searched for patients who underwent an MRE examination between October 1999 and January 2007 in our tertiary care medical center. After identifying 1582 MRE examinations during this period, we selected all patients in this group with histologically and clinically proven IBD, resulting in 801 MRE examinations. Based on these 801 cases we evaluated all patients who had a HR-US examination within a 14-d period before or after MRE. Based on this selection we chose 250 consecutive cases for further analysis.

MRE examination

MRE was performed using a 1.5-Tesla MRI unit (Magnetom Symphony and Magnetom Sonata, Siemens Healthcare, Erlangen, Germany) using a circular polarized 6-channel phased array body coil supplemented with a spine array coil. The dark lumen technique with an oral contrast of 1.5-2 L of water mixed with methylcellulose was applied. A coronal True-fast imaging with steady state precession sequence and an axial T2-weighted half-Fourier acquired single-shot turbo spin echo sequence were acquired. Subsequently, 0.1 mmol/kg body weight of Gd-DTPA (different vendors during the examination period) was injected intravenously. A fat-suppressed 3D gradient echo sequence and a fat-suppressed T1-weighted 2D gradient echo sequence with axial and coronal orientation were acquired with a delay of 70 s after the start of the contrast injection.

If the clinical examination or anamnesis suggested fistulas, additional sequences with 4 mm slice thickness (axial and coronal T2 short tau inversion recovery and T1-weighted sequences before and after Gd-DTPA iv) were acquired. The MRE examinations were evaluated by a board certified radiologist and an experienced resident, resulting in a written and electronically documented report based on a consensus decision.

HR-US

HR-US of the bowel was performed in our interdisciplinary ultrasound department by a resident in internal medicine, surgery or radiology or by a consultant in internal medicine. To evaluate the influence of the individual experience of the examiner on the examination results in ultrasound, we subdivided the examiners into groups with moderate and high experience. Examiners with high experience were board certified with more than 2000 documented ultrasound examinations per year.

For a clinical routine ultrasound examination of the bowel, a 3.5 MHz convex transducer was applied first. A high-resolution 5 to 10 MHz transducer was then used for bowel imaging. During the evaluation period we used several ultrasound devices: SonolineElegra (Siemens, Erlangen, Germany), Logiq 9 (General Electric, Solingen, Germany) or EUB-8500 (Hitachi, Tokyo, Japan). Ultrasound images were documented in soft prints as part of the patient record. An electronically documented report of the examination was available for all patients.

Data analysis

We retrospectively evaluated the electronically documented reports of MRE and US examinations without reviewing the original imaging data. The 250 cases were evaluated in a patient-based analysis with regard to the following categories: affected bowel wall, bowel stenosis, abscess, fistula, local lymphadenopathy and local fat injection or comb sign. The evaluation of these categories was based on the written report of each modality.

For MRE, the bowel wall was deemed affected when having a diameter of at least 3 mm and/or showing increased contrast uptake. For ultrasound examinations a bowel wall diameter larger than 3 mm was also considered as “thickened bowel”. A thickened bowel wall larger than 3 mm without any data on contrast enhancement of vascularization is a very unspecific finding. For routine HR-US examinations of the bowel, an intravenous contrast application is currently not an established standard. Vascularization information based on Power Doppler imaging was integrated into the ultrasound findings. The mention of an “accentuated” or “slightly thickened” bowel wall in a written ultrasound report is not very specific. For our comparison we separately evaluated the assessment of the bowel wall of each modality regarding the terms “affected” or “inflamed”, which represents a clear clinical statement, and “accentuated” and “slightly thickened”, which is an unspecific statement without immediate clinical consequences.

For bowel stenosis in MRE and US the term “stenosis” as well as “narrowed bowel lumen” was considered as a positive finding. For the diagnosis of an abscess or a fistula the term had to be mentioned in the report. Local lymphadenopathy was not restricted to a certain size or amount of lymph nodes. In this study, the descriptive term “lymphadenopathy” as well as “enlarged” or “multiple” lymph nodes represented the diagnosis of local lymphadenopathy. The diagnosis of a local fat injection was made when terms such as “injected mesenteric fat” were used. The comb sign as an indirect inflammation criterion caused by enlarged vasa recta surrounding the affected bowel was diagnosed with the mention of “comb sign” or the finding of enlarged or accentuated local vessels^[4].

In the patient-based analysis we focused on all cases with identified pathological findings in only one modality, which were not noted in the other modality report. Possible reasons for discrepant findings were analyzed and noted.

Statistical significance was calculated using the Chi-test by McNemar. P -values ≤ 0.05 were considered statistically significant while P values ≤ 0.01 were considered highly significant. Descriptive statistics were calculated using Excel (Office: Mac 2008, Version 12.2.5; Microsoft, Redmond, WA, USA). Significance levels were calculated using SPSS for Windows 16 (SPSS, Chicago, IL, USA).

RESULTS

We evaluated a total of 250 consecutive cases of patients

Table 1 Contingency table of all evaluated 250 cases (patient based) n (%)

Any bowel wall changes (including unspecific changes such as “accentuated wall”, etc.)	Ultrasound	
	Negative	Positive
Magnetic resonance enterography		
Negative	21 (8.4)	28 (11.2)
Positive	38 (15.2)	163 (65.2)

Negative findings means no bowel wall change found in the modality (magnetic resonance enterography or ultrasound) report.

with known IBD, where a MRE and a HR-US examination of the bowel were performed within 14 d. The 250 cases were based on 207 patients. The 207 patients had a mean age of 35.6 years (range 14–77 years; 55% female, 45% male). The evaluated patients suffered from Crohn’s disease (CD; 84.5%, $n = 175$) or ulcerative colitis (UC; 15.5 %, $n = 32$).

In 69% of all evaluated cases ultrasound was performed before MRE, in 16% MRE and ultrasound were performed on the same day, while in 15% MRE was performed before ultrasound. On average, HR-US was performed 1.9 d before MRE. In 90% of all cases the time period between MRE and ultrasound was less than 7 d, in 72% both examinations were performed within 3 d.

During the evaluation period we found a total of 100 different examiners for high-resolution bowel ultrasound. Of these examiners, 13 were considered highly experienced (13%) while 87% had moderate experience in ultrasound. The highly experienced examiners performed 48% ($n = 119$) of all bowel examinations.

Affected bowel wall

In the 250 cases evaluated both modalities described no bowel wall pathology in 21 cases. In the remaining 229 cases, a total of 673 changes of the bowel wall were described with 439 changes described by MRE and 405 changes described by US. The described changes included subtle and unspecific findings such as “accentuated bowel wall” without any specific diagnostic statement. With regard to all bowel wall changes, there was no statistically significant difference between MRE and US (Table 1). MRE and ultrasound had similar results in 163 cases, while MRE detected more lesions in 38 cases and US found more affected bowel wall segments in 28 cases. When analyzing the 28 cases with more lesions found on US, we had no explanation for the negative MRE findings in 10 of the 28 cases. For 12 cases, possible explanations were subtle US findings describing a wall thickness of 3 mm as an accentuated bowel wall. In 6 patients the MRE report stated inferior image quality due to breathing and motion artifacts.

When analyzing the subgroup of 28 cases with more US findings with regard to the examiner’s experience, 43% ($n = 12$) of the cases were examined by moderately experienced examiners (9 cases with possible explanations and 3 cases with no explanation), while 57% ($n = 16$) where

Table 2 Influence of the experience of the ultrasound examiner on diagnosis

Pathological changes	Ultrasound examiner with moderate experience (%)	Ultrasound examiner with high experience (%)
MRI found more lesions than ultrasound		
Stenosis (<i>n</i> = 72)	62	38
Abscess (<i>n</i> = 16)	46	46
Fistula (<i>n</i> = 32)	59	41
Ultrasound found more lesions than MRI		
Stenosis (<i>n</i> = 8)	0	100
Abscess (<i>n</i> = 4)	50	50
Fistula (<i>n</i> = 2)	0	100

The percentage indicates the ratio of moderate or experienced ultrasound examiners in this scenario. MRI: Magnetic resonance imaging.

Table 3 Contingency table of all evaluated 250 cases (patient based) *n* (%)

Statement “inflammation” or “bowel wall affection” in the report	Ultrasound	
	Negative	Positive
Magnetic resonance enterography		
Negative	83 (33.2)	6 (2.4)
Positive	132 (52.8)	29 (11.6)

Negative findings means no bowel wall affection or inflammation assessed based on the report (magnetic resonance enterography or ultrasound).

examined by the highly experienced group (9 cases with possible explanations and 7 cases with no explanation). Based on these data, there was no relevant tendency or statistical relevance regarding the examiner's experience (Table 2).

For the 38 cases with a superior MRE examination we had no explanation for the inferior US in 18 cases. For the remaining cases, restricted image quality was stated in 14 cases. Additionally, in 12 cases (in some cases more than one explanation for the same case was reported) the pathological changes were in anatomical areas with a difficult access by US (lesser pelvis, rectum). The 38 cases were performed by 13 highly experienced examiners and 25 moderately experienced examiners.

Different results emerged when analyzing diagnostic statements such as “inflammation” and “affected bowel wall” only (Table 3). Restricted to these terms, which represent a definite diagnostic statement with therapeutic consequences, we found only 29 cases with similar results. There were six cases, where US detected more lesions than MRE and 132 cases (53%) where MRE described inflamed and affected bowel wall without US findings.

Stenosis

In 170 of 250 cases (68%) there was a consensus between US and MRE, while in 80 cases (32%) the findings regarding a stenosis were different (Table 4). In 72 cases (29%) the MRE examination described a stenosis, while

the US was negative. For 18 of 72 cases there was no explanation for the negative ultrasound findings. In 54 cases we found possible explanations. In 23 patients the stenosis was localized in an area which was difficult to assess by US. In 20 patients, the US image quality deteriorated due to residual bowel air. In 19 cases an explanation can be assumed because of a subtle finding in the MRE. The MRE findings were just reported as a “narrowing of the lumen” without mentioning the word “stenosis” or describing indirect signs such as “pre-stenotic dilatation”.

The moderately experienced examiners performed 44 of the 72 cases with a superior MRE result, while the highly experienced examiners performed 28 of these examinations (Table 2). In 8 cases (3%) a stenosis was described in the US report having a negative MRE. The 8 cases with more US findings were examined only by highly experienced examiners.

Abscess

In the majority of all cases (221 cases, 88%) we did not find any abscesses (Table 4). In 16 cases (6%) MRE detected an abscess which was not described by US and in 4 cases (2%) US described an abscess not mentioned in the MRE report. These 4 cases were assessed by 2 moderately and 2 highly experienced examiners.

For the 16 cases with an abscess described by MRE and not by US there was no explanation in 1 case, where several abscesses with a diameter of 1 cm in the region of the cecum and terminal ileum were described in the MRE report. For 13 cases, a possible explanation was assumed to be difficult access mostly in the lesser pelvis, and for another 2 cases reduced image quality. Highly experienced examiners (*n* = 9) and moderately experienced examiners (*n* = 7) were equally distributed in these 16 cases.

Fistula

Two hundred and eleven of 250 cases (84%) showed no fistulas in either of the modalities (Table 4). In 5 cases (2%) both modalities detected a fistula in the same patient while US identified 2 fistulas (1%), which were not described in the MRE report. We identified 32 cases (13%) where MRE identified fistulas not described by US. The 2 cases with more US findings were performed by a highly experienced examiner.

Thirty two cases of fistula were detected by MRE which were not identified by US. In 24 cases a possible explanation was difficult access for US, while in 9 cases reduced image quality was stated in the US report. Nineteen moderately experienced and 13 highly experienced examiners performed these 32 examinations. There was no statistical significance in the examiners experience.

Local lymphadenopathy

Because the exact measured diameter was mentioned in just 4 cases for US and MRE we were unable to compare these data quantitatively. Based on both modalities, local lymphadenopathy was reported in 63 of 250 cases (25%). In 4 cases, MRE as well as US described enlarged or mul-

Table 4 Diagnostic performance of ultrasound and magnetic resonance enterography regarding bowel wall changes, diagnosis of bowel inflammation, stenosis, abscess, fistula and indirect inflammation signs such as local lymphadenopathy and fat injection or comb sign *n* (%)

	Bowel wall changes	Diagnosis "bowel wall inflammation"	Stenosis	Abscess	Fistula	Local lymph-adenopathy	Mesenteric fat injection/comb sign
US = MRE (no pathological change)	21 (8)	83 (33)	150 (60)	221 (84)	211 (88)	187 (75)	182 (73)
US = MRE (pathological change)	163 (65)	29 (12)	20 (8)	9 (4)	5 (2)	4 (2)	4 (2)
US > MRE	28 (11)	6 (2)	8 (3)	4 (2)	2 (1)	15 (6)	4 (2)
MRE > US	38 (15)	132 (53)	72 (29)	16 (6)	32 (13)	44 (18)	60 (24)

US: Ultrasound; MRE: Magnetic resonance enterography. In "US = MRE" the number (and percentage) of cases, when both modalities found the same amount of pathological features (patient based) are identical. For "US > MRE" the reports based on ultrasound described more pathological changes compared to the MRE, while for "MRE > US" more findings in the MRE reports were detected (because of truncation the percentage values in a column can exceed 100%).

Table 5 Possible explanations for misdiagnosis of all evaluated features (wall affection, stenosis, abscess, fistula, lymph nodes and indirect inflammation signs) for ultrasound and magnetic resonance enterography for all 250 evaluated cases

	HR-US	MRE
Reduced imaging quality (obesity, breathing artifacts, residual bowel gas, etc.)	25	7
Region difficult to access (lesser pelvis)	72	N/A
Subtle findings	15	31

Magnetic resonance enterography (MRE) missed most of the diagnoses made by ultrasound (US) because of subtle US findings (*n* = 31), while the most common reason for a misdiagnosis based on the US report was an anatomical region difficult to assess (*n* = 72). N/A: Not available.

multiple local lymph nodes, while lymphadenopathy was reported in 15 cases solely by US (6%) and in 44 cases solely by MRE (18%).

Local fat injection and comb sign

A total of 68 cases (27%) with signs of indirect inflammation such as local fat injection or comb sign were detected based on both modalities. In 4 cases, US and MRE identified these signs, while US described 4 additional cases (2%), which were not described by MRE. In the MRE reports, 60 cases (24%) with positive findings of indirect inflammatory signs were found, which were not described in the US report.

Reasons for discrepant findings

When analyzing all these results, when one modality described a pathological finding, which was not described by the other modality, we detected several circumstances and possible reasons for this misdiagnosis (Table 5). In most cases (*n* = 72) a possible reason for misdiagnosis was the anatomical region, which was difficult to access by US such as the lesser pelvis and peri-rectal tissue. In particular, the localization of fistulas in the pelvis is frequently an obstacle for abdominal US diagnosis. Other reasons were reduced image quality on US due to obesity or residual bowel air in 25 patients and inadequate bowel distension or breathing artifacts in 7 patients on MRE examination.

In most evaluations, a higher ratio of detected lesions by US was found for examiners with a higher level of experience, however, this influence was not demonstrated with any statistical significance (Table 2).

DISCUSSION

Our study retrospectively evaluated the results of reports based on MRE and HR-US examinations in 250 consecutive patients with known IBD.

By analyzing the bowel wall changes on a patient basis we obtained different results when evaluating different key words in the reports. When we evaluated all the terms regarding bowel wall changes including unspecific terms such as "accentuated bowel wall" or "wall thickening" we did not show statistically relevant differences between HR-US and MRE. In this scenario, the HR-US misses totaled 38 cases (15%) and the MRE misses totaled 28 cases (11%), which were described in the opposite modality, respectively. Assuming that MRE depicts the whole abdomen including the bowel wall quite objectively, one of the main reasons for these differences could be, that HR-US sometimes subjectively overestimates bowel wall thickening without giving exact measurements. In this study, we found this in at least 12 of the 28 cases, when US described a thickening without a correlation in MRE. A possible reason for this was a very subtle finding mentioning a wall thickness between 2 mm and 3 mm. In the MRE findings, there were no signs of inflammation such as increased contrast uptake or indirect inflammation features. Therefore, HR-US could theoretically sometimes show "false positive" results, especially when describing subtle findings such as "accentuated bowel walls". For diagnostic features such as "inflamed" or "affected" bowel wall there were statistically significant differences between HR-US and MRE. In this scenario, the US misses consisted of 132 cases (53%), while MRE misses consisted of just 6 cases (2%). Obviously MRE has some methodological advantages for decision making such as contrast media and better bowel distension because of the oral contrast application before the examination.

Comparing our results with other studies we found the same tendencies in a study by Potthast *et al.*^[5] carried out in 2002. This group retrospectively compared 46

patients undergoing MR enteroclysis with US and conventional enteroclysis, surgery or colonoscopy as a gold standard. For bowel wall changes they calculated 22% false negative results for US and 2.4% false negative results for MRE. Martínez *et al*^[6] in their prospective study of 30 patients with CD published different results. They compared US and MRI with regard to the extension and transmural complications in CD. For localization of affected bowel wall, US was not statistically significantly superior to MRI having a sensitivity of 91%, while MRI detected changes with a sensitivity of 83%. There are several major limitations of this study. In addition to the small number of patients, the authors accepted a time period of up to 3 mo between the examinations and the standard of reference. Also their choice of the standard of reference is very questionable. In addition to surgery they accepted conventional barium studies and small bowel follow-through examinations as a standard of reference. In several studies these examinations showed a poorer sensitivity and accuracy than MRE^[7].

A meta-analysis from 2008 on the diagnostic accuracy of studies based on US, MRI, scintigraphy, CT and PET imaging in IBD, did not reveal any statistically significant differences between the modalities^[3]. The authors calculated that on a patient basis, US had a sensitivity of 90% (range 78%-96%) and MRE had a sensitivity of 93% (range 82%-100%).

It is not possible to compare our results directly using sensitivity values, because we did not have any standard of reference in our study and just compared the positive results from the two modalities performed in the same patient. Based on our data, MRE (11% missed cases) detected slightly more lesions than HR-US (15% missed cases). A certain problem in this study was the vagueness of the diagnostic statements with regard to the affection and inflammation of bowel wall segments. Because of the lack of bowel distension and intravenous contrast application in standard HR-US examinations it seems inherent to the system that US examinations were frequently describes as an unspecific bowel wall thickening without calling a finding an inflammation or affection. In our study, US reports missed 53% of the cases when evaluating for terms such as “inflammation” or “affection”, while MRE just missed 2% in this scenario.

With regard to terms such as “lumen narrowing” and “stenosis” we found a highly significant difference between HR-US, which missed 29% of the described stenoses, and MRE, which missed just 3%. There was certainly a problem with the exact definition of stenosis for HR-US and MRE. US has the advantage of dynamic real-time imaging, which allows an assessment of peristalsis with consecutive appreciation of a functional stenosis *vs* lumen narrowing. In MRE reports, a lumen narrowing of more than 50% can be called a stenosis. MRE is not a real-time imaging modality using a standard MR protocol. The only way to identify a functional stenosis is to evaluate all MRI sequences along the time line, which are

performed over 25 min. Theoretically, MRI could lead to an overstaging of lumen narrowing calling it a “stenosis” because of the lack of functional real-time image data. In 17 of the 72 cases with a stenosis diagnosed by MRE and not by US, the stenosis was not called functionally relevant in the MRE report, while US described a wall thickening in the same bowel segments. The localization of misdiagnosed stenoses by US was responsible for 23 of the 72 discrepant cases. These stenoses were especially located in the lesser pelvis, which is difficult to assess by transabdominal US. Our results are in contrast to the prospective results of 48 patients published by Schmidt *et al*^[8] in 2003. In this study, conventional enteroclysis was considered the standard of reference with data acquisition between 1999 and 2000. They calculated a sensitivity of 56% for detecting stenosis when using US with a specificity of 97%, while MRI had a sensitivity of 44% with a specificity of 100%. On the other hand, the retrospectively performed comparison by Potthast *et al*^[5] had a sensitivity of 58% for US with a false negative rate of 16%, while MRI had a sensitivity of 100% with a false negative rate of 2%. In our study, 6 (75%) of the 8 examinations with a superior US result were performed by a highly experienced examiner, while in the group of superior MRI results just 28 (39%) of the 72 examinations were performed by the highly experienced group.

For abscess diagnosis the differences between HR-US (16 missed abscesses) and MRE (4 missed abscesses) were statistically significant ($P < 0.05$). Considering the fact that all 4 abscesses diagnosed by US and not by MRE were most likely not present at the time of MRE examination ($n = 3$) due to treatment or a false positive US result ($n = 1$; probably hemorrhagic ovarian cyst), MRE seems to be the superior modality for detecting abscesses in the abdomen.

For 13 (81%) of the 16 cases with a missed abscess by HR-US, the localization of the abscess in the lesser pelvis and peri-rectal region, which is difficult to assess by US, was the most plausible reason. Our results were mirrored in the study by Potthast *et al*^[5], which showed a sensitivity of 100% for MRI and 89% for US. Another study from the same hospital attributed MRI with a sensitivity of 83%, and US with a sensitivity of 67%^[9].

For fistula detection, the differences between MRI, which missed 2 cases (1%), and HR-US, which missed 32 cases (13%), were highly significant. The localization of the fistula in the lesser pelvis and peri-rectal region was a possible explanation in 24 of the 32 cases. The experience of the examiners did not have any influence on the results. It should be noted that HR-US examinations were restricted to transabdominal US examinations. A HR-US examination performed transperineal or trans-rectally would most probably improve the results of US dramatically. When comparing dedicated US examinations such as endoscopic endorectal US to MRI, both modalities were found to have similar sensitivities and specificities^[10-12]. The study by Potthast *et al*^[5] supports our data,

showing a false negative rate of 26% for transabdominal US and 5% for MRI.

The mention of a local lymphadenopathy is an indirect unspecific sign of an inflammatory process. Maconi *et al.*^[13] found an unspecific lymphadenopathy in 25% of their patients ($n = 240$) with CD using US. They detected more enlarged lymph nodes in young patients and in patients with fistulas and abscesses. In our study, MRE missed 15 cases (6%) with lymphadenopathy while US missed 44 cases (18%). There was an identical tendency in the evaluation of local mesenteric fat injection and comb sign with a missed rate of 2% in MRI and 24% in HR-US.

One of the major restrictions of our study is the retrospective approach used as well as the long data acquisition period of 7 years using different equipment and different examination protocols. In addition there was no standard of reference for the evaluated parameters. The retrospective evaluation of consecutive patients was deliberately chosen to assess the actual quality of MRE and US without influencing the examiners using both modalities. The quality improvements in HR-US over time were accompanied by improvements in MR imaging and protocols. However, basically identical protocols (fast T2 weighted sequences as well as gradient echo sequences after contrast application) were used during the evaluation time period. Having a standard of reference for all features evaluated in this study was extremely difficult. For small bowel assessment, MRI together with multidetector CT is already considered an accepted standard of reference with superior sensitivity and specificity compared to conventional enteroclysis and follow-through examinations^[2,3]. When considering surgery as the standard of reference this would reduce the number of patients included in the study dramatically. Therefore, we considered a pathological finding mentioned in one report as a real finding, which was also to be described in the other modality. The analysis of 32 of 250 examinations, which were performed in patients with UC makes the patients evaluated more inhomogeneous, but does not influence our results significantly. On the other hand, we intended to analyze a realistic scenario in patients suffering from IBD.

In the recent literature, there are no major studies evaluating the diagnostic quality of HR-US and MRI with regard to bowel wall affections as well as transmural complications such as abscesses or fistulas. In our study, MR imaging was superior to US for all evaluated features. The difference was statistically significant for the diagnosis and detection of an abscess and highly significant for stenosis and fistula evaluation.

In conclusion, transabdominal HR-US can miss a certain number of pathological changes in patients with IBD. The localization of the pathological lesions in anatomical regions, which are difficult to assess by US, such as the lesser pelvis or the peri-rectal region, is one of the most frequent reasons for a missing a diagnosis. When

the clinical suspicion of bowel affection or complications is mentioned, an MRI examination should be performed after an US examination without relevant findings.

COMMENTS

Background

Patients suffering from inflammatory bowel disease (IBD) require a complete bowel assessment for primary diagnosis or follow-up, when their symptoms are increasing. Guidelines recommend high-resolution ultrasound (HR-US) or magnetic resonance imaging (MRI) as imaging modalities.

Research frontiers

Ultrasound and MRI are diagnostic modalities without ionizing radiation. In this study, the authors compared the findings of MRI as well as ultrasound examinations in the same patients. They evaluated the limitations of ultrasound imaging for whole bowel assessment in patients with IBD.

Innovations and breakthroughs

Currently there are no major studies comparing state of the art MRI with ultrasound in bowel imaging for IBD. Our study shows, that relevant complications such as fistulas, stenoses or abscesses are often missed when using ultrasound imaging only.

Applications

By understanding the limitations of ultrasound imaging due to difficult to assess anatomical areas, algorithms for IBD imaging can be improved.

Peer review

This is an interesting retrospective manuscript on the comparison of HR-US and magnetic resonance enterography in a high number of patients with IBD. The comparison of competing imaging methods is of high clinical relevance therefore the topic of the manuscript is of clinical importance.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

Subcellular distribution of nitric oxide synthase isoforms in the rat duodenum

Petra Talapka, Nikolett Bódi, Izabella Battonyai, Éva Fekete, Mária Bagyánszki

Petra Talapka, Nikolett Bódi, Izabella Battonyai, Éva Fekete, Mária Bagyánszki, Department of Physiology, Anatomy and Neuroscience, University of Szeged, 6726 Szeged, Hungary
Author contributions: Talapka P, Bódi N and Battonyai I contributed equally to this work; Fekete É and Bagyánszki M designed and performed the research; Bódi N and Bagyánszki M analyzed the data; Bódi N, Fekete É and Bagyánszki M wrote the paper.

Supported by Hungarian National Grant Agency, Grant#F46201 to Bagyánszki M

Correspondence to: Mária Bagyánszki, PhD, Lecturer in Zoology, Department of Physiology, Anatomy and Neuroscience, University of Szeged, 6726 Szeged, Hungary. bmarsci@bio.u-szeged.hu

Telephone: +36-62-544123 Fax: +36-62-544291

Received: September 2, 2010 Revised: November 3, 2010

Accepted: November 10, 2010

Published online: February 28, 2011

Abstract

AIM: To study the cell-type specific subcellular distribution of the three isoforms of nitric oxide synthase (NOS) in the rat duodenum.

METHODS: Postembedding immunoelectronmicroscopy was performed, in which primary antibodies for neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), were visualized with protein A-gold-conjugated secondary antibodies. Stained ultrathin sections were examined and photographed with a Philips CM10 electron microscope equipped with a MEGAVIEW II camera. The specificity of the immunoreaction in all cases was assessed by omitting the primary antibodies in the labeling protocol and incubating the sections only in the protein A-gold conjugated secondary antibodies.

RESULTS: Postembedding immunoelectronmicroscopy revealed the presence of nNOS, eNOS, and iNOS immunoreactivity in the myenteric neurons, the enteric smooth muscle cells, and the endothelium of capillaries

running in the vicinity of the myenteric plexus of the rat duodenum. The cell type-specific distributions of the immunogold particles labeling the three different NOS isozymes were revealed. In the control experiments, in which the primary antiserum was omitted, virtually no postembedding gold particles were observed.

CONCLUSION: This postembedding immunoelectron-microscopic study provided the first evidence of cell-type-specific differences in the subcellular distributions of NOS isoforms.

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Key words: Postembedding immunoelectronmicroscopy; Subcellular distribution; Neuronal nitric oxide synthase; Endothelial nitric oxide synthase; Inducible nitric oxide synthase

Peer reviewer: Guancun Huang, MD, PhD, Department of Center for Clinical and Translational Research, The Research Institute at Nationwide Children's Hospital, 700 Childrens Drive, Center for Clinical and Translational Research, Research II, WA 2112, Columbus, OH 43205, United States

Talapka P, Bódi N, Battonyai I, Fekete É, Bagyánszki M. Subcellular distribution of nitric oxide synthase isoforms in the rat duodenum. *World J Gastroenterol* 2011; 17(8): 1026-1029
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1026.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1026>

INTRODUCTION

In the enteric nervous system, endogenous nitric oxide (NO) has an important role in mediating non-adrenergic, non-cholinergic (NANC) relaxation of the intestinal smooth muscle^[1]. There are three genetically different isoforms of NO synthase (NOS) that account for NO production: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)^[2]. Of the three NOS

isoforms, nNOS constitutes the predominant source of NO in neurons, while eNOS is the predominant source in the endothelium^[3,4]. It has been demonstrated, however, that the myenteric neurons in the mouse colon express all three NOS isoforms^[5], and NO synthesized by nNOS, eNOS, and iNOS regulates motility by acting as an inhibitory neurotransmitter^[6,7]. NO cannot be stored in the cells; therefore, it depends on new synthesis to exert its functional properties. The subcellular compartmentalization of NOS is therefore determinative in NO production^[8]. However, the tissue specificity and subcellular distribution of NOS isoforms continue to be subject to debate^[9]. Accordingly, the aim of the present study was to establish the presence and subcellular distribution of NOS isoforms in the duodenum of adult rats.

MATERIALS AND METHODS

All the experiments were approved by the Local Ethics Committee for Animal Research Studies at the University of Szeged. Healthy adult male Wistar rats weighing 350-400 g were used throughout the experiments. The animals were killed by cervical dislocation and segments of the duodenum were dissected, rinsed in 0.05 mol/L phosphate buffer (PB, pH 7.4), and processed for postembedding immunohistochemistry.

The gut segments were cut along the mesentery, pinched flat, and fixed overnight at 4°C in 2% paraformaldehyde and 2% glutaraldehyde solution, buffered with 0.1 mol/L PB. The samples were then washed and further fixed for 1 h in 1% OsO₄. After fixation, the gut segments were rinsed in 0.1 mol/L PB, dehydrated in increasing alcohol concentrations (50, 70, 96% and absolute ethanol) and acetone, and embedded in Epon epoxy resin. The Epon blocks were used to prepare ultrathin (70 nm) sections, which were mounted on Formvar-coated nickel grids and processed for immunogold labeling. All steps were performed at room temperature. Sections were preincubated in 1% bovine serum albumin solution for 30 min, incubated overnight in the primary antibodies (Table 1), followed by protein A-gold-conjugated anti-mouse (18 nm gold particles, Jackson ImmunoResearch, USA) or anti-rabbit (10 nm gold particles, Sigma-Aldrich, USA) secondary antibodies for 3 h. Sections were counterstained with uranyl acetate and lead citrate, and then examined and photographed with a Philips CM10 electron microscope equipped with a MEGAVIEW II camera. The specificity of the immunoreaction was assessed in all cases by omitting the primary antibodies from the labeling protocol and incubating the sections only in the protein A-gold-conjugated secondary antibodies. The distributions of the gold particles coding for nNOS, iNOS, and eNOS were determined in 12 ultrathin sections of the duodenum from three different animals.

RESULTS

Postembedding immunoelectronmicroscopy revealed the presence of nNOS (Figures 1-3), eNOS (Figures 4-6)

Table 1 Primary antibodies and working dilutions applied in the experiments

Primary antibody	Clone	Host	Company	Working dilution
nNOS	Monoclonal IgG1, NOS-B1	Mouse	Sigma-Aldrich (USA)	0.18055556
eNOS	Monoclonal IgG1 clone 3	Mouse	Transduction Laboratories (Canada)	0.21527778
iNOS	Polyclonal IgG	Rabbit	Santa Cruz Biotechnology (USA)	0.38888889

nNOS: Neuronal nitric oxide synthase; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase.

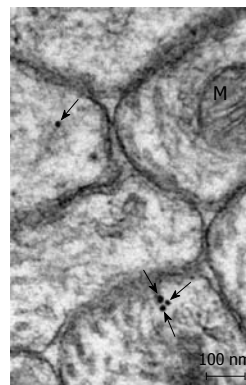


Figure 1 Postembedding immunogold labeling for neuronal nitric oxide synthase in a myenteric ganglion of the rat duodenum. The majority of the gold particles are above the structureless cytoplasm (arrows). M: Mitochondria.

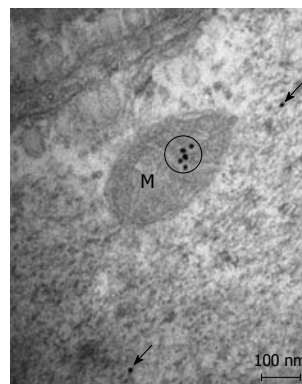


Figure 2 Postembedding immunogold labeling for neuronal nitric oxide synthase in an enteric smooth muscle cell. Gold particles coding for neuronal nitric oxide synthase accumulated in the mitochondria (M, encircled). Only a few gold particles are seen above the structureless cytoplasm (arrows).

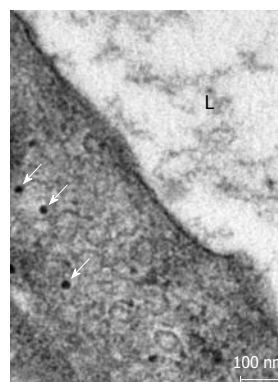


Figure 3 Postembedding immunogold labeling for neuronal nitric oxide synthase in the capillary endothelium in the vicinity of the myenteric plexus of the rat duodenum. The majority of the gold particles are bound to the vesicular membranes or are situated inside vesicles (arrows). L: capillary lumen.

and iNOS (Figures 7 and 8) immunoreactivity in myenteric neurons, in enteric smooth muscle cells, and in the endothelium of the capillaries running in the vicinity of

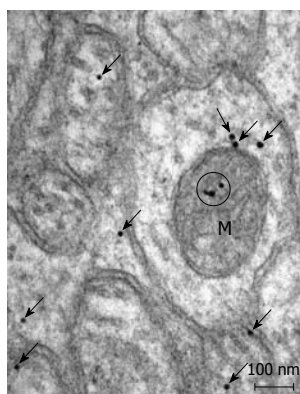


Figure 4 Postembedding immunogold labeling for endothelial nitric oxide synthase in a myenteric ganglion of the rat duodenum. The majority of the gold particles are distributed evenly (arrows); however, mitochondrial accumulation of endothelial nitric oxide synthase-coding gold particles (M, encircled) is also observed.

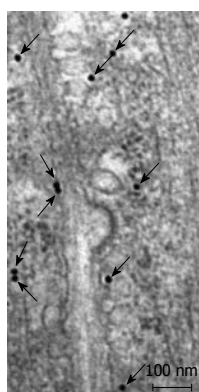


Figure 5 Postembedding immunogold labeling for endothelial nitric oxide synthase in an enteric smooth muscle cell. Gold particles are evenly distributed (arrows) and avoid caveolae.

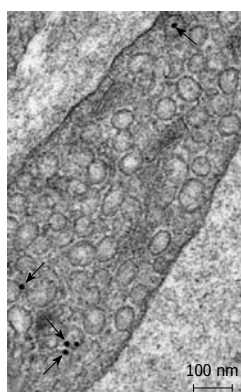


Figure 6 Postembedding immunogold labeling for endothelial nitric oxide synthase in an endothelial cell. The gold particles are bound to the vesicular membranes or are situated inside vesicles (arrows).

the myenteric plexus of the rat duodenum. The cell type-specific distributions of the immunogold particles labeling the three different NOS isozymes were revealed. Gold particles labeling nNOS (Figure 3), eNOS (Figure 6), or iNOS (Figure 8) in the local endothelium were localized almost exclusively to vesicle membranes or the interior of vesicles. The majority of the gold particles labeling nNOS in enteric muscle cells (Figure 2), or eNOS in nerve terminals (Figure 4), accumulated in the mitochondria. The gold particles labeling nNOS in myenteric neurons (Figure 1) or eNOS in enteric smooth muscle cells (Figure 5) were dispersed above the structureless cytoplasm. iNOS was evenly distributed in enteric muscle cells and also in myenteric neurons (Figure 7). In the control experiments in which the primary antiserum was omitted, virtually no postembedding gold particles were observed (not shown).

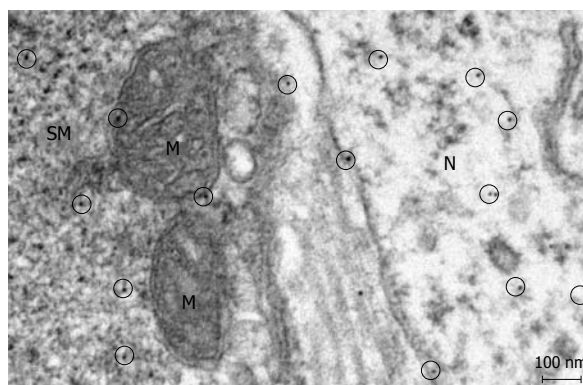


Figure 7 Postembedding immunogold labeling for inducible nitric oxide synthase. Gold particles (encircled) are randomly distributed in the enteric muscle cells (SM) and also in the myenteric neurons (N). M: Mitochondria.

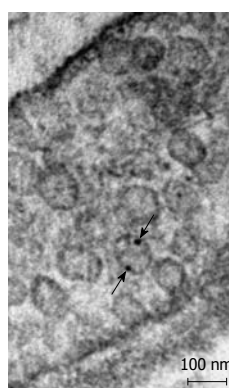


Figure 8 Postembedding immunogold labeling for inducible nitric oxide synthase in the endothelial cell of a capillary in the vicinity of the myenteric plexus of the rat duodenum. The majority of the gold particles were bound to the vesicular membranes or are situated inside vesicles (arrows).

DISCUSSION

The present postembedding immunoelectronmicroscopic study has provided the first evidence of cell-type-specific differences in the subcellular distributions of NOS isoforms in myenteric neurons, enteric muscle cells, and the endothelium of local capillaries in the rat duodenum. We confirmed previous results^[5] that all three NOS isoforms are present in myenteric neurons. However, the subcellular locations that we verified here for the NOS isoforms in the different cellular elements of the duodenal wall of rat contrasted with results of previous localization studies, where nNOS and iNOS were considered to be cytosolic^[3] and to be eNOS membrane-associated^[4]. To date, there has been no explanation as to why the three different NOS isoforms are expressed in myenteric neurons, enteric muscle cells, and the capillary endothelium. It appears, conceivable, however, that, in consequence of the differences in compartmentalization, the different NOS isoforms are not simultaneously active in NO synthesis in these cells. Hence, the presence of three NOS isoforms with similar functions in the same cells might reflect a functional plasticity, in which one NOS isoform can replace another under different pathophysiological conditions. The rearrangement of the subcellular NOS compartments under pathological conditions will therefore be the subject of our further investigations.

COMMENTS

Background

Despite being a simple molecule, nitric oxide (NO) has fundamental roles in the fields of neuroscience, physiology and immunology. In the intestine, NO has an important role in the gastrointestinal motility.

Research frontiers

The presence of the three nitric oxide synthase (NOS) isoforms in the same cells might reflect a functional plasticity.

Innovations and breakthroughs

The present study provided the first direct evidence of cell-type-specific differences in the subcellular distributions of NOS isoforms in the rat duodenum.

Applications

The presence of three NOS isoforms with similar functions in the same cells might reflect a functional plasticity, in which one NOS isoform can replace another under different pathophysiological conditions. Further research will focus on the potential rearrangement of the subcellular NOS compartments under pathological conditions. The regulatory elements of this rearrangement might offer attractive targets for therapeutic approaches.

Terminology

There are three genetically different isoforms of NOS, which account for NO production: neuronal, endothelial, and inducible NOS.

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NO is a highly active molecule; therefore, the subcellular compartmentalization of the three NOS is determinative in NO production. Using postembedding immunoelectronmicroscopy it has been demonstrated that neurons, smooth muscle cells, and also endothelial cells in the gut wall express all three NOS isoforms.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Zheng XM

NSAID-induced deleterious effects on the proximal and mid small bowel in seronegative spondyloarthropathy patients

Mihai Rimbaş, Mădălina Marinescu, Mihail Radu Voiosu, Cristian Răsvan Băicuș, Simona Caraiola, Adriana Nicolau, Doina Nițescu, Georgeta Camelia Badea, Magda Ileana Pârvu

Mihai Rimbaş, Mădălina Marinescu, Mihail Radu Voiosu, Department of Gastroenterology, "N.Gh.Lupu" Clinic of Internal Medicine, "Carol Davila" University of Medicine and Pharmacy, Colentina Clinical Hospital, 020125 Bucharest, Romania
 Cristian Răsvan Băicuș, Simona Caraiola, Adriana Nicolau, Doina Nițescu, Georgeta Camelia Badea, Department of Internal Medicine, "N.Gh.Lupu" Clinic of Internal Medicine, "Carol Davila" University of Medicine and Pharmacy, Colentina Clinical Hospital, 020125 Bucharest, Romania
 Magda Ileana Pârvu, Department of Rheumatology, Colentina Clinical Hospital, 020125 Bucharest, Romania

Author contributions: All authors contributed to this work; Rimbaş M and Voiosu MR designed the research; Caraiola S, Nicolau A, Nițescu D, Badea GC and Pârvu MI recruited the patients; Rimbaş M and Marinescu M performed the research; Rimbaş M and Băicuș CR analyzed the data; Rimbaş M wrote the paper.

Supported by CNCIS-UEFISCSU, Romanian Ministry of Education and Research, project number PNII-IDEI 320/2007, and is part of a study registered on ClinicalTrials.gov with the number NCT00768950

Correspondence to: Mihai Rimbaş, MD, Teaching Assistant, Department of Gastroenterology, "N.Gh.Lupu" Clinic of Internal Medicine, "Carol Davila" University of Medicine and Pharmacy, Colentina Clinical Hospital, 19-21 Ștefan cel Mare Street, 020125 Bucharest, Romania. mrmbas@yahoo.com
 Telephone: +40-21-3180604 Fax: +40-31-8162376

Received: August 15, 2010 Revised: November 11, 2010

Accepted: November 18, 2010

Published online: February 28, 2011

and demographic data were taken, as well as the history of non-steroidal anti-inflammatory drug (NSAID) consumption. After reading each VCE recording, a capsule endoscopy scoring index for small bowel mucosal inflammatory change (Lewis score) was calculated. Statistical analysis of the data was performed.

RESULTS: The Lewis score for the whole cohort was 397.73. It was higher in the NSAID consumption subgroup ($P = 0.036$). The difference in Lewis score between NSAID users and non-users was reproduced for the first and second proximal tertiles of the small bowel, but not for its distal third (P values of 0.036, 0.001 and 0.18, respectively). There was no statistical significant difference between the groups with regard to age or sex of the patients.

CONCLUSION: The intestinal inflammatory involvement of SpA patients is more prominent in NSAID users for the proximal/mid small bowel, but not for its distal part.

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Key words: Videocapsule endoscopy; Lewis score; Non-steroidal anti-inflammatory drugs; Small bowel mucosal injury; Spondyloarthropathy

Peer reviewer: Dr. Fen Liu, University of Minnesota, 6-155 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455-0375, United States

Abstract

AIM: To investigate the small bowel of seronegative spondyloarthropathy (SpA) patients in order to ascertain the presence of mucosal lesions.

METHODS: Between January 2008 and June 2010, 54 consecutive patients were enrolled and submitted to a video capsule endoscopy (VCE) examination. History

Rimbaş M, Marinescu M, Voiosu MR, Băicuș CR, Caraiola S, Nicolau A, Nițescu D, Badea GC, Pârvu MI. NSAID-induced deleterious effects on the proximal and mid small bowel in seronegative spondyloarthropathy patients. *World J Gastroenterol* 2011; 17(8): 1030-1035 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1030.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1030>

INTRODUCTION

Spondyloarthropathies (SpAs) are a group of related disorders with common clinical and genetic characteristics and a global prevalence between 0.5% and 1%^[1]. Entities included in the group of SpAs are ankylosing spondylitis, reactive arthritis, psoriatic arthritis, undifferentiated SpA, juvenile onset SpA and SpA in patients with inflammatory bowel disease (IBD). Some researchers also include within this concept Behçet's disease-associated SpA^[2].

Over 20 years ago, Mielants *et al*^[3] showed that a substantial number of these patients have subclinical ileal inflammation. They reported finding macroscopic ileal abnormalities in up to 30% of SpA patients, including erythema, edema, ulceration, granulation, and a cobblestone appearance of the ileal mucosa. Moreover, inflammatory lesions macroscopically resembling those found in SpAs are induced by non-steroidal anti-inflammatory drug (NSAID) consumption, and about two thirds of NSAID users demonstrate intestinal abnormalities^[4]; this is important, since many of the SpA patients use NSAIDs in the treatment of their disease.

Video capsule endoscopy (VCE) is a diagnostic tool that has recently made non-invasive imaging of the entire small bowel possible. This technique has been demonstrated as the first-line diagnostic tool for detecting small bowel pathologies^[5], and we have considered it to be suited for the evaluation of small bowel mucosal lesions.

The aim of this study was to evaluate the pattern, frequency, and severity of small bowel mucosal injury in patients with SpAs as assessed by VCE, and to clarify the role of NSAIDs in the occurrence of the lesions, given the inflammatory involvement of the small bowel that already exists in the SpA control group^[6].

MATERIALS AND METHODS

This is a single-center observational study in a tertiary referral teaching hospital in Bucharest, Romania, conducted from January 2008 to June 2010. All consecutive adult patients evaluated by the study team with a form of seronegative SpA (as defined by an Amor score ≥ 6 ^[1]) in whom no intestinal stenosis or obstruction was suspected were included, if they agreed to take part in the experiment. Exclusion criteria were also: pregnancy, swallowing disorders or the presence of cardiac pace-makers, and history of IBD. The study was conducted according to the Declaration of Helsinki, Good Clinical Practice (GCP) and local regulations. The protocol was approved by the institutional ethics committee and all the patients agreed to participate in the study and signed the informed consent before enrolling.

The patients were investigated using VCE examination. The preparation for the procedure included a fasting period of 12 h and a routine PEG-based bowel preparation (Endofalk, Dr. Falk Pharma GmbH, Freiburg, Germany) with 2 L administered in the evening before and 1 L on the morning of the procedure. Simethicone 80 mg was given orally 15 to 20 min prior to the initia-

Table 1 Data regarding the patients in the study

Diagnosis	NSAID users	Non NSAID users
Ankylosing spondylitis (<i>n</i> = 36)		
Males	13	7
Females	9	7
Psoriatic spondyloarthropathy (<i>n</i> = 3)		
Males	1	1
Females	1	0
Undifferentiated spondyloarthropathy (<i>n</i> = 9)		
Males	2	2
Females	2	3
Behçet-associated spondyloarthropathy (<i>n</i> = 6)		
Males	1	0
Females	2	3

NSAID: Non-steroidal anti-inflammatory drug.

tion of VCE examination (Espumisan L, Berlin Chemie AG, Berlin, Germany). Video capsule examination was performed with PillCam SB2 capsules (Given Imaging, Yokneam, Israel). The RAPID™ versions that were used for reading the VCE recordings were 5.0, and, starting with December 2009 recordings, 6.0.

Patients were allowed to drink clear liquids 2 h after VCE ingestion and were free to engage in their normal daily activities. They were allowed to eat a light lunch 4 h after capsule ingestion and returned for removal of the recorder 8-9 h after ingestion. No adverse effects were reported and all the capsules were excreted.

Two endoscopists (MR and MM) with experience in VCE examination reviewed the findings of VCE. Capsule endoscopy results were interpreted, and the Lewis score was calculated for every patient by one endoscopist (MR) in each tertile of the small bowel (stenosis score included), and then for the whole bowel.

Data regarding patient demographics and treatment taken were also retrieved, with emphasis on concurrent NSAID consumption. Collected data were recorded on a preformed questionnaire and introduced afterwards in the SPSS database. Results are expressed as frequencies for categorical variables (further analyzed by Fisher's exact test), mean and standard deviation for normal continuous variables (analyzed by Student's *t* test), and median and extremes for non-normal continuous variables (analyzed by Mann-Whitney *U* test). Hypothesis testing was 2-tailed, with *P* < 0.05 considered statistically significant.

The statistical software package SPSS for Windows Version 16.0 (SPSS Inc., Chicago, IL) was used to analyze the data.

RESULTS

Fifty-four patients (27 males and 27 females) were enrolled. The rheumatologic disease was ankylosing spondylitis in 36 patients (66%), psoriatic SpA in 3 (6%), undifferentiated SpA in 9 (17%) and 6 patients concurrently satisfied the criteria for Behçet's disease and SpA (11%) (Table 1).

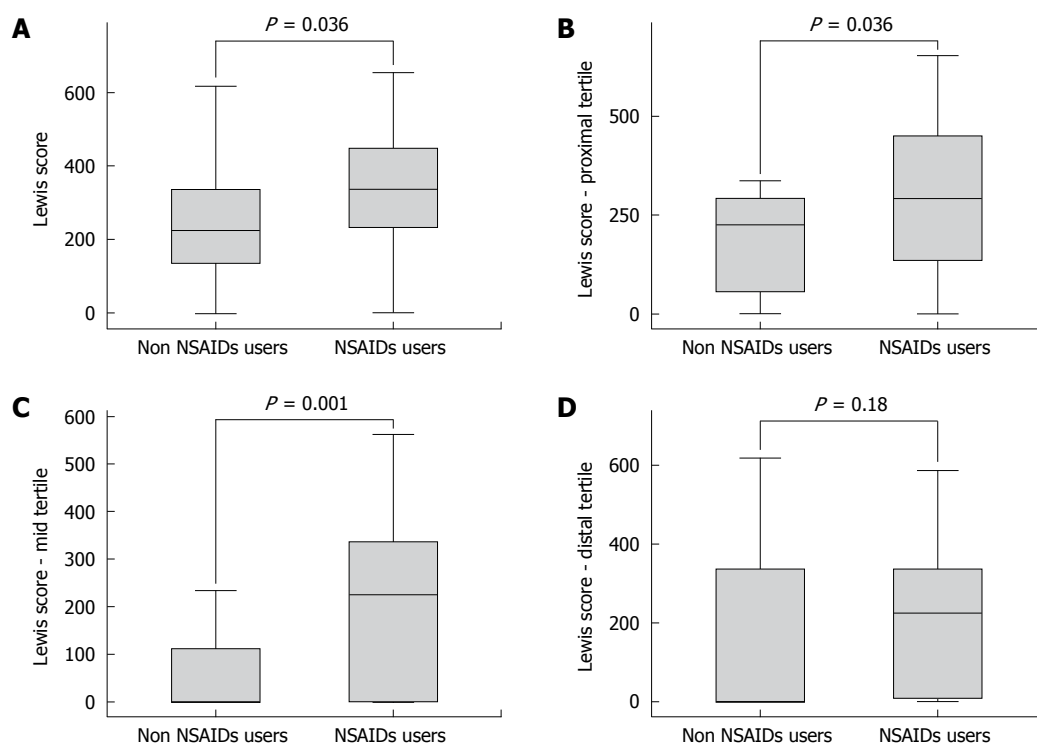


Figure 1 Comparison between the Lewis scores for the entire small bowel (A), proximal tertile of the small bowel (B), mid tertile of the small bowel (C) and distal tertile of the small bowel (D) in the groups of non-steroidal anti-inflammatory drug users and non-users (the median of values and quartiles represented). NSAID: Non-steroidal anti-inflammatory drug.

Table 2 Lewis score characteristics for the study patients. [mean; median (min, max)]

	Non NSAID users	NSAID users	P value
Whole small bowel (n = 51)	344; 225 (0, 1630)	438; 337 (0, 1462)	0.036 ^a
Proximal tertile (n = 53)	226; 225 (0, 1462)	428; 292 (0, 1690)	0.036 ^a
Mid tertile (n = 51)	63; 0 (0, 562)	196; 225 (0, 562)	0.001 ^a
Distal tertile (n = 51)	217; 0 (0, 1630)	215; 225 (0, 586)	0.186

^aStatistically significant. NSAID: Non-steroidal anti-inflammatory drug.

Thirty-one patients (57%) were concomitantly treated with at least one NSAID. The mean age was 38.65 ± 11.58 years for controls *vs* 37.87 ± 10.63 years for NSAID users ($P = 0.79$, *T*-Test, independent samples test). There were more males in the NSAID user (54.8%) *vs* non-NSAID user subgroup (43.5%) ($P = 0.41$, *T*-Test, paired samples correlations).

There was a large heterogeneity regarding the type of drug [either cyclooxygenase-2 (COX-2) selective or non-selective, or a combination of them], the doses taken (some patients were only taking acetylsalicylic acid in antiaggregant doses) and the duration of NSAID use (weeks to years).

The Lewis score for the whole small bowel could be calculated in 52 patients (96%). In one of these patients,

although the score could not be calculated for the distal tertile, the extremely high value in the mid tertile was extrapolated for the whole bowel; regardless, due to the fact that this subject had a very high discordant value of the Lewis score (4846), he was not considered for the descriptive and statistical analysis of the data. Approximating the capsule final position in the remaining 2 patients, the Lewis score could be estimated only for the proximal third of the small bowel because of the late passage of the video capsule through the pyloric ring into the small bowel.

The intraobserver reproducibility^[7] for the Lewis score was assessed in 20 patients, and was considered to be high-coefficient of variation reached 3.52%.

The mean value of the Lewis score for the whole cohort was 397.73 (range 0-1630, standard deviation 401.46), with mean values of 340.64, 138.92 and 215.63 for the proximal, mid and distal tertiles of the small bowel, respectively.

There was a significant difference in the Lewis scores for the whole small bowel between the subgroups with and without NSAID consumption ($P = 0.036$) (Table 2 and Figure 1A). The difference was reproduced for the proximal and mid small bowel tertiles, but not for the distal tertile (P values of 0.036, 0.001 and 0.18, respectively) (Table 2 and Figure 1B-D).

DISCUSSION

The term SpA indicates a group of related diseases, all

sharing common clinical features which are HLA-B27 positivity, sacroiliitis, inflammatory low back pain and oligoarticular asymmetric synovitis. The need for a standardized approach led to the development of the classification criteria proposed by Amor *et al*^[1] which consider clinical and historical symptoms, radiologic findings, genetic background and response to treatment. Given the high diagnostic sensitivity (85%) and specificity (95%), we used these criteria for our study; a patient was considered to have SpA if the sum of the criteria scores was at least 6.

SpAs are associated with several extra-articular manifestations including inflammatory gut lesions, the latter being reported in 25%-75% of patients, depending on the subtype of SpA^[8]. Although some of these patients may evolve to overt IBD, the immunological link between SpA and IBD is still poorly understood^[9], the genetic or environmental factors determining the progression within this cascade being largely unknown^[10].

A number of studies using different methodologies have evaluated the potential deleterious effects of NSAIDs on the small bowel. Considered together, they suggest that mild NSAID-related intestinal injury is common^[11]-up to two-thirds of NSAID users demonstrate intestinal inflammation on VCE examination^[12-14].

Having the above background, our aim was to investigate, using VCE examination, the possibility that NSAIDs could determine supplementary small bowel injury in SpA patients, these subjects being already predisposed to having mild inflammatory involvement of the bowel^[15].

The design of the study included calculation of the Lewis score, facilitated by latest versions of the RAPID™ software. The Lewis score represents a capsule endoscopy scoring index developed for quantification of small intestinal mucosal disease activity^[16]. It evaluates the aspect of the mucosal villi and the presence of ulcerative lesions and stenoses of the bowel lumen. A score is calculated for each tertile of the small bowel (proximal, mid, distal-resulting from dividing into three the time the capsule spent in the small bowel), and the total score equals the highest of these values plus the stenosis score. The Lewis score can range from 0 to 7840, and values below 136 are considered within normal.

A problem of the present study is represented by the large heterogeneity in the type of NSAID drug, the doses taken and the duration of NSAID use, therefore we had to group the subjects only according to NSAID concurrent use. This meant that we included in the same group of NSAID consumption patients those who ingested COX-2 selective and non-selective drugs. This might have been not so very wrong, because recent studies demonstrate that COX-2 inhibitors may not be more protective than some non-selective NSAIDs^[17]. The NSAID doses also varied widely in our study, as well as the amount of time over which the drugs were taken. Therefore, we could not evaluate the effects of different doses and durations of treatment on the small bowel. Another limitation of the present study is represented by the fact that many



Figure 2 Video capsule endoscopy image from the mid jejunum of a 39-year-old male spondyloarthropathy patient, non-steroidal anti-inflammatory drug user, showing a group of small aphthoid ulcerations in a circumscribed area of erythema (blue circle).

of the patients also received as part of their treatment immunosuppressive drugs, disease-modifying anti-rheumatic drugs, anti-tumor necrosis factor α therapy, or a combination of these.

In the present study, mild inflammatory involvement of the small bowel has been found in the whole cohort of SpA patients, given that the mean value of the Lewis score was 397.73. Subjects with NSAID consumption had more significant inflammatory lesions when the evaluation was performed for the whole small bowel. This result was expected because of the NSAID potential to induce small bowel inflammatory lesions. The above difference was reproduced for the proximal and mid small bowel tertiles but, surprisingly, not for the distal tertile, where there was no difference in bowel involvement between the NSAID concomitant users and non-users.

As it has already been established that NSAID intake predisposes to lesions in the distal small bowel^[11], partly because of the use of enteric-coated, sustained-release, or slow-release NSAIDs^[18], and because of the potential of NSAIDs to exacerbate the intestinal inflammation in Crohn's disease-an entity related to the concept of SpA and considered to be located mainly in the distal small bowel-we were expecting to find more lesions in the NSAID consumption group in the distal tertile of the small bowel.

Other studies have already been performed to detect small bowel mucosal abnormalities in patients with SpAs using endoscopy techniques. Significant small bowel findings (erythema, mucosal breaks, aphthous or linear ulcers, and erosions) were detected by capsule endoscopy in one third of patients in a small SpA recent series^[19], but in this study patients who were on NSAIDs in the 2 mo prior to enrollment were strictly excluded and the Lewis score was not calculated.

Up till now, the participation of the NSAIDs in the generation of bowel lesions in SpAs could only be postulated^[20]. At the mucosal level, predicted mechanisms of NSAID injury are inhibition of protective prostaglandins, alterations in blood flow, and increased small intestinal

permeability with subsequent invasion by luminal factors. The mucosal damage may lead to inflammation and ulceration^[11], but there is nothing endoscopically specific about NSAID-induced gut lesions^[12] (Figure 2); in the differential diagnosis could be included infectious etiologies, IBD, ischemia, radiation enteritis, vasculitides, and other drugs^[11].

The present study is not intended to delineate which of the bowel lesions are due to NSAID intake and which to the rheumatologic disease. It only aims to identify the pattern of involvement of the small bowel when the two conditions superimpose. It is worth mentioning that the statistically significant differences between the NSAID users and non-users regarding bowel lesions found in our study were actually small (differences in the mean values of the Lewis scores of 93.92, 201.69 and 133.43 for the whole small bowel, and proximal and mid tertiles, respectively). It is not clear why the distal part of the small bowel is not influenced by NSAID consumption in this subset of patients.

In our view, there remains a great deal of uncertainty regarding the place of invasive methods, such as push- or double-balloon enteroscopy, in providing histological assessment of the abnormalities found on VCE. Another unresolved issue is represented by the persistence of these lesions, when stopping or continuing NSAID intake. Further studies are needed in the era of biological therapy to elucidate the clinical relevance of the lesions that we have detected and their possible influences on prognosis and further management of SpA patients.

COMMENTS

Background

The term spondyloarthropathy (SpA) indicates a group of related diseases, all sharing common clinical features such as the presence of HLA-B27 antigen and particular rheumatologic involvement (sacroiliitis, inflammatory low back pain and oligoarticular asymmetric synovitis). Entities included in the group of SpAs are ankylosing spondylitis, reactive arthritis, psoriatic arthritis, undifferentiated SpA, juvenile onset SpA and SpA in patients with inflammatory bowel disease. SpAs are associated with several extra-articular manifestations, including inflammatory gut lesions. However, non-steroidal anti-inflammatory drugs (NSAIDs), frequently used to treat these patients, have the potential of inducing intestinal injury.

Research frontiers

Having the above background, our aim was to investigate, using videocapsule examination, the possibility that NSAIDs could determine supplementary small bowel injury in the subgroup of patients with a form of SpA, these subjects being already predisposed to having mild inflammatory involvement of the bowel.

Innovations and breakthroughs

We found a mild inflammatory involvement of the small bowel in all SpA patients. Subjects with NSAID consumption had more significant inflammatory lesions in the whole small bowel. The above difference was reproduced for the proximal and mid small bowel tertiles but, surprisingly, not for the distal tertile, where there was no difference in bowel involvement between the NSAID concomitant users and non-users.

Applications

In our view, there remains a great deal of uncertainty regarding the place of invasive methods, such as push- or double-balloon enteroscopy, in providing histological confirmation of the abnormalities found on capsule endoscopy. Another unanswered question is how stable these lesions are, when stopping or continuing NSAID intake. Further studies will be needed in this era of biological therapy to elucidate the clinical relevance of the lesions that we detected

and their possible influence on prognosis and further management of the SpA patients.

Peer review

Based on the background that NSAID intake has potential deleterious effects on the small bowel, and SpAs are associated with inflammatory gut lesions, the authors designed this statistical study, trying to provide more evidence for the participation of NSAIDs in generation of bowel lesions in SpA patients. The results confirmed the inflammatory involvement of small bowel, especially in the proximal and mid tertiles, in SpA patients that have been treated with NSAIDs. The conclusion is of interest.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

miR-200 family expression is downregulated upon neoplastic progression of Barrett's esophagus

Cameron M Smith, David I Watson, Mary P Leong, George C Mayne, Michael Z Michael, Bas PL Wijnhoven, Damian J Hussey

Cameron M Smith, David I Watson, Mary P Leong, George C Mayne, Damian J Hussey, Department of Surgery, Flinders University, Bedford Park, South Australia 5042, Australia
Michael Z Michael, Department of Gastroenterology and Hepatology, Flinders University, Bedford Park, South Australia 5042, Australia

Bas PL Wijnhoven, Department of Surgery, Erasmus Medical Centre, PO Box 2040, 3000 CA, Rotterdam, The Netherlands

Author contributions: Hussey DJ and Smith CM conceived the experiments and analyzed the data; Smith CM, Leong MP and Mayne GC carried out the experiments; Watson DI, Wijnhoven BPL and Michael MZ contributed to study design and analyzed the data; all authors were involved in writing the paper and had final approval of the submitted version.

Supported by National Health and Medical Research Council, Australia

Correspondence to: Dr. Damian J Hussey, Department of Surgery, Flinders University, Flinders Medical Centre, Room 3D213, Bedford Park, South Australia 5042, Australia. damian.hussey@flinders.edu.au

Telephone: +618-82046091 Fax: +618-82046130

Received: July 21, 2010 Revised: September 13, 2010

Accepted: September 20, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate miR-200 family expression in Barrett's epithelium, gastric and duodenal epithelia, and esophageal adenocarcinoma.

METHODS: Real-time reverse transcriptase-polymerase chain reaction was used to measure miR-200, *ZEB1* and *ZEB2* expression. Ingenuity Pathway Analysis of miR-200 targets was used to predict biological outcomes.

RESULTS: Barrett's epithelium expressed lower levels of miR-141 and miR-200c than did gastric and duodenal epithelia ($P < 0.001$). *In silico* analysis indicated roles for the miR-200 family in molecular pathways that distinguish Barrett's epithelium from gastric and duodenal

epithelia, and which control apoptosis and proliferation. All miR-200 members were downregulated in adenocarcinoma ($P < 0.02$), and miR-200c expression was also downregulated in non-invasive epithelium adjacent to adenocarcinoma ($P < 0.02$). The expression of all miR-200 members was lower in Barrett's epithelium derived high-grade dysplastic cell lines than in a cell line derived from benign Barrett's epithelium. We observed significant inverse correlations between miR-200 family expression and *ZEB1* and *ZEB2* expression in Barrett's epithelium and esophageal adenocarcinoma ($P < 0.05$).

CONCLUSION: miR-200 expression might contribute to the anti-apoptotic and proliferative phenotype of Barrett's epithelium and regulate key neoplastic processes in this epithelium.

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Key words: miRNA; Barrett's esophagus; Esophageal adenocarcinoma; miR-200; Epithelial to mesenchymal transition; Apoptosis; Proliferation; Epithelium

Peer reviewer: Joseph J Cullen, MD, Professor, Department of Surgery, University of Iowa Carver College of Medicine, 4605 JCP, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242, United States

Smith CM, Watson DI, Leong MP, Mayne GC, Michael MZ, Wijnhoven BPL, Hussey DJ. miR-200 family expression is downregulated upon neoplastic progression of Barrett's esophagus. *World J Gastroenterol* 2011; 17(8): 1036-1044 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1036.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1036>

INTRODUCTION

Barrett's esophagus is characterized by the replacement of the normal stratified squamous esophageal epithelium

with metaplastic columnar epithelium. Metaplasia to Barrett's esophagus is probably an adaptive response to the insult from reflux of gastric acid and duodenal bile salts into the esophagus, which occurs in chronic gastroesophageal reflux^[1]. Barrett's esophagus epithelium possesses secretory and absorptive cell types, and these closely resemble those found in normal gastric and intestinal epithelia^[2,3]. mRNA expression profiling studies confirm its similarity to gastric and duodenal epithelia^[4-6]. However, Barrett's esophagus epithelium also expresses a specific cluster of genes, including those associated with alterations in cell cycle/proliferation, apoptosis, stress response, and cellular migration pathways, and these distinguish it from all related gastrointestinal mucosae^[4]. Other studies have confirmed unique phenotypic characteristics of Barrett's epithelium that correspond with this specific gene expression cluster. For example, unlike gastric and duodenal epithelia, cellular proliferation in Barrett's esophagus continues along the upper crypt and at the luminal surface, possibly due to abnormal cell cycle entry or exit^[7]. Furthermore, Barrett's esophagus epithelium expresses unusually high levels of anti-apoptotic proteins^[8] and can mount a unique anti-apoptotic and proliferative response to reflux^[9-12].

Barrett's esophagus is clinically important because it is the only visibly identifiable precursor to esophageal adenocarcinoma^[13]. Progression to dysplastic stages involves increased abnormalities in the cell cycle and overall proliferation^[13]. Neoplastic progression commonly occurs without obvious symptoms, and at the time of diagnosis, most patients with esophageal adenocarcinoma have local invasion or metastases^[14]. Several lines of evidence suggest that epithelial to mesenchymal transition is required for local invasion and metastasis^[15]. Epithelial to mesenchymal transition involves inhibition of E-cadherin expression and transition from epithelial to fibroblastic cell type, with associated alterations in cellular adhesion and migration^[15]. Other studies have presented immunohistochemical, gene expression and cell line data that suggest a role for epithelial to mesenchymal transition in esophageal adenocarcinoma^[16,17].

miRNAs downregulate target gene expression at the post-transcriptional level through the binding of their "seed" sequences with complementary sites in the 3'-untranslated region of target mRNAs^[18]. The miR-200 family of miRNAs (miR-141, 200a, 200b, 200c and 429) are key regulators/inhibitors of epithelial to mesenchymal transition, and act to maintain the epithelial phenotype by targeting the expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2^[19-21]. Accordingly, the number of studies reporting downregulation of miR-200 family expression in cancer is increasing^[19-25]. In addition, members of the miR-200 family have recently been shown to affect other cell behaviors including proliferation, cell cycle and apoptosis^[26-28].

Given the unique gene expression profile and cellular behavior in Barrett's esophagus epithelium, the phenotypic features that characterize its neoplastic progression, and the potential relevance of epithelial to mesenchymal transition to esophageal adenocarcinoma, we sought to determine the expression of miR-200 family members in gastric, duodenal and Barrett's esophagus epithelium, and

to assess their expression with neoplastic progression of Barrett's esophagus. We hypothesized that Barrett's esophagus epithelium may possess a miR-200 expression profile different to gastric and duodenal epithelia, and that downregulation of miR-200 family expression may occur upon progression to esophageal adenocarcinoma.

MATERIALS AND METHODS

Tissue collection and processing

Tissues from patients diagnosed with either Barrett's esophagus ($n = 17$) or esophageal adenocarcinoma ($n = 20$) were collected at endoscopy or after surgical resection. The clinical research ethics committees of Flinders University and Erasmus Medical Centre approved the protocol for this study. Details of the collection process, information about the clinical characteristics of the patients, and RNA isolation from tissues have been published in full elsewhere^[29]. In brief, endoscopic biopsy samples were obtained from the second part of the duodenum, proximal stomach, and distal esophagus. All biopsies were immediately stored in RNAlater (Ambion, Austin, TX, USA) and frozen at -20°C until required. All biopsy samples used in this study were collected from the most distal level of endoscopically visualized Barrett's esophagus epithelium, which was confirmed by concurrent histopathology to be from columnar mucosa with intestinal metaplasia. In individuals with esophageal adenocarcinoma, a similar biopsy collection protocol was used for endoscopic biopsy. Samples were obtained from the second part of the duodenum, proximal stomach and the adenocarcinoma. Samples from surgical resection specimens were obtained from the normal upper stomach, and the tumor site, and immediately stored in RNAlater (Ambion) and frozen at -20°C until required. If any Barrett's esophagus epithelium was present proximal to an esophageal adenocarcinoma, this was also sampled using the same protocols. Samples from patients with adenocarcinoma of the esophagus were always obtained before any neoadjuvant chemotherapy or radiotherapy was commenced, if clinically indicated.

The stored endoscopic biopsies and resection tissues were thawed in RNAlater as required. Thirty percent of each endoscopic biopsy sample, or a small portion of the resection samples, was dissected from the thawed tissue sample, fixed in formalin, embedded in paraffin, and processed for conventional histopathology. This was done to confirm that the biopsy contained only the appropriate tissue type. The remaining tissue had any remaining RNAlater removed, and was then processed in Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction. RNA was also extracted from cell lines derived from benign Barrett's esophagus (Qh) and high grade dysplastic (Ch and Gi) epithelium^[30].

Quantitative reverse transcriptase-polymerase chain reaction analysis of miR-200 family, ZEB1 and ZEB2 expression

miR-200 expression was determined using commercially available TaqMan[®] miRNA assays specific for each member of the miR-200 family (Applied Biosystems, Foster City, CA,

Table 1 Relative miRNA expression in Barrett's esophagus, gastric and duodenal mucosal tissues

MiRNA	Duodenal (n = 10)	Barrett's esophagus (n = 17)	Gastric (n = 15)	P value (Kruskal-Wallis test)
miR-141	0.076 (0.039, 0.167)	0.026 (0.023, 0.036)	0.051 (0.042, 0.092)	0.0002 ^{1,2}
miR-200a	0.148 (0.067, 0.340)	0.148 (0.126, 0.177)	0.247 (0.154, 0.509)	0.0314 ²
miR-200b	0.796 (0.606, 1.276)	0.833 (0.750, 0.993)	1.233 (1.089, 1.963)	0.0011 ²
miR-200c	2.700 (1.890, 3.511)	1.049 (0.929, 1.170)	2.335 (1.792, 2.773)	< 0.0001 ^{1,2}
miR-429	0.095 (0.042, 0.070)	0.070 (0.061, 0.087)	0.078 (0.072, 0.153)	0.259

Relative expression for each epithelial tissue type. Relative expression values are median (95% CI). The group *P* value was the result of a Kruskal-Wallis test across the three tissue groups. Significant differences were identified by post hoc testing by the Holm-Bonferroni method for: ¹Duodenal versus Barrett's esophagus mucosa - miR-141 (*P* = 0.0008) and miR-200c (*P* < 0.0001); ²Gastric versus Barrett's esophagus mucosa - miR-141 (*P* = 0.0004) miR-200a (*P* = 0.0078), miR-200b (*P* = 0.0001), and miR-200c (*P* < 0.0001).

USA). *ZEB1* and *ZEB2* mRNA expression was assessed using the Quantiscript® RT kit for reverse transcription and the Quantitect® SYBRGreen mastermix for polymerase chain reaction (PCR). Primer details are available upon request. miRNA expression was normalized using RNU44, and mRNA expression was normalized using 18S rRNA. Data were analyzed quantitatively using Q-Gene software^[31]. Apparent differences in gene expression between the tissues were assessed for statistical significance using the Kruskal-Wallis test (significance cut-off *P* < 0.05). If significance was reached for this analysis, then the *post hoc* Holm-Bonferroni test was used for pairwise comparisons. Statistical testing was performed using Microsoft Excel. Spearman rank order correlation tests between miRNA and mRNA expression were conducted on-line (<http://www.wessa.net/rankcorr.wasp>). In addition to miR-200 expression, we also tested miR-215 because we have previously demonstrated down-regulation of this miRNA in esophageal adenocarcinoma^[29], and it was recently shown to target *ZEB2* directly^[32].

miRNA target prediction and pathway analysis

Target prediction using miRecords (<http://mirecords.biolead.org/>)^[33] and a core analysis using Ingenuity Pathway Analysis (www.ingenuity.com) were combined to elucidate possible implications of reduced miRNA expression. Predicted targets used in this analysis were required to be predicted by at least five databases in the miRecords search engine. Ingenuity Pathway Analysis parameters were set to assess a knowledge base derived from direct and indirect associations between genes in human experiments, and also in epithelial cell lines. In the Ingenuity Pathway Analysis, genes are grouped according to function and are allocated to top associated networks and cellular functions. Ingenuity Pathway Analysis uses a right-tailed Fisher's exact test to assign *P* values to each grouping, testing each result against a result from random groups of input predicted genes. Networks and cellular functions are ranked according to their score with the highest scoring networks representing the greatest statistical significance.

RESULTS

miR-200 family expression analysis in Barrett's esophagus, gastric and duodenal epithelia

Taqman Quantitative reverse transcriptase-PCR (qRT-

PCR) revealed that expression levels of miR-141 and 200c were significantly lower in Barrett's esophagus epithelium compared with gastric and duodenal epithelia (Table 1). miR-200a and miR-200b expression was significantly lower in Barrett's esophagus epithelium than in gastric epithelium, but did not differ in expression between Barrett's esophagus and duodenal epithelia. The expression level of miR-429 was not significantly different across these three epithelial types.

Predicted implications of reduced miR-200 family expression in Barrett's esophagus

Core Ingenuity Pathway Analysis on predicted targets of miR-141 and miR-200c were performed to determine the probable biological effects of their reduced expression. The collection of target predictions from multiple algorithms, presented in miRecords, listed 272 gene targets for miR-141 and 429 targets for miR-200c. The top associated biological networks for these targets are illustrated in Figure 1, and the biological functions associated with these targets are listed in Table 2.

miR-200, ZEB1 and ZEB2 expression in Barrett's esophagus and esophageal adenocarcinoma

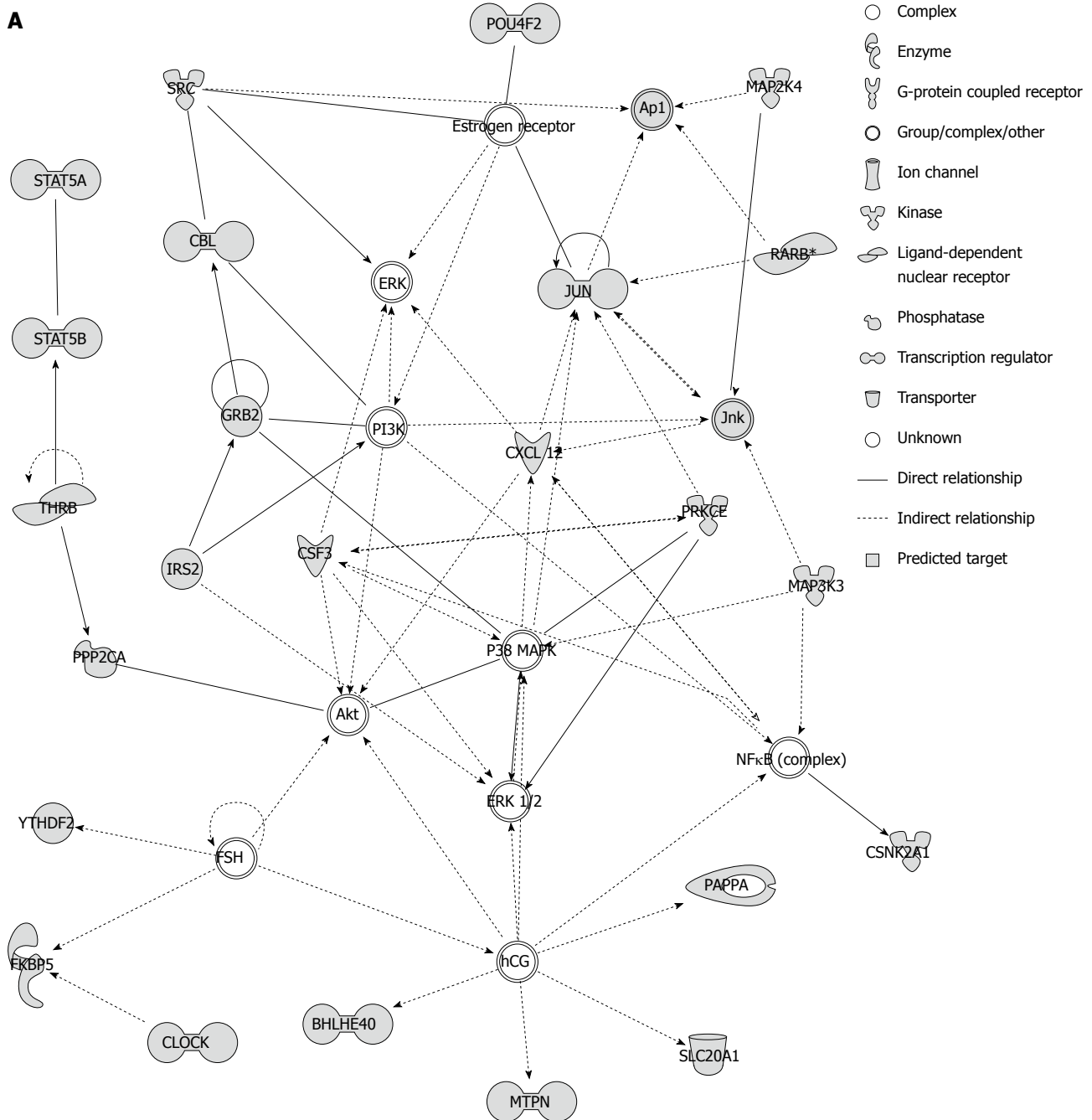
Expression of all members of the miR-200 family was significantly lower in esophageal adenocarcinoma compared with Barrett's esophagus epithelium (Table 3). The median expression of all miR-200 family members was lower in Barrett's esophagus epithelium proximal to esophageal adenocarcinoma than in Barrett's epithelium from patients without cancer or dysplasia. However, after *post hoc* analysis, only the difference in miR-200c expression was statistically significant. To determine whether this could indicate downregulation of miR-200 expression in dysplasia, prior to the development of adenocarcinoma, miR-200 family expression was further assessed in a non-dysplastic Barrett's esophagus derived cell line (Qh), and two cell lines derived from Barrett's esophagus with high-grade dysplasia (Ch, Gi). Figure 2 shows that the expression of all miR-200 members was markedly reduced in both dysplastic cell lines compared to expression in the benign cell line.

To determine whether the miRNA switch for epithelial to mesenchymal transition might be active in esophageal adenocarcinoma development, we also assessed the mRNA expression of miR-200 targets *ZEB1* and *ZEB2*.

Table 2 Top molecular and cellular functions of miR-200 gene targets

miRNA	Molecular and cellular functions	Molecules involved	P value
miR-141	Cell cycle	41	1.90E-07-2.82E-02
	Gene expression	53	4.94E-07-2.82E-02
	Cellular movement	39	2.45E-05-2.82E-02
	Cellular assembly and organization	61	2.88E-04-2.82E-02
	Cellular growth and proliferation	68	2.88E-04-2.28E-02
miR-200c	Gene expression	84	1.37E-09-2.32E-02
	Cellular growth and proliferation	108	5.65E-05-2.32E-02
	Cell cycle	53	9.17E-05-2.32E-02
	Cell death	95	9.62E-05-2.32E-02
	Cellular assembly and organization	55	1.19E-04-2.32E-02

The top five functions associated with the predicted targets of miR-141 and miR-200c, as determined by Ingenuity Pathway Analysis, are shown in this table. This analysis used a right-tailed Fisher's exact test to calculate the probability (P value above) that each cellular function ascribed to the predicted target gene list was due to chance alone. For each miRNA, the molecular and cellular functions are ranked according to their P value.



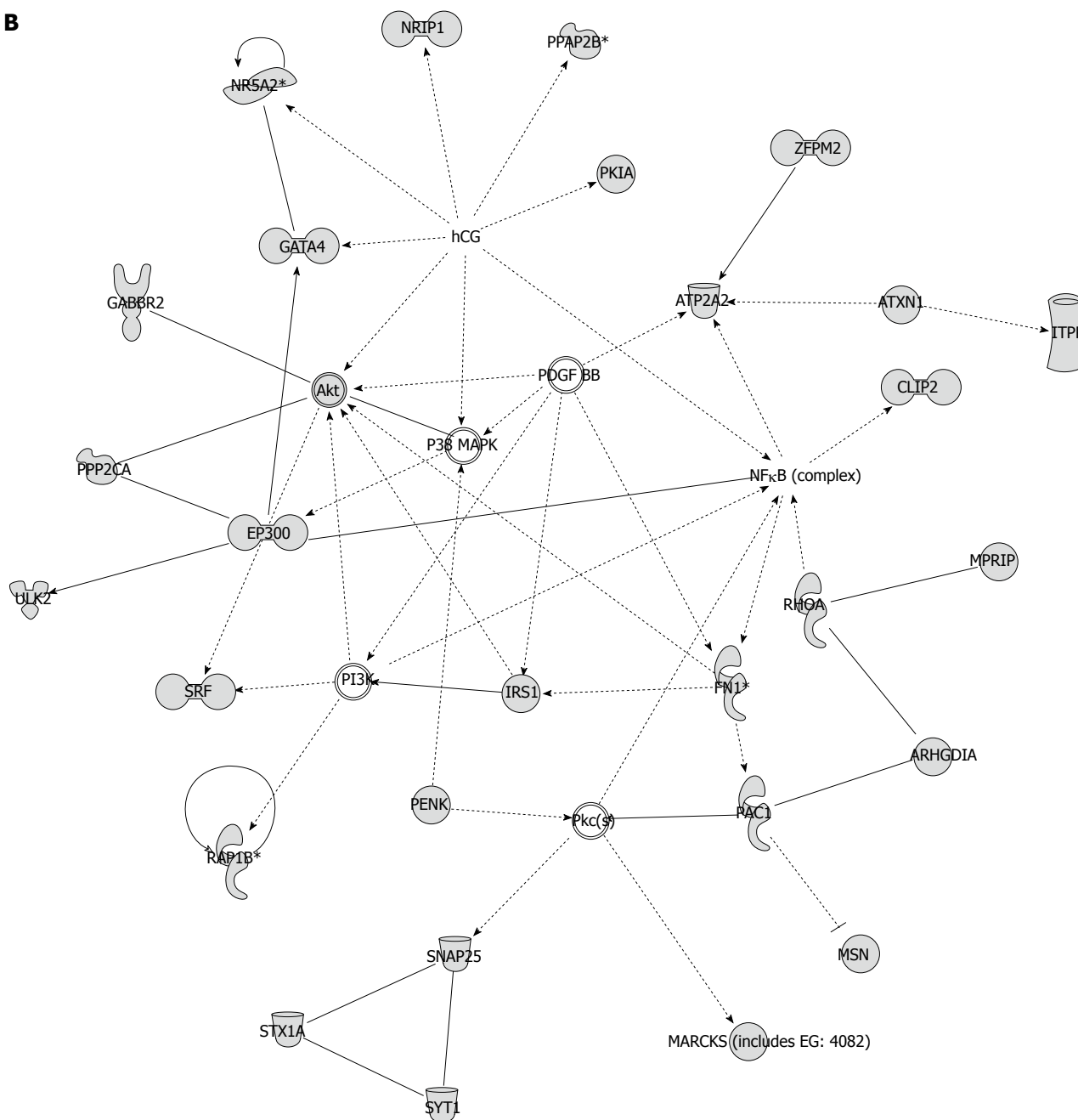
B

Figure 1 Top associated network functions for miR-141 and miR-200c. A: Ingenuity Pathway Analysis predicted that the top associated network functions for miR-141 were gene expression, cell death and cell cycle ($P = 1 \times 10^{-35}$). B: The top associated network functions for miR-200c were cell morphology, cellular assembly and organization, cellular function and maintenance ($P = 1 \times 10^{-38}$). Predicted targets of miR-141 or miR-200c are highlighted in grey. Uncolored entries represent molecules that are associated with the pathway but are not predicted miR-141 or miR-200c targets. The P values were derived from a right-tailed Fisher's exact test to calculate the probability that each predicted miRNA target matches the ascribed network function due to chance alone.

ZEB1 and *ZEB2* expression was significantly higher in esophageal adenocarcinoma compared to Barrett's esophagus epithelium from patients without cancer or dysplasia (Table 4). There were significant inverse correlations between the expression of *ZEB1/ZEB2* and the expression of some miR-200 members. miR-215 and *ZEB2* expression were inversely correlated (Table 4).

DISCUSSION

We found that miR-141 and miR-200c were expressed at

lower levels in Barrett's esophagus epithelium, compared to normal gastric and duodenal epithelia. Bioinformatics analysis indicated that this might contribute to the cell cycle, stress response (proliferation, apoptosis), and cellular migration behavior, which are known to make Barrett's esophagus epithelium different to gastric and duodenal epithelia^[4,7-12,34]. The reduced miR-200 levels that we observed in Barrett's esophagus epithelium adjacent to adenocarcinoma, and Barrett's esophagus with high-grade dysplasia derived cell lines suggested an association between down-regulation of miR-200 expression and neoplastic progres-

Table 3 miRNA expression in Barrett's esophagus and esophageal adenocarcinoma

miRNA	BE (n = 17)	Adeno carcinoma (n = 20)	BEC (n = 9)	P value (Kruskal-Wallis test)
miR-141	0.026 (0.023, 0.036)	0.012 (0.011, 0.025)	0.015 (0.01, 0.04)	0.03398 ¹
miR-200a	0.148 (0.126, 0.177)	0.057 (0.013, 0.042)	0.08 (0.017, 0.201)	0.00079 ¹
miR-200b	0.833 (0.75, 0.993)	0.387 (0.316, 0.572)	0.399 (0.258, 1.13)	0.00068 ¹
miR-200c	1.05 (0.929, 1.170)	0.551 (0.461, 0.939)	0.662 (0.438, 0.965)	0.00323 ^{1,2}
miR-429	0.07 (0.062, 0.087)	0.039 (0.029, 0.06)	0.042 (0.036, 0.09)	0.01355 ¹

Relative expression for each tissue type. Relative expression values are median (95% CI). BE = Barrett's esophagus epithelium from individuals without cancer, BEC = Barrett's epithelium taken proximal to adenocarcinoma and confirmed by histology to be free of invasive cancer. The group P value was the result of a Kruskal-Wallis test across the three tissue groups. Significant differences were identified by post hoc testing by Holm-Bonferroni method for: ¹Adenocarcinoma versus Barrett's esophagus mucosa from individuals without cancer - miR-141 ($P = 0.0126$), miR-200a ($P = 0.0001$), miR-200b ($P < 0.0001$), and miR-200c ($P = 0.0014$) and miR-429 ($P = 0.0031$); ²Barrett's esophagus mucosa from individuals with versus without cancer - miR-200c ($P = 0.0191$).

Table 4 ZEB1 and ZEB2 expression in Barrett's epithelium and esophageal adenocarcinoma

	Fold ↑	miR-141	miR-200a	miR-200b	miR-200c	miR-429	miR-215
ZEB1	2.9	R = -0.2	R = -0.4	R = -0.5	R = -0.5	R = -0.3	R = -0.3
	$P < 0.0001$	$P = 0.341$	$P = 0.046$	$P = 0.009$	$P = 0.006$	$P = 0.159$	$P = 0.152$
ZEB2	1.5	R = -0.3	R = -0.3	R = -0.5	R = -0.5	R = -0.4	R = -0.4
	$P = 0.029$	$P = 0.101$	$P = 0.085$	$P = 0.008$	$P = 0.015$	$P = 0.067$	$P = 0.044$

The fold increase (↑) in median ZEB1 and ZEB2 expression in esophageal adenocarcinoma versus Barrett's esophagus mucosa from individuals without cancer, and the P value derived using the Mann-Whitney test are given. Spearman correlations (R = Spearman's rho) between ZEB1/2 expression and each miR-200 member, with associated P values are also shown.

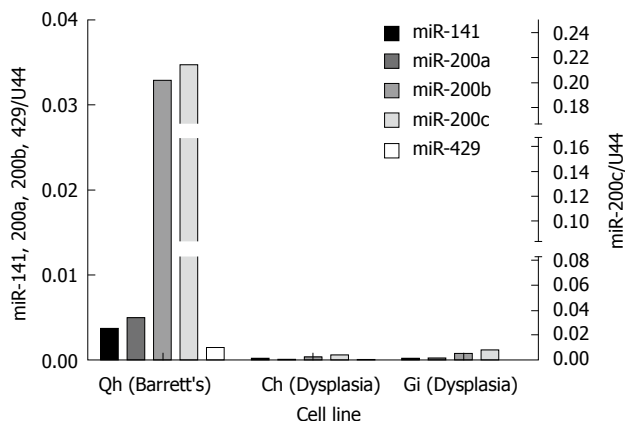


Figure 2 miR-200 family expression in benign Barrett's and dysplastic cell lines. Relative expression of all miR-141, miR-200a, miR-200b and miR-429, normalized to U44 expression is shown on the left hand y axis. Relative expression of miR-200c is shown on the right hand y axis. The pattern of relative expression of miR-200 members in the Qh (benign Barrett's) cell line closely resembled that in benign Barrett's esophagus mucosa (see relative expression values in Table 1).

sion in Barrett's esophagus. The increased expressions of ZEB1 and ZEB2 in esophageal adenocarcinoma, and their inverse correlations with miR-200 expression, are consistent with induction of epithelial to mesenchymal transition mediated by loss of miR-200 expression. Taken together with the known biological functions of the miR-200 family^[26-28], our study provides evidence for their influence in patterning the known unique gene expression^[4] and phenotypic characteristics^[4,7-12,34] of benign Barrett's esophagus epithelium, as well as features that characterize its neoplastic progression^[13]. Furthermore, our data indicate that the

miRNA switch for turning on epithelial to mesenchymal transition^[20] might be activated during the development of esophageal adenocarcinoma.

Although the miR-200 family has some redundancy in seed sequence, and therefore the genes they target, they act co-operatively to control the expression of their targets, and a change in expression of just one member is sufficient to alter target transcript levels^[20]. miR-141 and miR-200c are known to modulate apoptosis, cell cycle and proliferation, and cellular migration through regulation of their target genes^[26-28]. Several *in vivo* and *in vitro* studies have elucidated the molecules/complexes that are upregulated/activated in Barrett's epithelium and are responsible for its unique anti-apoptotic and proliferative responses to reflux-induced stress. These include three mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase, p38 and C-Jun N-terminal kinase^[35]; protein kinase C^[11]; phosphatidylinositol 3-kinases (PI3K) and downstream Akt^[36]; and transcription factors nuclear factor (NF)- κ B^[37] and activator protein-1^[36]. Our combined target prediction and Ingenuity Pathway Analysis predicted indirect (or direct in the case of Akt) targeting of all of the aforementioned molecules/complexes in the top associated network functions of miR-141 and/or miR-200c. Overall, this suggests that reduced miR-141 and miR-200c expression in Barrett's esophagus epithelium (*vs* gastric and duodenal epithelia) might activate molecular pathways that are known to promote the specific response of Barrett's esophagus epithelium to the insult of gastroesophageal reflux.

Our analysis predicted direct targeting and downregulation of fibronectin by miR-200c, and this was evident in the top associated network. A recent study has shown that fibronectin expression is reduced in cell lines in direct

response to miR-200c expression^[38]. Therefore, it is reasonable to expect that fibronectin expression should be higher in Barrett's esophagus epithelium than in gastric and duodenal epithelia; due at least partly to reduced miR-200c expression in Barrett's esophagus. In agreement with this, fibronectin is in the gene expression cluster that separates Barrett's esophagus epithelium from gastric and duodenal epithelium^[4]. Fibronectin has key roles in cellular adhesion and migration^[39]. In the development of Barrett's esophagus, its expression is proposed to help facilitate expansion of epithelial cell populations in order to replace areas of denuded squamous epithelium with metaplastic cells^[4]. Furthermore, fibronectin expression contributes to increased proliferative and anti-apoptotic behavior through NF- κ B and PI3K signaling^[40]. Taken together, these observations lend support to the biological validity of our bioinformatics-based approach, and further promote a likely role for reduced miR-200c expression in Barrett's esophagus.

The development of high-grade dysplasia significantly increases the risk of progression to esophageal adenocarcinoma^[41,42]. Features of dysplastic epithelium that make it distinct from benign Barrett's esophagus epithelium include increased proliferative and anti-apoptotic behavior^[13]. We found that miR-200c expression is downregulated in Barrett's esophagus sampled from patients with a concurrent esophageal cancer, and the entire miR-200 family was downregulated in cell lines derived from patients with high-grade dysplasia. Our pathway analysis indicates that this could result in the molecular events known to contribute to the features of dysplastic Barrett's esophagus, including increased signaling through MAPK pathways^[43], increased activation and expression of NF- κ B^[44,45], and increased activation of Akt^[43,46]. Activation of Akt is stimulated by interaction of the obesity-related hormone leptin and its receptor, and this results in increased proliferation and resistance to apoptosis in Barrett's esophagus derived cancer cell lines^[46]. The leptin receptor is downregulated *in vitro* in response to miR-200c expression^[38], and our miRecords searches predicted direct targeting of the leptin receptor by miR-200c. We speculate that reduced miR-200 family expression in dysplasia could be an important mechanism for leptin receptor mediated activation of Akt, and this could contribute to the established link between obesity and an increased risk of esophageal adenocarcinoma development^[47]. In further support of the direct relevance of decreased miR-200 expression to the molecular features of neoplastic progression of Barrett's esophagus, we found that three gene transcripts (*EGR3*, *HS3ST1* and *RPS6KB1*), listed in miRecords to be targets of miR-200c, were present in a panel of 18 transcripts that are upregulated in Barrett's epithelium, from which cancer has arisen *versus* benign Barrett's esophagus epithelium^[48].

Downregulation of E-cadherin expression *via* transcriptional repression is a central mechanism for epithelial to mesenchymal transition^[15]. ZEB1 and ZEB2 are amongst a group of transcription factors that repress transcription of E-cadherin^[15]. ZEB1 and ZEB2 are targets of the miR-200 family, and an increase in ZEB1 and ZEB2 activity caused by downregulation of miR-200 expression is sufficient to

induce epithelial to mesenchymal transition^[20]. Epithelial to mesenchymal transition that is promoted by downregulation of the miR-200 family has now been implicated as an important mechanism for invasion and metastasis in several cancers^[20,22-25]. Previous epithelial and mesenchymal cell marker studies have provided evidence for the involvement of epithelial mesenchymal transition in the development of esophageal adenocarcinoma^[16,17], but to the best of our knowledge, no studies have investigated loss of miR-200 mediated control of ZEB1 and ZEB2 expression as a possible mechanism for epithelial to mesenchymal transition in this disease. We found downregulation of the entire miR-200 family and upregulation of ZEB1 and ZEB2 transcription levels upon progression of Barrett's esophagus to adenocarcinoma. We observed significant inverse correlations between expression of miR-200 members and ZEB1/ZEB2 transcripts. miR-215 expression, which we have previously reported as downregulated in neoplastic progression of Barrett's esophagus^[29], was also inversely correlated with ZEB2 expression. miR-215 has recently been shown to target ZEB2 expression directly in kidney cells^[32], and our results suggest the same in Barrett's esophagus. Together, these results suggest that the miR-200 family contributes to control of ZEB1 and ZEB2 expression in Barrett's esophagus, and this might be important for maintaining the epithelial phenotype. They add to the current evidence for the involvement of epithelial to mesenchymal transition in esophageal adenocarcinoma, and indicate downregulation of miR-200 expression as a potential mechanism for this.

With regard to the potential clinical relevance of our findings, we hypothesize that the miR-200 family might be useful biomarkers for identifying patients with Barrett's esophagus who are at increased risk of adenocarcinoma. They may also be helpful for assessing the potential antineoplastic effects of medical and surgical treatment of Barrett's esophagus. Further studies are required to evaluate this hypothesis. Recent advances in delivery of small RNAs in a clinical setting^[49], and the demonstrated *in vitro* antineoplastic effects of endogenous miR-200 expression^[38,50], suggest a possible future role for the therapeutic use of this family of miRNAs in treating early cancer in Barrett's epithelium.

Our study had some limitations. First, we used Barrett's esophagus epithelium proximal to cancer, and cell lines derived from dysplastic epithelium, to determine evidence for reduced miR-200 expression in dysplasia. We did not demonstrate miR-200 downregulation in dysplastic epithelium from patients who did not have invasive cancer, and this is an important area for investigation in future studies. Second, although our study provides evidence for effects of miR-200 expression on known gene-expression and phenotypic features of benign and dysplastic Barrett's esophagus, we did not expand this evidence using functional studies.

In summary, we showed that the miR-200 expression profile in Barrett's esophagus distinguished it from gastric and duodenal epithelia, and that downregulation of miR-200 expression was associated with dysplasia and adenocarcinoma. Further investigation is warranted to evaluate whether changes in the expression of these miRNAs can

be used to identify patients with Barrett's esophagus who are at risk of progression to esophageal adenocarcinoma.

ACKNOWLEDGMENTS

We thank Dr. Peter Rabinovitch for kindly providing the Barrett's esophagus and dysplastic cell lines Qh, Ch and Gi. We thank Associate Professor Greg Goodall and Dr. Tina Bianco-Miotto for helpful discussions. This study was funded by a grant from the National Health and Medical Research Council of Australia. Cameron Smith was supported by a scholarship from the New South Wales Cancer Council.

COMMENTS

Background

Barrett's esophagus is a precursor to esophageal adenocarcinoma and develops in response to chronic gastroesophageal reflux. Barrett's esophagus epithelium closely resembles that of the stomach and small intestine. However, some genes are expressed uniquely in Barrett's esophagus, and this is associated with pronounced anti-apoptotic and proliferative behavior in response to reflux. Esophageal adenocarcinoma development may involve an epithelial to mesenchymal transition-mediated acquisition of invasive phenotype. The miR-200 family of miRNAs can regulate cell death and proliferation, and control epithelial to mesenchymal transition through targeting of *ZEB1* and *ZEB2* gene expression.

Research frontiers

miRNAs have been implicated in almost every cellular process investigated. Recent evidence highlights a role for the miR-200 family members in numerous cellular processes including cell proliferation, cell death and epithelial to mesenchymal transition. All of these processes have been implicated in Barrett's esophagus and esophageal adenocarcinoma. We identified aberrant expression of the miR-200 family in Barrett's esophagus and esophageal adenocarcinoma and hypothesize that this differential expression contributes to aspects of neoplastic progression.

Innovations and breakthroughs

This study is the first to identify differential expression of the miR-200 family in Barrett's esophagus and esophageal adenocarcinoma. The miR-200 family expression data in Barrett's esophagus paired with functional studies in other cell types provides evidence that aberrant miR-200 family member expression may contribute to the increased proliferation and decreased cell death associated with Barrett's esophagus. The complete downregulation of the miR-200 family and subsequent upregulation of mRNA targets *ZEB1* and *ZEB2* in esophageal adenocarcinoma mimics what is seen in epithelial to mesenchymal transition and other invasive solid tumors, which suggests that the miR-200 family is involved in invasive esophageal adenocarcinoma development.

Applications

The authors hypothesize that the miR-200 family might be useful biomarkers for identifying patients with Barrett's esophagus who are at increased risk of adenocarcinoma. They may also be helpful for assessing the potential antineoplastic effects of medical and surgical treatment of Barrett's esophagus.

Terminology

Barrett's esophagus is a precursor to esophageal adenocarcinoma and develops in response to chronic gastroesophageal reflux. The miR-200 family of miRNAs can regulate cell death and proliferation, and control epithelial to mesenchymal transition through targeting of *ZEB1* and *ZEB2* gene expression.

Peer review

This is a well-written manuscript that describes miRNAs in Barrett's epithelium. The authors' conclusions are supported by the data.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Arterial-phase contrast-enhanced ultrasonography for evaluating anti-angiogenesis treatment: A pilot study

Keiko Yoshida, Tomihiko Hirokawa, Fuminori Moriyasu, Longzhong Liu, Guang-Jian Liu, Masahiko Yamada, Yasuharu Imai

Keiko Yoshida, Tomihiko Hirokawa, Fuminori Moriyasu, Longzhong Liu, Guang-Jian Liu, Masahiko Yamada, Yasuharu Imai, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo 160-0023, Japan

Author contributions: Liu L, Hirokawa T and Moriyasu F designed the study; Yoshida K, Hirokawa T, Liu L and Liu GJ performed the experiments; Yoshida K, Hirokawa T, Yamada M, and Imai Y analyzed the data; Yoshida K and Hirokawa T wrote the manuscript; Moriyasu F supervised the study.

Correspondence to: Keiko Yoshida, MD, Department of Gastroenterology and Hepatology, Tokyo Medical University, 6-7-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. keiyoshida01@gmail.com

Telephone: +81-3-53256838 Fax: +81-3-53256840

Received: August 14, 2010 Revised: September 13, 2010

Accepted: September 20, 2010

Published online: February 28, 2011

Abstract

AIM: To verify whether arterial-phase contrast-enhanced ultrasonography (CEUS) of tumor parenchymal tissue is useful for evaluation of anti-angiogenesis agents.

METHODS: Rabbits with liver tumor were subjected to CEUS, and images of the nodular maximal diameter in vascular phase were recorded. Image analysis was performed to plot the time intensity curve (TIC) at the tumor parenchyma, which set the diameter of the region of interest of intensity measurement. The TIC was calculated to obtain the time to peak intensity (TPI) and the magnitude of PI. Rabbits were randomly assigned to a treatment group with sorafenib and a control group. Two weeks later, the same ultrasound examination was repeated followed by pathological testing to assess the effect of sorafenib on the liver tumor.

RESULTS: In four rabbits in the treatment group, the rate of change of tumor size was decreased compared

with that of the control (the rate 2.3 vs 7.9, $P = 0.02$). The TPI of the treatment group elongated significantly (the rate 3.1 vs 1.1, $P = 0.07$ for SonoVue, 2.0 vs 0.88, $P = 0.09$ for Sonazoid). The magnitude of PI showed no significant changes. In pathological examination, capillary diameters in the treatment group were significantly smaller than those in the control group (26.4 vs 42.8 μm , $P = 0.013$).

CONCLUSION: Analysis of the TIC in the arterial phase of tumor tissue could evaluate the efficacy of anti-angiogenesis drug treatment in liver tumor.

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Key words: Liver tumor; Ultrasound agent; Contrast-enhanced ultrasonography; Sorafenib; Animal model

Peer reviewer: London Lucien Ooi, Professor, Chairman, Division of Surgery, Singapore General Hospital, 1 Hospital Drive, 169608 Singapore, Singapore

Yoshida K, Hirokawa T, Moriyasu F, Liu L, Liu GJ, Yamada M, Imai Y. Arterial-phase contrast-enhanced ultrasonography for evaluating anti-angiogenesis treatment: A pilot study. *World J Gastroenterol* 2011; 17(8): 1045-1050 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1045.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1045>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth largest cause of neoplasms worldwide^[1]. Although effective treatment for early-stage HCC exists in the form of surgery and locally destructive techniques such as radiofrequency ablation, there are few treatments for advanced HCC. Recent randomized control trials of the anti-angiogenesis drug sorafenib have shown effectiveness for the first time. Time to radiological progression and overall survival were

prolonged in patients with advanced HCC who received sorafenib. However, some problems remain for anti-angiogenesis treatment. One of them is how to assess clinically whether the drugs are effective, because some tumors develop tolerance. Oncologists have to change the therapy as soon as they find that the liver tumor has become refractory to anti-angiogenesis therapy^[2].

Enhanced computed tomography (CT) and magnetic resonance imaging (MRI) are generally used for this screening. They are convenient to assess the response of anti-angiogenesis therapy, because tumor size, necrotic lesions, vascularity and perfusion are evaluated at the same time. However, these modalities sometimes make incorrect assessments, because tumor perfusion is difficult to evaluate. Perfusion is more important than the size for anti-angiogenesis drug therapy^[3].

Ultrasound examination can evaluate tumor size and necrotic lesions. Moreover, contrast-enhanced ultrasonography (CEUS) can evaluate tumor blood flow and perfusion more easily at the bedside than can CT and MRI with contrast agents^[4]. Recently, CEUS has been used for differential diagnosis of liver tumor and to determine the degree of differentiation of HCC^[5].

Previously, the usefulness of CEUS for the evaluation has been reported. However, a long time is needed for the ultrasound examination^[6]. In response to this, we tried to simplify the evaluation method with CEUS. The purpose of this study was to probe whether the perfusion information from tumors at the arterial phase of CEUS is useful for monitoring the early-phase response of anti-angiogenesis treatment.

MATERIALS AND METHODS

In situ tumor model

Twenty male Japanese white rabbits (body weight 2.5–5.0 kg, aged 12–16 wk) were used in this study. After habituation, implantation of Vx-2 tumor into the liver was performed. The Vx-2 tumor is a product of a virus-induced papilloma of rabbits, and the implanted nodules in liver are often used as an HCC model^[7,8]. Rabbits were anesthetized by intravenous injection of 8 mg/kg pentobarbital. Each rabbit received a single percutaneous injection of about 1 mm × 1 mm × 2 mm of Vx-2 tumor directly into the liver parenchyma from a donor rabbit, with a fine 14 G needle guided by ultrasound. Every 2 wk after implantation, ultrasound examination was repeated until the tumor grew to 7–10 mm and was available for ultrasound evaluation. Tumors were successfully grown in 10 animals. Six rabbits were assigned randomly to receive sorafenib and the others were assigned to a control group. The study was approved by the animal ethical committee of Tokyo Medical University.

Administration of sorafenib

Sorafenib was used as an anti-angiogenesis drug. It inhibits multiple kinases and signaling pathways of angiogenesis including vascular endothelial growth factor (VEGF), and induces apoptosis of tumor cells^[2]. Sorafenib was

suspended into a carboxymethyl cellulose sodium salt solution and orally administered to six rabbits at a dose of 30 mg/kg once daily (30 mg/kg per day) for 14 d. The other four rabbits received no medication.

Conventional ultrasound and CEUS examination

Ultrasonography was performed after placement of an intravenous catheter, and monitoring of respiration and heartbeat under anesthesia. Before injection of contrast agents, the livers were scanned in fundamental imaging mode. Ultrasound settings were as follows: diagnostic ultrasound system, SSA-770A (Toshiba Medical Systems, Japan) with PLT-1204AT (12 MHz) or PLT-704AT (7.5 MHz) probe, 2D mode for the abdomen, gain 80, dynamic range 65, rate 50 frames/s.

CEUS examination was performed by manually stabilizing the probe at the plane including its maximum diameter. Contrast agents were injected intravenously: SonoVue and Sonazoid. SonoVue is commercially available in Europe and China, and Sonazoid in Japan. A bolus of 0.05 mL/body of SonoVue was administered intravenously. After recording of images with SonoVue, 0.03 mL/body Sonazoid was given in the same way. For each agent, contrast-enhanced imaging of the tumor and surrounding parenchyma was performed for 3 min from just after injection, from the arterial to the portal phase, and for 7–8 min at the late phase.

Ultrasound settings were as follows: diagnostic ultrasound system, SSA-770A with PLT-1204AT (12 MHz) or PLT-704AT (7.5 MHz) probe, harmonic mode for CEUS, gain 70, dynamic range 50, rate 15 frames/s, mechanical index 0.23 for SonoVue and 0.32 for Sonazoid in harmonic imaging.

In the sorafenib group, the ultrasound examinations were performed on d 1 and 14 after sorafenib administration. In the control group, the same examinations were performed at 14 d after implantation and were repeated at 14 d after the first examination.

Images were recorded digitally on an optical disc and analyzed offline. Average video intensity (VI) of the region of interest (ROI) on every frame was measured automatically by the software Image Lab (Toshiba Corporation, Japan), after the ROI that included the most markedly enhanced area of a whole tumor region was identified. Necrotic regions, vessels, and liver parenchyma surrounding the tumor were excluded from the ROI. The corresponding time intensity curve (TIC) was plotted. The following parameters of blood flow were measured from the TIC: baseline intensity of ROI (VI₀), maximal signal of the ROI (VI_{max}), peak intensity (PI) that shows the difference between VI_{max} and VI₀ (VI_{max}–VI₀), and time to peak intensity (TPI) – time required from the onset of tumor contrast enhancement to VI_{max} (Figure 1)^[6]. Variation of TPI was the ratio of the post-treatment TPI to the pre-treatment TPI.

Pathological examination

Animals were euthanized by injection of a lethal dose of pentobarbital just after the second ultrasound examina-

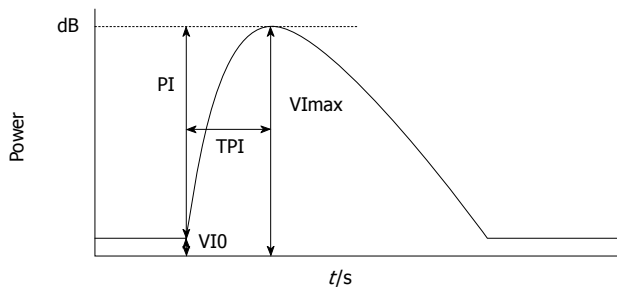


Figure 1 Time intensity curve. V0 is the baseline intensity of ROI; VImax is the maximal signal of the ROI; PI is the difference between VImax and VIO (VImax-VIO); time to peak intensity (TPI) is the time required from the onset of tumor contrast enhancement to reach VImax.

tion. Their livers were excised and examined pathologically to assess the response to sorafenib and its effects. After the examination of gross features, hematoxylin-eosin staining and immunological staining by anti-VEGF antibody were performed. Specimens were fixed in 10% formalin. Paraffin-embedded 5- μ m-thick tissue sections were de-waxed, dehydrated, washed in distilled water, and rinsed in PBS, and then incubated with VEGF antibody (clone JH121; Thermo Fisher Scientific, CA, USA), which has species reactivity for humans and rabbits. Positive staining was identified when brown staining was found in the cytoplasm of the vascular endothelial cells.

Measurement of vascular diameter

Cross-sectional images of the tumors were recorded in their maximum diameter as TIFF files at 40 \times magnification using a BX50 microscope (Olympus, Tokyo, Japan) and Micropublisher 5.0 imaging system (Q Imaging, Canada). Blood vessels were identified in the image, and the diameters of the five largest vessels were measured macroscopically with Q Capture pro. 5.0 software (Q Imaging). The mean blood vessel diameters were compared. Vessels in the surrounding parenchyma and necrotic lesions and vessels that connected to the capsules were excluded because they were thought to correlate poorly with enhancement of the tumor.

Statistical analysis

SPSS version 16 software (SPSS Inc, Chicago, IL, USA) was used for all statistical analysis. The differences between the anti-angiogenesis treatment and control groups were compared using the Mann-Whitney test for tumor size and *t* test for other parameters. $P < 0.10$ was considered statistically significant.

RESULTS

Two rabbits in the treatment group died of systemic metastasis of the implanted tumor. The other four rabbits were used for evaluation.

Tumor size was calculated by multiplying the longest diameter by the shortest. In the sorafenib group, the tumor grew more slowly than in the control group. Pre-treatment tumor size was a mean 107.5 mm² (range: 58.6-192.9 mm²),

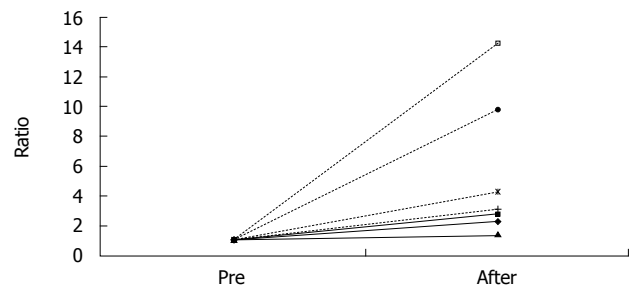


Figure 2 Tumor size variation. Solid lines show the size variation of the sorafenib-treated group. Interrupted lines show the size variation of the control group. The control group showed greater tumor enlargement than the sorafenib group did.

Table 1 Tumor size

	Pre-treatment	Post-treatment	Ratio
Sorafenib 1	69.4	159.0	2.3
Sorafenib 2	58.6	165.3	2.8
Sorafenib 3	109.0	150.9	1.4
Sorafenib 4	192.9	547.2	2.8
Control 1	49.0	210.3	4.3
Control 2	24.5	240.0	9.8
Control 3	78.8	246.1	3.1
Control 4	30.6	436.6	14.0

and post-treatment size was a mean 255.6 mm² (150.9-547.2 mm²) in the sorafenib group. In the control group, tumor size increased more rapidly. Baseline tumor size was a mean 45.7 mm² (range: 24.5-78.8 mm²) and that at 14 days was a mean 283.2 mm² (range: 210.3-436.6 mm²). Ratio (post-/pre-treatment) of the tumor size was a mean 2.3 (1.4-2.8) in the sorafenib group and 7.9 (3.1-14.2) in the control group ($P = 0.02$) (Figure 2, Table 1).

There were no significant differences in variation of PI in the tumor. In SonoVue imaging, variation in PI in the sorafenib group was a mean 2.1 (range: 0.5-5.5) and that of the control group was a mean 1.3 (range: 0.9-1.7) ($P = 0.84$). In Sonazoid imaging, variation in PI in the sorafenib group was a mean 1.6 (range: 1.2-2.5) and that in the control group was a mean 1.2 (range: 1.0-1.4) ($P = 0.28$, Table 2).

Variation in TPI in the tumor was prolonged in the sorafenib group. In contrast, variation in TPI in the tumor did not change or was shortened in the control group. In SonoVue imaging, variation in TPI in the sorafenib group was a mean 3.1 (range: 1.1-4.8) and that in the control group was a mean 1.1 (range: 0.56-1.4) ($P = 0.07$). In Sonazoid imaging, variation in TPI in the sorafenib group was a mean 2.0 (range: 1.0-3.2) and that in the control group was a mean 0.88 (range: 0.42-1.33) ($P = 0.09$, Figures 3 and 4, Table 2).

Pathological examination showed peritoneal dissemination and multiple metastases outside the liver. Some of the rabbits also showed ascites. Tumors appeared round, yellow/white, and separate from the surrounding parenchyma, with large necrotic lesions inside.

In vascular measurement, tumors in the sorafenib group had smaller vessels than in the control group. In

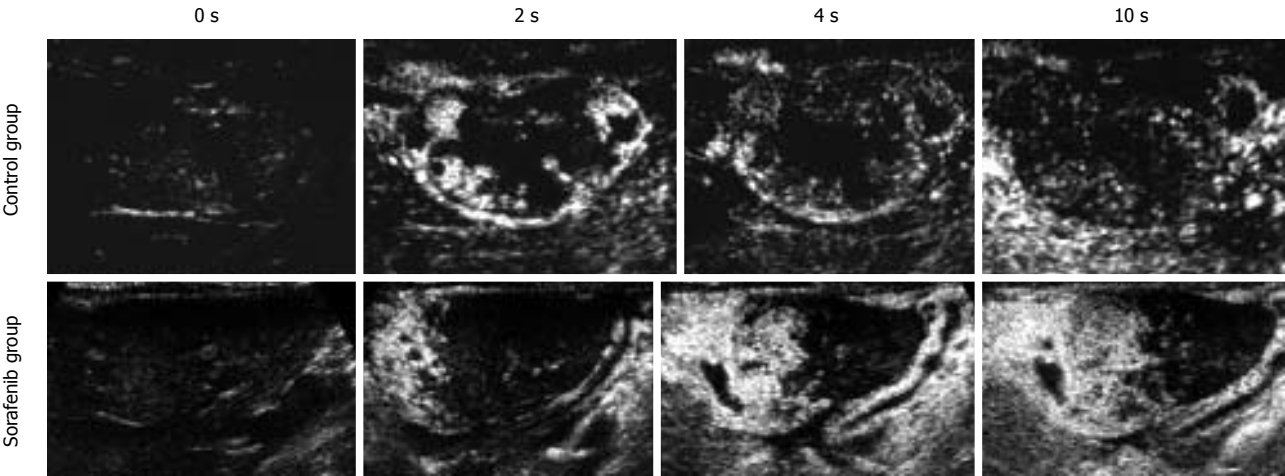


Figure 3 Contrast-enhanced ultrasonography examination showed that time to peak intensity was delayed in the sorafenib-treated group.

Table 2 Time intensity curve							
		VO	VI _{max}	TPI	PI	Variation	
						PI	TPI
SonoVue							
Sorafenib 1	0W	-44.8	-22.3	1.00	24.5		
	2W	-48.3	-13.6	3.40	34.7	1.4	3.4
Sorafenib 2	0W	-45.5	-19.6	0.27	25.8		
	2W	-48.4	-35.3	0.87	13.1	0.51	3.2
Sorafenib 3	0W	-49.6	-43.7	1.00	5.9		
	2W	-49.1	-16.8	1.13	32.3	5.5	1.1
Sorafenib 4	0W	-39.6	-14.9	0.86	24.7		
	2W	-45.6	-18.8	4.14	26.8	1.1	4.8
Control 1	0W	-46.4	-25.0	2.00	21.5		
	2W	-45.3	-8.7	1.13	36.6	1.7	0.57
Control 2	0W	-48.5	-14.6	2.53	34.0		
	2W	-47.8	-16.0	2.60	31.8	0.94	1.0
Control 3	0W	-46.9	-12.4	2.07	34.4		
	2W	-49.0	-18.1	2.93	30.9	0.90	1.4
Control 4	0W	-49.9	-37.2	1.06	12.7		
	2W	-50.0	-28.5	1.47	21.5	1.7	1.4
Sonazoid							
Sorafenib 1	0W	-49.1	-21.9	2.27	27.2		
	2W	-48.1	-13.5	3.67	34.5	1.3	1.6
Sorafenib 2	0W	-47.8	-21.4	1.00	26.3		
	2W	-49.7	-19.0	2.13	30.7	1.2	2.1
Sorafenib 3	0W	-34.5	-19.1	2.60	15.4		
	2W	-47.9	-10.0	2.67	37.9	2.5	1.0
Sorafenib 4	0W	-35.8	-13.0	1.33	22.8		
	2W	-41.8	-9.5	4.20	32.3	1.4	3.2
Control 1	0W	-42.5	-16.2	2.33	26.3		
	2W	-48.6	-13.0	2.80	35.6	1.4	1.2
Control 2	0W	-37.8	-7.4	4.27	30.4		
	2W	-44.0	-12.8	1.80	31.2	1.0	0.42
Control 3	0W	-41.7	-14.7	1.60	27.0		
	2W	-49.2	-18.0	2.14	31.2	1.2	1.3
Control 4	0W	-46.7	-27.7	2.87	18.9		
	2W	-49.8	-23.7	1.60	26.1	1.4	0.56

TPI: Time to peak intensity.

the sorafenib group, the diameter of the tumor vessels was a mean 26.4 μm (range: 23.9-26.7 μm) and that in the control group was a mean 42.8 μm (range: 34.2-50.1 μm) ($P = 0.013$, Figure 5).

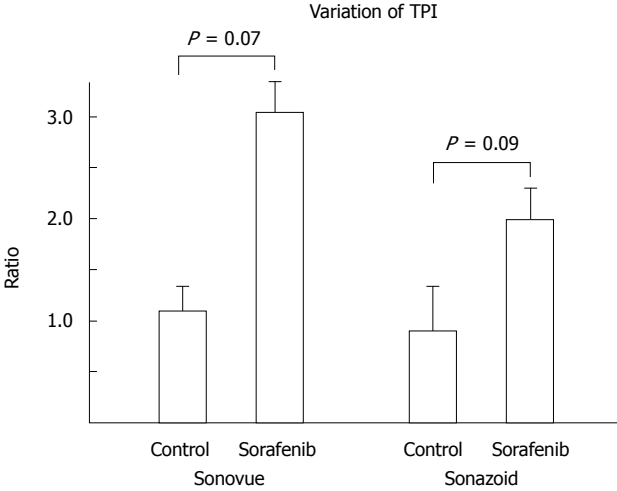


Figure 4 Time to peak intensity in the tumor was significantly prolonged in the sorafenib group with both SonoVue and Sonazoid imaging. Time to peak intensity (TPI) in the tumor did not change or was shortened in the control group.

VEGF immunostaining examination presented no significant difference between both groups.

DISCUSSION

In the present study, prolonged TPI in arterial phase CEUS was shown during treatment with anti-angiogenesis agent.

Lavisse *et al*^[6] have reported that TPI of tumor was elongated and PI was decreased in an anti-angiogenesis treatment group compared with a control group. Tumors induce new vessels to obtain oxygen and nutrition for their growth, and total blood flow of tumors increases, so-called angiogenesis. According to the study of Wilhelm *et al*^[9], the anti-angiogenesis agent sorafenib inhibits angiogenesis and reduces microvessel density. This should be the reason why PI is reduced and TPI is prolonged in the sorafenib group.

In our study, TPI of tumors in the sorafenib group was similarly prolonged significantly, but no significant

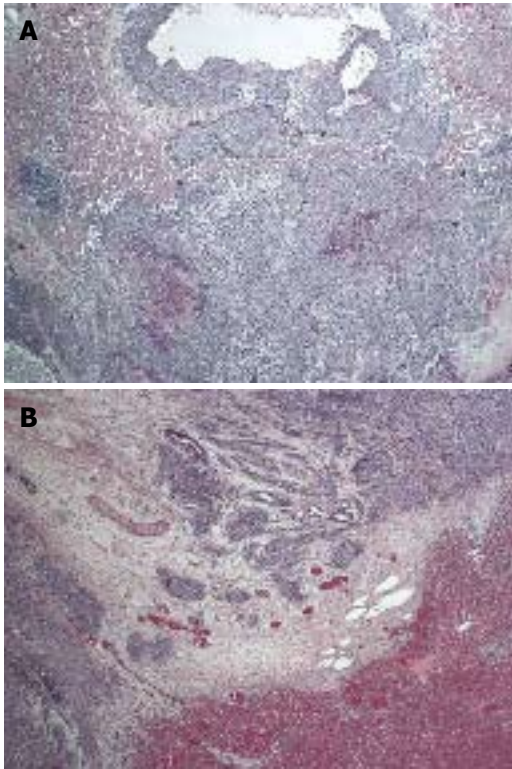


Figure 5 Pathological examination (HE stain, × 40). A: Sorafenib group: vessels were relatively small and not obvious; B: Control group: dilated vessels were shown in the parenchyma of the tumor.

difference was detected in PI. In a previous study, entire tumors were estimated as the ROI, but in the present study, part of the viable region of the tumor was estimated as the ROI to simplify the measurement procedure. The analysis software that we used could not cover the entire tumor for the ROI. That was why no significant difference was detected in PI in our study.

The fact that we used rabbits in this study, unlike many previous studies that used mice and rats, is considered to have influenced the difference. First, rabbit liver is sufficiently large to be scanned by ultrasonography and more appropriate than that of mice and rats. The liver of mice is so small that we have to perform ultrasonography under different conditions. We implanted Vx-2 tumor in rabbit liver to satisfy conditions, because intrahepatic tumor and ectopic tumor behave differently in terms of proliferation. The blood supply and the biochemical environment are different in the skin and liver. Furthermore, there are enzymes that metabolize drugs in the liver. As a result, tumor responses to drugs differ depending on its location^[10]. Second, we wanted to examine our simplified method, so nodules were implanted in the left lobe of the rabbits, where fixing the scan plane is difficult because of the movement of the heart and lungs. If a significant difference is shown under difficult conditions by a given method, then that method can be performed under most conditions and considered to be useful. As mentioned above, the TPI was significantly prolonged with both ultrasound contrast agents.

Ultrasound examination has less reproducibility and integrity than CT and MRI, because it needs more expertise and stable scanning fields. Our simplified method needs less examination time and no fixing probe or expensive location system. If the tumor has a rich parenchymal vascular network due to increased angiogenic activity, dilation of arterioles in the parenchyma can occur, and increased blood perfusion per unit volume should accelerate onset of tumor enhancement from ultrasound contrast agent. However, accurate blood flow, speed, and flux are difficult to evaluate, and TPI can show them better than other modalities can. When the administrated anti-angiogenesis drug induces a reaction in tumor vessels, the arterioles are not dilated, which results in a different enhancement pattern in CEUS in the arterial phase. TPI is considered to represent flux per unit volume of a scanning lesion. In spite of a lack of samples, the results of this study probably support this hypothesis.

In this study, we compared the dynamics of enhanced signals by a single dose of contrast agent. We did not use more accurate methods, such as the replenish curve or attenuation curve methods, because of complications^[11].

In pathological examination, we detected a reduction of tumor vessel density in some parts of the tumors. Sorafenib is an anti-angiogenesis agent that prevents formation of new vessels and affects pre-existing capillaries to cause morphological alterations. As a result, the number of tumor vessels and their area and volume are reduced^[12]. This is one of the explanations for prolongation of TPI in the sorafenib group. We could not evaluate endothelial cells because there is no appropriate antibody for CD34 immunostaining in rabbits. No significant difference was observed in VEGF immunostaining between the sorafenib and control groups, which is consistent with inhibition of VEGF signaling by sorafenib at the level of VEGF receptors.

Most HCC occurs in Eastern and Southeastern Asia, Africa and Melanesia. About 70% of patients are not eligible for curative treatment and prognosis is poor^[1]. Treatment with anti-angiogenesis drugs such as sorafenib will become widespread and effective assessment of treatment will be needed. Although efficacy of antitumor drugs has previously been evaluated mainly by variation in tumor size, it is complex and demanding to evaluate the size of tumors with necrotic lesions. Tumor markers do not correspond with efficacy of anti-angiogenesis drugs.

It is undeniable that a greater number of samples were required than we used. However, an adequate number of animals were used to allow for proper statistical analysis.

In conclusion, Analyzing the TIC of arterial phase CEUS in tumor parenchyma could be helpful for evaluation of the efficacy of anti-angiogenesis drug treatment of liver tumor.

ACKNOWLEDGMENTS

We would like to thank Dr. Longzhong Liu for his special contribution to planning and conducting the experi-

ments in this project, as well as Dr. Munire Rexiati for her management of the animals and tumor cells.

COMMENTS

Background

Although most patients with hepatocellular carcinoma are candidates for systemic therapy, conventional cytotoxic drugs yield poor therapeutic results. Recently, randomized control trials of the anti-angiogenesis drug sorafenib have shown effectiveness for the first time.

Research frontiers

Therapeutic efficacy has been assessed by time to radiological progression and overall survival in previous studies. Anti-angiogenesis treatment might produce necrosis and no tumor shrinkage, so that new imaging techniques are needed to assess antitumor effects. In this study, we verified that arterial phase contrast-enhanced ultrasonography (CEUS) of tumor parenchymal tissue is useful for evaluation for anti-angiogenesis treatment.

Innovations and breakthroughs

Enhanced computed tomography (CT) and magnetic resonance imaging (MRI) are generally used to assess treatment, but tumor perfusion is difficult to evaluate by these methods. CEUS with our simplified method can evaluate the blood flow and perfusion of tumor more easily than CT and MRI at the bedside.

Applications

This was a pilot study with an animal model. A clinical study with a large number of patients and application of sorafenib to clinical practice are awaited.

Terminology

Ultrasound contrast agents consist of microbubbles that visualize the blood flow. The time intensity curve is a graph that shows the variation in echo signal intensity with time. Peak intensity is the difference between maximal signal and baseline intensity, and correlates with blood flow volume. Time to peak intensity is the time to reach maximal intensity and correlates with blood flow volume per unit time. We can establish tumor perfusion by these parameters.

Peer review

This is an interesting paper that looks at the role of CEUS in assessing sorafenib response and the value of using only arterial phase as compared to triphasic CEUS for evaluation. The use of the rabbit model was good and the comparisons between Sonovue and SonoVue are useful. The initial tumor sizes for the treatment and control group however are too distinct, a mean 107.5 for the treatment group and 45.7 for the control group, which could have affected the validity of the observations at 14 d. Despite this, the study did demonstrate a significant reduction in vascular diameter in the sorafenib-treated group at 14 d compared to the controls.

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S- Editor Sun H L- Editor Kerr C E- Editor Ma WH

Detection of early gastric cancer using hydro-stomach CT: Blinded vs unblinded analysis

Ki Jeong Park, Min Woo Lee, Ji Hyun Koo, Yulri Park, Heejung Kim, Dongil Choi, Soon Jin Lee

Ki Jeong Park, Min Woo Lee, Ji Hyun Koo, Yulri Park, Heejung Kim, Dongil Choi, Soon Jin Lee, Department of Radiology and Center for Imaging Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Ilwon-Dong, Kangnam-Ku, Seoul 135-710, South Korea

Author contributions: Park KJ and Lee MW performed the majority of this study and wrote the manuscript; Lee MW and Choi D designed the study and performed research; Park KJ, Koo JH, Park Y, Kim H and Lee SJ were involved in the acquisition of data and in editing the manuscript.

Correspondence to: Dr. Min Woo Lee, Department of Radiology and Center for Imaging Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Ilwon-Dong, Kangnam-Ku, Seoul 135-710,

South Korea. leeminwoo0@gmail.com

Telephone: +82-2-34101380 Fax: +82-2-34100049

Received: June 16, 2010 Revised: November 26, 2010

Accepted: December 3, 2010

Published online: February 28, 2011

RESULTS: The respective sensitivities and specificities [reviewer 1: blinded, 20% (22/110) and 98% (215/220); unblinded, 27% (30/110) and 100% (219/220)/reviewer 2: blinded, 19% (21/110) and 98% (216/220); unblinded, 25% (27/110) and 98% (215/220)] were not significantly different. Although intraobserver agreements were good (weighted $\kappa = 0.677$ and 0.666), interobserver agreements were fair (blinded, 0.371) or moderate (unblinded, 0.558). For both univariate and multivariate analyses, the tumor size and invasion depth were statistically significant factors affecting visibility.

CONCLUSION: The diagnostic performance of hydro-stomach CT to detect an EGC was not significantly different between blinded and unblinded analysis. The tumor size and invasion depth were independent factors for visibility.

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Abstract

AIM: To evaluate the difference in diagnostic performance of hydro-stomach computed tomography (CT) to detect early gastric cancer (EGC) between blinded and unblinded analysis and to assess independent factors affecting visibility of cancer foci.

METHODS: Two radiologists initially blinded and then unblinded to gastroscopic and surgical-histological findings independently reviewed hydro-stomach CT images of 110 patients with single EGC. They graded the visibility of cancer foci for each of three gastric segments (upper, middle and lower thirds) using a 4-point scale (1: definitely absent, 2: probably absent, 3: probably present, and 4: definitely present). The sensitivity and specificity for detecting an EGC were calculated. Intraobserver and interobserver agreements were analyzed. The visibility of an EGC was evaluated with regard to tumor size, invasion depth, gastric segments, histological type and gross morphology using univariate and multivariate analysis.

Key words: Stomach neoplasm; Computed tomography; Water; Early detection of cancer; Sensitivity

Peer reviewer: Dr. Wei-Dong Tong, MD, MS, BS, Associate Professor, Department of General Surgery, Veterans Affairs Medical Center, Medical college of Wisconsin, Milwaukee, WI 53295, United States

Park KJ, Lee MW, Koo JH, Park Y, Kim H, Choi D, Lee SJ. Detection of early gastric cancer using hydro-stomach CT: Blinded vs unblinded analysis. *World J Gastroenterol* 2011; 17(8): 1051-1057 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1051.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1051>

INTRODUCTION

With the introduction of a screening test for stomach cancer, the incidence of early gastric cancer (EGC) has increased more than 40% in South Korea^[1,2]. Since the

prognosis of EGC after curative resection is highly favorable (5-year survival rate > 90%), early detection of stomach cancer is essential to improve prognosis^[3-5].

Due to the limited role of computed tomography (CT) for the detection of EGC, preoperative stomach CT imaging has been principally used for N and M staging^[6-8]. However, considering that an EGC lesion sometimes lacks a tactile sensation on the operative field, especially for laparoscopic surgery, the exact detection and localization of a cancer focus on preoperative stomach CT imaging is of great help to omit preoperative gastroscopic clipping for localization of a cancer focus, as well as for surgical planning. Therefore, preoperative CT imaging is important for not only N and M staging, but also for detection and localization of EGC.

Traditionally, both air and tap water have been used as oral contrast agents to achieve adequate gastric distension. As compared to the use of CT gastrography with air-distension, stomach CT with water-distension (hydro-stomach CT) is less hindered by artifacts caused by air in the lumen. Thus, detailed mucosal enhancement of cancer foci is well demonstrated on hydro-stomach CT^[9,10]. However, despite the use of multiplanar reconstruction (MPR) images, the detection rate of EGC on hydro-stomach CT is still low, ranging from 36% to 48%^[9,11,12]. Therefore, we have postulated that hydro-stomach CT is intrinsically limited for the detection of EGC. If this assumption is correct, the detection rate of EGC on an unblinded analysis will not improve as compared to that of a blinded analysis.

The purpose of this study was to (1) evaluate the difference in diagnostic performance of hydro-stomach CT to detect EGC between a blinded analysis and unblinded analysis with reference to gastroscopic and surgical-histological findings and to (2) assess factors affecting visibility of cancer foci on hydro-stomach CT imaging.

MATERIALS AND METHODS

Patients and enrollment criteria

The institutional review board approved this retrospective study and informed consent of patients was waived. Between July 2008 and August 2008, 159 consecutive patients with a pathologically proven single EGC in our institution, a tertiary care hospital, were enrolled in the study. Surgical or endoscopic submucosal dissection was performed within 1 mo after CT image acquisition. Of the 159 patients, 49 patients were excluded from the analysis for one of the following reasons: (1) CT scanning obtained at other hospital ($n = 31$); (2) post-endoscopic clipping state ($n = 10$); (3) post-endoscopic submucosal dissection state ($n = 1$); (4) no available MPR images ($n = 2$); (5) the presence of a synchronous advanced gastric cancer at another site ($n = 1$); (6) the presence of a synchronous gastric polyp at another site ($n = 1$); and (7) improper gastric distension ($n = 3$). Ultimately, 110 patients comprised the study population. The patients consisted

of 74 men and 36 women (age range, 22-86 years; mean age, 57 years).

CT acquisition

Hydro-stomach CT was performed with a 64-detector row CT scanner (LightSpeed VCT; GE Healthcare, Milwaukee, WI, USA) with the patient in the prone position on the CT table. With this protocol, non-dependent side of the stomach (i.e. the fundus for a patient in the prone position) was frequently distended by air rather than by water. In order to avoid air distension, if the cancer was located in the gastric fundus, a CT scan was performed with the patient in the supine position. Before CT scanning, each patient had fasted for over 6 h. A total of 500-1000 mL tap water was administered orally to obtain gastric distension just prior to scanning. A CT scan was obtained 70 s after the injection of a dose of 2 mg/kg of nonionic contrast material iopromide (Ultravist 300; Schering, Berlin, Germany) at a rate of 4 mL/s using an automated power injector. The scanning ranged from the diaphragm to the lower end of the symphysis pubis. The CT parameters used were as follows: 40 mm beam collimation (0.625 mm \times 64); pitch, 0.984; kVp/mA, 120/300 with automatic exposure control using both Auto mA and Smart mA; gantry rotation time, 0.6 s. Axial images (slice thickness/interval, 5 mm/5 mm) and sagittal and coronal MPR images (slice thickness/interval, 3 mm/3 mm) were obtained with the use of isotropic raw data (slice thickness/interval, 0.625 mm/0.625 mm).

Image analysis

Two gastrointestinal radiologists (D.C., 11 years experience; M.W.L., 6 years experience) evaluated the visibility of EGC by interpretation of both axial and MPR images on a 2K \times 2K picture archiving and communication system (PACS, GE Medical Systems Integrated Imaging Solutions, Mt Prospect, IL, USA) monitor (MDL9DLB020; Totoku, Tokyo, Japan), independently. At the first interpretation session, although the radiologists were aware that each patient was confirmed to have an EGC, the radiologists were blinded to the gastroscopic and surgical-histological findings. The stomach was divided into three segments along the longitudinal axis (from gastroesophageal junction to pyloric canal): upper, middle and lower thirds. For 330 gastric segments, both reviewers graded the likelihood of the presence of an EGC focus for each segment based on the use of a 4-point scale as follows: (1) definitely absent; (2) probably absent; (3) probably present; and (4) definitely present. The presence of an EGC was defined as focal plaque-like wall thickening compared to adjacent gastric wall with or without prominent enhancement of the gastric inner layer^[12]. In addition, the radiologists marked the lesion with an arrow during image analysis. A study coordinator (Park KJ) captured and stored the marked images into a JPEG file whenever one reviewer finished the interpretation of the CT images. Then the study coordinator erased the mark on the CT

Table 1 Comparison of cancer characteristics between visible and invisible early gastric cancers

Characteristic	Visible EGC (<i>n</i> = 39)	Invisible ECC (<i>n</i> = 71)	<i>P</i> value at univariate analysis	Multiple logistic regression analysis		
				<i>P</i> value	Odds ratio	95% CI
Size (cm)	3.59 ± 1.91	2.20 ± 1.37	< 0.001	0.002	1.573	1.185-2.088
Depth of invasion			0.001	0.018	2.923	1.201-7.116
Mucosa	17	54				
Submucosa	22	17				
Involved segment			0.378			
Upper 1/3	6	5				
Middle 1/3	14	28				
Lower 1/3	19	38				
Type of histology			0.862			
Tubular adenocarcinoma, well differentiated	10	15				
Tubular adenocarcinoma, moderately differentiated	14	21				
Tubular adenocarcinoma, poorly differentiated	8	17				
Poorly differentiated carcinoma	1	3				
Signet ring cell carcinoma	6	15				
Gross morphology of tumor			0.541			
I	1	2				
II a	8	7				
II b	7	17				
II c	23	44				
III	0	1				

EGC: Early gastric cancer.

images before the initiation of the next interpretation session.

Two weeks after the first blinded interpretation session, a second interpretation was performed. For the second session, the reviewers were not blinded to the clinical information of patients and were provided with all data including the gastroscopic, surgical and pathological findings.

Evaluation and statistical analysis

The two reviewers and a study coordinator assessed whether the indicated lesions on CT images were in accord or not in consensus based on the gastroscopic, surgical, and pathological findings. If at least one of the two reviewers correctly indicated an EGC lesion, it was regarded as a visible EGC. If both reviewers missed an EGC lesion, it was regarded as an invisible EGC.

The scores 1 and 2 for the likelihood of the presence of an EGC focus for each segment were regarded as an absence of an EGC focus. The scores 3 and 4 were regarded as a presence of an EGC focus. The sensitivity and specificity for the detection of an EGC focus on CT images were calculated and compared between the blinded analysis and unblinded analysis. Intraobserver and interobserver agreements were analyzed with the use of weighted κ statistics.

Reasons of visibility for the subjective assessment by the radiologists were analyzed. Visible EGCs were compared with invisible EGCs for tumor size, depth of invasion, involved gastric segments, type of histology and type of gross morphology by use of univariate analysis and multivariate analysis. The size of an EGC was based on the maximal diameter as measured on a gross specimen. The univariate association between individual

variables for visibility was tested using the χ^2 test for categorical variables (depth of invasion, involved gastric segments, type of histology and type of gross morphology). The size of a tumor between visible and invisible EGC was compared using the unpaired *t* test. In order to assess independent factors that affected the visibility of an EGC on hydro-stomach CT images, multiple logistic regression analysis was used to test the significance of adjusted factors. Variables with *P* < 0.05 determined by univariate analysis were chosen as variables for multiple logistic regression analysis. For both univariate analysis and multiple logistic regression analysis, *P* < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Hydro-stomach CT

No significant technical failure, such as a severe artifact that interfered with image interpretation of hydro-stomach CT, was noted for all patients. Thus, 330 segments (110 segments with EGC and 220 segments without EGC) were included and were analyzed.

Pathological findings

The pathological findings of 110 EGCs are summarized in Table 1. Seventy-one EGCs were confined to the mucosa and the other 39 EGCs invaded the submucosal layer. There were 18 elevated (type I and IIa), 24 flat (type II b) and 68 depressed (type II c and III) EGCs.

Diagnostic performance for EGC

The sensitivity and specificity of the blinded analysis and unblinded analysis are summarized in Table 2. Based on a consensus review, 39 EGCs were indicated by at least one

Table 2 Diagnostic performance of early gastric cancer on hydro-stomach computed tomography *n* (%)

	Reviewer 1			Reviewer 2		
	Blinded	Unblinded	<i>P</i> value	Blinded	Unblinded	<i>P</i> value
Sensitivity	22/110 (20)	30/110 (27)	> 0.05	21/110 (19)	27/110 (25)	> 0.05
Specificity	215/220 (98)	219/220 (100)	> 0.05	216/220 (98)	215/220 (98)	> 0.05

Data in parenthesis are numbers of lesions. McNemar test was used for statistical analysis.

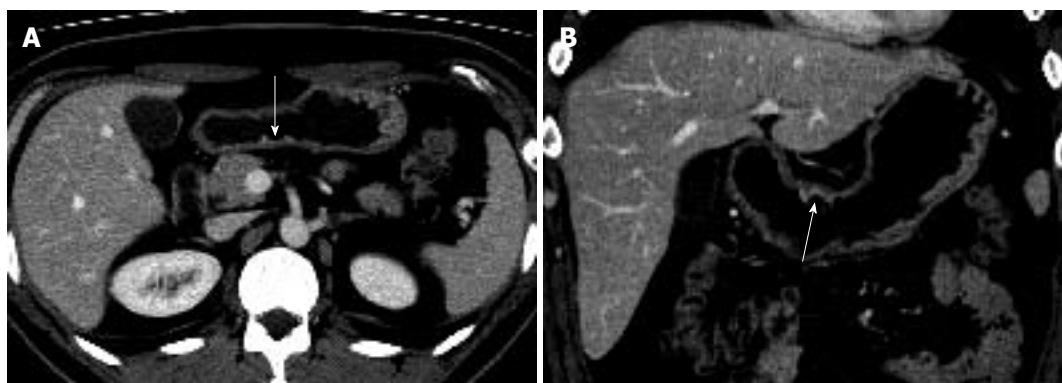


Figure 1 Early gastric cancer in a 45-year-old man. The size of tumor was 3.8 cm at the longest diameter and the tumor extended to submucosal layer. The transverse (A) and coronal multiplanar (B) images show an ulcerative, thickened wall with enhancement in posterior wall of gastric antrum (arrows). It was graded as 4 at lower 1/3 segment of stomach on both blinded and unblinded analysis by both reviewers.

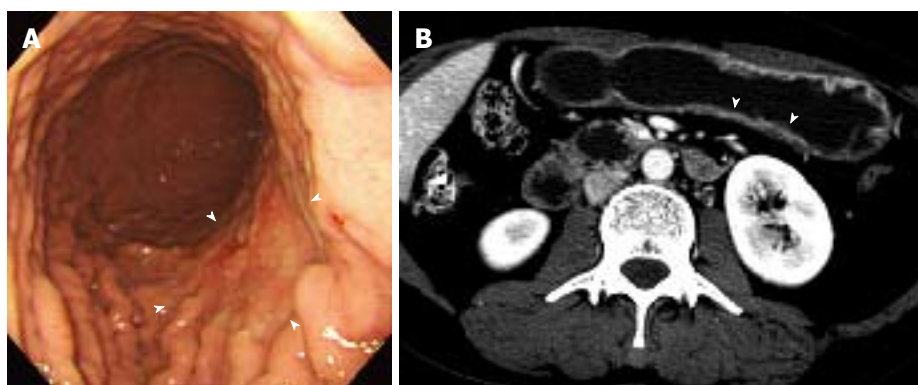


Figure 2 Early gastric cancer in a 45-year-old woman. The size of tumor was 9.5 cm at the longest diameter and the tumor extended to submucosal layer. A: The gastroscopy shows a large ill-defined lesion with abnormal convergence of gastric folds in posterior wall of gastric mid body (arrowheads); B: The transverse computed tomography scan shows equivocal enhancement and thickening (arrowheads) in posterior wall of gastric mid body. Although one reviewer indicated this lesion on both blinded and unblinded analysis, the other did not find this lesion even on unblinded analysis.

reviewer and were regarded as visible EGCs. The other 71 EGCs were regarded as invisible. There were frequent false positives at blinded analysis for both reviewers (five for reviewer 1 and four for reviewer 2). The sensitivity and specificity of both reviewers were not significantly different between the blinded analysis and unblinded analysis (Figures 1-3). Despite an unblinded analysis, the detection rates were not improved for both reviewers. For mucosal cancer ($n = 71$), the sensitivity and specificity for reviewer 1 blinded to the findings were 17% (12/71) and 98% (139/142), respectively, and were 21% (15/71) and 100% (142/142), respectively, for reviewer 1 unblinded to the findings. The sensitivity and specificity for reviewer 2 blinded to the findings were 10% (7/71) and 98%

(139/142), respectively, and were 14% (10/71) and 100% (142/142), respectively, for reviewer 2 unblinded to the findings. For both reviewers, differences were not significant (McNemar test, each $P > 0.05$). The intraobserver agreements were good for both reviewers (weighted $\kappa = 0.677$ for reviewer 1 and 0.666 for reviewer 2). However, interobserver agreements were fair (blinded, 0.371) or moderate (unblinded, 0.558).

Comparison of cancer characteristics between visible and invisible EGCs

The baseline characteristics of 110 EGCs are summarized in Table 1. For both univariate analysis and multivariate analysis, the size and depth of invasion of the tu-

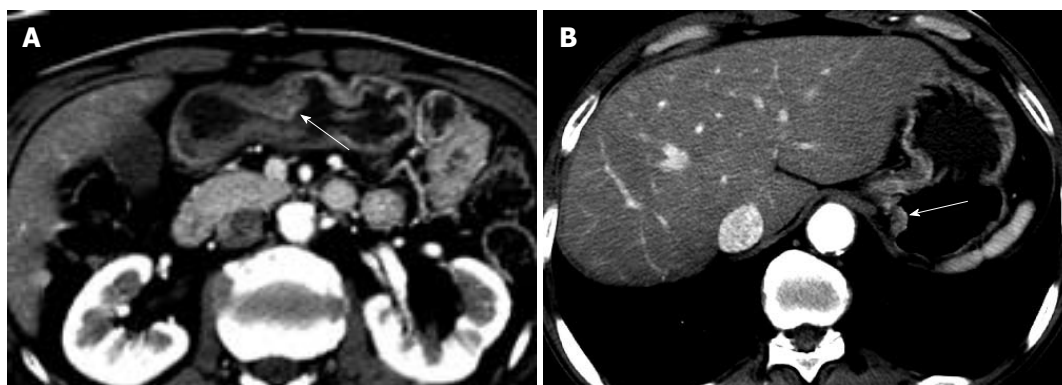


Figure 3 Early gastric cancer in a 62-year-old man. The size of tumor was 2.2 cm at the longest diameter and the tumor extended to submucosal layer. A: The transverse computed tomography scan shows thickened wall with enhancement in greater curvature of gastric antrum (arrow), which was true lesion based on gastroscopy and pathologic examination; B: Reviewer 1 indicated a lesion (arrow) as a cancer focus because thickened wall was suspicious on blinded analysis. However, on unblinded analysis, focal lesion (arrow) in greater curvature of gastric antrum in lower gastric 1/3 segment was indicated correctly.

mor were statistically significant factors affecting visibility (Table 1).

DISCUSSION

Our study demonstrated that EGCs are poorly visible on hydro-stomach CT images even though both axial and MPR (sagittal and coronal) images were evaluated. The detection rate was not significantly different between blinded and unblinded analysis for both reviewers, which indicated that EGC detection on hydro-stomach CT imaging is intrinsically limited due to poor detection performance. These findings correspond well with results of an earlier study by Yu *et al*^[13]. In that study, almost all primary gastric cancers (43/44, 98%) not visualized on preoperative hydro-stomach CT images were identified as EGCs.

In comparison, CT gastrography with air-distension has enabled the ability to obtain various three-dimensional (3D) rendering images such as virtual gastroscopy, surface shaded display, volume rendering and transparent rendering images^[8,14-17]. On CT gastrography, combined interpretation of axial and various 3D reconstructed images have shown an improved detection rate of EGC in many previous studies^[8,15-17]. The detection rate of EGC by the use of CT gastrography has been reported to range from 73% to 96%^[8,15-17], which seems to be superior to that of hydro-stomach CT (36% to 48%)^[9,11,12]. Using both two-dimensional (2D) and various 3D rendering images of CT gastrography, mucosal nodularity, a malignant fold change and the presence of a depressed or elevated lesion could be demonstrated^[15,16]. Furthermore, virtual gastroscopy can be used as a complimentary study to conventional gastroscopy, as the modality provides endoluminal images with a wider field of view, resulting in no blind spots unlike those which occur with the use of conventional gastroscopy^[16]. However, as compared to conventional gastroscopy, virtual gastroscopy is limited in the detection of superficial flat lesions, as the method cannot provide discoloration of a flat EGC lesion.

Although hydro-stomach CT has been known to provide improved visualization of the gastric wall and gastric tumors without image degradation by artifacts derived from intraluminal air^[6,18], only 2D-based images (axial and MPR images) are used for the interpretation of preoperative evaluation of stomach cancer with the use of hydro-stomach CT. Although the addition of MPR images to conventional axial images has offered improved detection and localization of EGC^[11], detection of EGC in the absence of thickened wall and enhancement is still difficult with the use of 2D images only. In addition, partial volume averaging artifacts can be problematic for the evaluation of the gastric wall, especially in a case located on the antrum or angle where the gastric wall is tangent to an axial scan^[11]. Nevertheless, hydro-stomach CT has been widely utilized for the preoperative staging work-up of stomach cancer due to increased workload of radiologists and time-consuming postprocessing of CT gastrography.

In our study, the detection of EGC was highly related to the depth of invasion. Mucosal cancer is less visible as compared to submucosal cancer, a finding similar to the results of previous studies^[11,12]. As compared to the EGC detection rate (48%, 34/71) determined by Kim *et al*^[9], although the exact proportion of mucosal and submucosal cancer in that study was not known, the detection rate of EGC based on the blinded analysis (20%, 22/110, reviewer 1; 19%, 21/110, reviewer 2) in our study was inferior. This finding could be explained by the fact that the proportion of mucosal cancer that is known as difficult to detect on hydro-stomach CT was as high as 65% (71/110) in our study. The sensitivity for mucosal cancer as determined by the two reviewers was only 17% and 10% in our study. These results are in close agreement with sensitivity values of 17% (3/18) reported by Shimizu *et al*^[11] and 16% (11/69) reported by Woo *et al*^[12].

In addition to the depth of invasion, the size of a tumor was another cause of invisibility in our study (Table 1). The size (3.59 ± 1.91 cm) of a visible EGC was larger as compared to an invisible EGC (2.20 ± 1.37 cm). Howev-

er, this result is in disagreement with that (2.80 ± 1.35 cm *vs* 2.18 ± 1.85 cm) reported in a study by Woo *et al*^[12] in which the size was not significantly different between invisible and visible EGCs. This discrepancy between two studies could have been influenced by differences in the study population.

Gross morphology was not a factor that affected EGC visibility in our study. If lesions were grouped into two categories (depressed *vs* non-depressed lesions), visibility was not significantly different between the two categories (Table 1), which coincides well with a previous study^[12]. We believe that the perception of a shallow depressed lesion on 2D images is more difficult as compared to various 3D reconstructed images provided by CT gastrography, as the 3D images intuitively show malignant fold changes, mucosal nodularity and elevated or depressed lesions^[15,16].

This study has some limitations. Firstly, this study was a retrospective, single institution study over a defined period. The ability to detect an EGC lesion on a CT scan may be different, depending on factors such as the experience of the radiologists, CT protocol, patient population and tumor characteristics. In addition, we did not perform CT gastrography with air-distension. Therefore, although we compared our results with findings of previous investigations that used CT gastrography, a direct comparison is limited due to the different patient populations, type of CT equipment and experience of the radiologists. Thus, a further comparison study may be warranted between hydro-stomach CT and CT gastrography to detect EGC lesions. Secondly, we did not evaluate the visibility of EGC according to gastric distension. As a collapsed stomach could obscure a gastric lesion or simulate the pathology, detection of EGC could be influenced by gastric distension^[14]. In addition, as with the transverse colon as visualized on CT colonography^[19], the lower one-third segment of the stomach is more likely to be compressed than the upper one-third segment for a patient in the prone position. However, as oral administration of more than 500 mL of water has been widely used for optimal gastric distension on hydro-stomach CT^[9,12,13], gastric distension was not taken into consideration at the time of analysis. Thirdly, some of the EGC lesions marked by the reviewers were discordant between each other. In addition, there were frequent false positives at blinded analysis by both reviewers. Therefore, visible EGC lesions in our study have a possibility of false positives. However, this problem seems to be intrinsically inevitable for this style of study, since a fully reliable method was not available to confirm the location of EGC on CT images. Fourthly, since patient position at CT acquisition partially gives the information about the location of an EGC focus, this might have influenced the blinded image analysis of reviewers.

In conclusion, hydro-stomach CT imaging was not a reliable tool for the detection of EGC. The poor diagnostic performance of hydro-stomach CT to detect EGC was not significantly different between blinded and

unblinded analysis. The size and depth of invasion of an EGC were two independent factors for visibility.

COMMENTS

Background

The incidence of early gastric cancer (EGC) is currently rising faster than previously, correlating with the introduction of a screening test for stomach cancer. Considering the poor tactile sensation of an EGC lesion on the operative field, especially for laparoscopic surgery, the exact localization of a cancer focus on preoperative stomach CT imaging is important to omit preoperative gastroscopic clipping for localization of a cancer focus as well as for surgical planning.

Research frontiers

Despite introduction of multi-detector row CT techniques and the use of multiplanar reconstruction (MPR) images, the detection rate of EGC on hydro-stomach CT has still been unsatisfactory. In order to see whether the detection rate of EGC on unblinded analysis can be improved as compared to that of blinded analysis with reference to gastroscopic and surgical-histological findings, diagnostic performance of hydro-stomach CT to detect EGC was compared between blinded analysis and unblinded analysis. In this study, the authors demonstrate that the diagnostic performance of hydro-stomach CT to detect EGC was poor and was not significantly different between blinded and unblinded analysis.

Innovations and breakthroughs

Traditionally, both air and tap water have been used as oral contrast agents to achieve adequate gastric distension for preoperative CT imaging in patients with EGC. This study demonstrates that diagnostic performance of hydro-stomach CT for the detection of EGC is poor and unenhanced even when unblinded analysis is performed. The tumor size and invasion depth were independent factors for visibility of EGC on hydro-stomach CT.

Applications

Hydro-stomach CT imaging seems not to be a reliable tool for the detection of EGC. In this context, for the preoperative evaluation of patients with EGCs, it is time to consider CT gastrography which can offer not only 2D images but also various 3D reconstructed images showing malignant fold changes, mucosal nodularities and elevated or depressed lesions.

Terminology

Hydro-stomach CT refers to stomach CT with water-distension. Compared with CT gastrography with air-distension, hydro-stomach CT has been believed to be less affected by artifacts caused by intraluminal air and is likely to demonstrate detailed mucosal enhancement of cancer foci.

Peer review

This study was designed to evaluate the difference of diagnostic performance of hydro-stomach CT to detect EGC between blinded and unblinded analysis. The English is written well.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

Association between acute pancreatitis and peptic ulcer disease

Kang-Moon Lee, Chang-Nyol Paik, Woo Chul Chung, Jin Mo Yang

Kang-Moon Lee, Chang-Nyol Paik, Woo Chul Chung, Jin Mo Yang, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Gyeonggi-do 442-723, South Korea

Author contributions: Lee KM and Paik CN contributed to the study design, data interpretation, and wrote the manuscript; Chung WC and Yang JM contributed to the study design and critically reviewed the manuscript.

Correspondence to: Chang-Nyol Paik, MD, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, St. Vincent's Hospital, Ji-dong, Paldal-gu, Suwon-si, Gyeonggi-do 442-723, South Korea. cmcu@catholic.ac.kr
 Telephone: +82-31-2497138 Fax: +82-31-2538898

Received: August 19, 2010 Revised: September 26, 2010

Accepted: October 3, 2010

Published online: February 28, 2011

Abstract

AIM: To evaluate the relationship between peptic ulcer disease (PUD) and acute pancreatitis.

METHODS: A cohort of 78 patients with acute pancreatitis were included in this study. The presence of PUD and the *Helicobacter pylori* (*H. pylori*) status were assessed by an endoscopic method. The severity of acute pancreatitis was assessed using Ranson's score, the Acute Physiology and Chronic Health Evaluation (APACHE) II score, computed tomography severity index and the clinical data during hospitalization, all of which were compared between the patients with and without PUD. The risk factors for PUD were also evaluated.

RESULTS: Among 78 patients, 41 patients (52.6%) with acute pancreatitis suffered from PUD, but only 13 (31.7%) patients with PUD were infected by *H. pylori*. On univariate analysis, male gender, an etiology of alcohol-induced pancreatitis, a history of smoking or alcohol consumption, elevated triglyceride and C-reactive protein levels, and high APACHE II score were significantly associated with PUD. However, on multivariate logistic regression

analysis, the APACHE II score (odds ratio: 7.69; 95% confidence interval: 1.78-33.33; $P < 0.01$) was found to be the only independent risk factor for PUD.

CONCLUSION: Patients with acute pancreatitis are liable to suffer from PUD. PUD is associated with severe acute pancreatitis according to the APACHE II score, and treatment for PUD should be considered for patients with severe acute pancreatitis.

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Key words: Acute pancreatitis; Peptic ulcer disease; *Helicobacter pylori*; Acute Physiology and Chronic Health Evaluation II score

Peer reviewer: Shoichiro Sumi, MD, PhD, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan

Lee KM, Paik CN, Chung WC, Yang JM. Association between acute pancreatitis and peptic ulcer disease. *World J Gastroenterol* 2011; 17(8): 1058-1062 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1058.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1058>

INTRODUCTION

Acute pancreatitis is a common disease that has shown an increased incidence in the past two decades^[1-3]. Acute pancreatitis is caused by an acute inflammatory response resulting from unregulated activation of pancreatic enzymes, which can lead to extrapancreatic complications due to the persistence of hypovolemia, a decreased intravascular volume and multiorgan dysfunction. In fact, patients with acute pancreatitis may complain of various abdominal symptoms such as nausea, vomiting and pain, and these symptoms are sometimes confused with dyspeptic symptoms^[4,5]. In a recent study, 65% of patients with acute

pancreatitis were found to have acute gastrointestinal mucosal lesions^[6]. We hypothesized that the decrease in intravascular volume, and the stress response that diminishes the blood flow could result in upper gastrointestinal ischemia or inflammation, and cause peptic ulcer disease (PUD). The aims of this study were to evaluate the prevalence of PUD among patients with acute pancreatitis, and to compare the clinical characteristics and severity of acute pancreatitis according to the presence of PUD.

MATERIALS AND METHODS

The study was conducted at St. Vincent's Hospital, a teaching hospital of the Catholic University of Korea. The medical records, charts and the digitalized picture archived images of consecutive patients who were admitted with acute pancreatitis between February 2008 and August 2009 were collected. The study was approved by the Institutional Review Board of the Catholic University of Korea. The patients included in this study were all older than 17 years of age, had visited our clinic within 2 d of the occurrence of abdominal symptoms, and underwent endoscopic gastroduodenoscopy during hospitalization. The patients with acute pancreatitis due to endoscopic retrograde cholangiopancreatography were excluded. The diagnosis of acute pancreatitis was based on the presence of two of the following three features^[7]: (1) acute onset of typical abdominal pain; (2) serum amylase and/or lipase level ≥ 3 times the upper limit of normal; and (3) characteristic findings of acute pancreatitis on an abdominal computed tomography (CT) scan or on ultrasonography. Gallstone pancreatitis was diagnosed by CT or ultrasonography in the absence of another etiology such as excessive alcohol consumption^[6,9]. Hyperlipidemia was diagnosed in cases with a serum triglyceride level above 500 mg/mL^[6]. The exclusion criteria were previous abdominal surgery, a previous history of acute or chronic pancreatitis, a diagnosis of PUD in the previous 3 mo, a history of taking drugs such as non-steroidal antiinflammatory drugs, aspirin, anticoagulants and/or antiplatelet drugs in the previous month, and incomplete medical records. Our institute routinely recommended endoscopy to confirm and treat acute mucosal lesions in the stomach or duodenum in patients with acute pancreatitis before the patient was permitted any oral intake, and determination of the severity score of acute pancreatitis for evaluating and managing the patients. An ulcer was defined as a lesion with loss of mucosal integrity (a whitish exudate was observed) and the lesion was > 5 mm in size with apparent depth determined by endoscopy^[10]. The status of *Helicobacter pylori* (*H. pylori*) was evaluated in patients with PUD, who underwent antral or body biopsy for histopathology or a rapid urease test (CLO test). The prevalence of PUD and *H. pylori* in the patients with acute pancreatitis were evaluated. The severity of acute pancreatitis were evaluated by laboratory data and scores such as Ranson's score, the Acute Physiology and Chronic Health Evaluation (APACHE) II score and the CT severity index (CTSI) during hospitalization. The clinical characteristics including demographic, labora-

tory, or radiologic data and the severity of acute pancreatitis in the patients with and without PUD were compared.

Statistical analysis

The primary end points of the study were the prevalence of PUD associated with acute pancreatitis. The secondary end points were the risk factors for PUD in the patients with acute pancreatitis. The continuous data were expressed as mean \pm SE (standard error of the mean) determined using the independent sample Student *t*-test, while categorical variables were expressed as quantities and were analyzed using the χ^2 test. Multiple stepwise logistic regression analysis was used to identify the risk factors for PUD. The analyses were performed with a statistical software package (SPSS, version 15.0; SPSS Inc). A *P*-value < 0.05 was considered significant for all tests. This research adhered to the principles of the Declaration of Helsinki.

RESULTS

During the study period, a total of 123 consecutive patients with acute pancreatitis and who were not related were enrolled. Patients who did not undergo endoscopic gastroduodenoscopy during hospitalization were excluded ($n = 30$). Of the remaining 93 patients, 15 were excluded due to a history of acute or chronic pancreatitis ($n = 10$), a diagnosis of PUD in the previous 3 mo ($n = 1$), a history of taking drugs such as aspirin in the previous month ($n = 3$), and incomplete medical records ($n = 1$).

A total of 78 patients were finally enrolled and included in the analysis. The mean age of the patients was 53.4 ± 1.8 years (range, 18-86 years) and 52 patients (66.7%) were male. The mean time to the endoscopic procedure after admission was 3.3 ± 0.3 d (range, 1-16 days). Forty one patients (52.6%) were found to have PUD. The characteristics of the patients with or without PUD are shown in Table 1. On univariate analysis, male gender ($P = 0.03$), an etiology of pancreatitis due to alcohol ($P = 0.03$), a history of smoking ($P = 0.05$), a history of excessive alcohol consumption ($P = 0.01$), an elevated triglyceride level ($P = 0.05$), an elevated C-reactive protein level ($P < 0.001$) and an elevated APACHE II score ($P = 0.001$), in particular an APACHE II score ≥ 6 ($P < 0.001$), were found to be significantly associated with PUD in patients with acute pancreatitis (Table 1). The receiver-operating characteristic (ROC) curve with a cutoff of an APACHE II score of 6 was selected as the highest sensitivity and specificity values for evaluating the factors for PUD [area under the curve (AUC), 0.75; 95% confidence interval (95% CI): 0.67-0.86] (Figure 1). On multivariate logistic regression analysis, only an APACHE II score ≥ 6 was found to be a significant risk factor (Table 2).

Among the 41 patients with PUD and acute pancreatitis, *H. pylori* was detected in 13 patients (31.7%). Any demographic differences were not found between the *H. pylori*-positive and -negative groups, except for the location of the ulcer. Among the 13 patients in the *H. pylori*-positive ulcer group, 10 (76.9%) patients revealed only a gastric ulcer, and a duodenal ulcer was not found. Howev-

Table 1 Characteristics of the patients with or without peptic ulcer disease (mean \pm SE) *n* (%)

	Ulcer (<i>n</i> = 41)	No ulcer (<i>n</i> = 37)	<i>P</i> value
Age (yrs)	56.6 \pm 2.3	49.8 \pm 2.9	0.07
Gender			
Male	32 (78.0)	20 (54.1)	0.03
Female	9 (22.0)	17 (45.9)	
BMI (kg/m ²)	23.9 \pm 0.5	23.3 \pm 0.8	0.48
Diabetes			
Yes	7 (17.7)	3 (8.1)	0.31
No	34 (82.9)	34 (91.9)	
Hypertension			
Yes	9 (22.0)	6 (16.2)	0.52
No	32 (78.0)	31 (83.8)	
Etiology			
Alcohol	22 (53.7)	9 (24.3)	0.03
Biliary stone	7 (17.1)	9 (24.3)	
Idiopathic	6 (14.6)	14 (37.8)	
Others	6 (14.6)	5 (13.5)	
Smoke			
Yes	20 (48.8)	10 (27.0)	0.05
No	21 (51.2)	27 (73.0)	
Alcohol			
Yes	29 (70.7)	16 (43.2)	0.01
No	12 (29.3)	21 (56.8)	
Laboratory			
BUN (mg/dL)	17.2 \pm 1.6	17.9 \pm 1.7	0.78
Cr (mg/dL)	1.1 \pm 0.1	1.0 \pm 0.1	0.34
Amylase (IU/L)	633.9 \pm 123.0	564.3 \pm 77.9	0.64
Lipase (IU/L)	405.1 \pm 75.2	518.5 \pm 82.6	0.31
Triglyceride (mg/dL)	261.5 \pm 71.5	111.0 \pm 15.6	0.05
ESR (mm/h)	46.6 \pm 5.5	36.3 \pm 4.6	0.16
CRP (mg/dL)	15.5 \pm 2.3	5.2 \pm 0.9	< 0.001
CTSI	2.1 \pm 0.3	1.6 \pm 0.2	0.21
Ranson score			
On admission	1.3 \pm 0.2	1.2 \pm 0.2	0.76
At 48 h	0.8 \pm 0.2	0.4 \pm 0.1	0.07
APACHE II score			
< 6	15 (36.6)	29 (78.4)	0.001
\geq 6	26 (63.4)	8 (21.6)	< 0.001
Time to endoscopy (d)	3.5 \pm 0.5	3.0 \pm 0.4	0.45
Death	0 (0)	0 (0)	

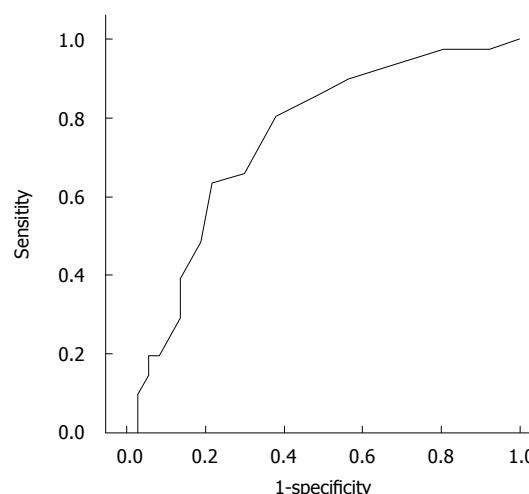
BMI: Body mass index; BUN: Blood urea nitrogen; Cr: creatinine; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; CTSI: Computed tomography severity index; APACHE: Acute Physiology and Chronic Health Evaluation.

er, for the 28 patients in the *H. pylori*-negative ulcer group, gastric ulcers were found in 12 patients (42.9%), duodenal ulcers in 12 patients (42.9%), and both gastric and duodenal ulcers in 4 patients (14.3%). The location of the ulcers was different according to the status of *H. pylori* (Table 3).

DISCUSSION

The current study showed that the prevalence of PUD in patients with acute pancreatitis was relatively high (52.6%), and the cause of may be related to the stressful condition of the underlying pancreatitis.

Although the previous literature has reported that the use of histamine 2 (H₂) receptor antagonists or proton pump inhibitors (PPI) could prevent stress ulcers in cases of severe pancreatitis^[7,11], the published clinical evidence is still scanty and controversial. Only one clinical study

**Figure 1** Receiver operating characteristic curve for Acute Physiology and Chronic Health Evaluation II score in predicting the severity of acute pancreatitis (receiver operating characteristic area under the curve, 0.75; 95% confidence interval: 0.64-0.86).**Table 2** Risk factors for peptic ulcer in patients with acute pancreatitis (multivariate analysis)

Variables	OR	95% CI	<i>P</i> value
Alcohol-induced	3.23	0.77-14.29	0.11
Smoking	2.86	0.65-12.5	0.16
APACHE II score \geq 6	7.69	1.78-33.33	< 0.01

OR: Odds ratio; CI: Confidence interval; APACHE: Acute Physiology and Chronic Health Evaluation.

Table 3 Infection rate of *Helicobacter pylori* in the patients with peptic ulcer disease (mean \pm SE) *n* (%)

	<i>H. pylori</i> -positive	<i>H. pylori</i> -negative	<i>P</i> value
<i>n</i> (%)	13 (31.7)	28 (68.3)	
Age (yrs)	61.2 \pm 4.5	54.5 \pm 2.5	0.17
Gender			
Male	10 (76.9)	22 (78.8)	0.91
Female	3 (23.1)	6 (21.4)	
BMI (kg/m ²)	24.1 \pm 1.1	23.8 \pm 0.6	0.76
Smoke			
Yes	7 (53.8)	14 (50.0)	0.82
No	6 (46.2)	14 (50.0)	
Alcohol			
Yes	8 (61.5)	21 (75.0)	0.38
No	5 (38.5)	7 (25.0)	
Location of ulcer			
GU only	10 (76.9)	12 (42.9)	0.02
DU only	0	12 (42.9)	
GU and DU	3 (23.1)	4 (14.3)	

H. pylori: *Helicobacter pylori*; BMI: Body mass index; GU: Gastric ulcer; DU: Duodenal ulcer.

reported that acute gastrointestinal lesions occurred in 65% of patients with acute pancreatitis^[6], and this rate was higher than that of our study (52.6%). Our strict selection of enrolled patients with only PUD, as demonstrated by endoscopy with definitive criteria, was the reason

why a relatively low rate was demonstrated in our study. Because the prevalence of PUD in the general population is known to be about 5% according to recent data^[12], the 52.6% prevalence of our data was high, and so PUD seems to be associated with acute pancreatitis.

The pathogenesis of PUD associated with acute pancreatitis is still not understood. However, gastric mucosal ischemia under the stressful condition of acute pancreatitis might be a major factor for peptic ulcer occurring together with acute pancreatitis^[6]. Acute pancreatitis may be complicated by the hypovolemic status of the pancreas or extrapancreatic ischemia due to the diminished effective blood volume or hypoperfusion^[4,7,11,13], which have been reported to be causative factors for stress ulcers^[14]. Another suggestion is that acidic conditions in the intestine develop because of reduced bicarbonate secretion by the pancreas, resulting in patients with pancreatitis becoming susceptible to a duodenal ulcer^[7]. Furthermore, intestinal ischemia increases intestinal permeability to bacteria, bacterial products and/or endotoxins, permitting a secondary pancreatic infection, and also stimulates cytokine release, and increases the level of nitric oxide, which serially contributes to ongoing pancreatic injury as well as organ failure^[15-17]. In our study, an association between PUD and acute pancreatitis was observed, and the use of antiulcer medication may have an impact on the treatment or prognosis of patients with acute pancreatitis.

PUD disease is a multifactorial disease that has been largely attributed to the presence of *H. pylori* infection^[18-20], and the presence of *H. pylori* infection in patients with PUD has been reported to range between 61% and 94%^[21-23]. However, in our data the prevalence of *H. pylori* infection was only 31.7%. The distinct difference in location of ulcers between *H. pylori*-positive and -negative groups was interesting. Why a low prevalence of *H. pylori* infection was revealed in patients with a duodenal ulcer was unclear. The suggested hypothesis is that inflammation of the pancreas affects sites nearer to the duodenum than the stomach. Because our study excluded patients with a recent drug history that could cause PUD, our study at least demonstrated that the main cause of PUD might be associated with acute pancreatitis.

An ulcer was clearly defined and selected to exclude other mucosal lesions such as erythema, erosion and edema that were seen on endoscopy. The reason is that by excluding subjective ambiguous mucosal lesions and including the clinically significant meaningful lesion, the prevalence or characteristics of the ulcer could be evaluated. Another reason is that in our country, patients who have PUD revealed on endoscopy can receive the medical benefits of health insurance for treatment with drugs such as PPI. The patients were not placed on ulcer prophylaxis at admission with acute pancreatitis because prescription of anti-ulcer medications such as PPI or H₂ blockers under health insurance need documentation of an ulcer by endoscopy.

The APACHE II score was the only independent risk factor associated with PUD in our study. Many prognostic factors for acute pancreatitis have been previously sug-

gested, such as laboratory markers, radiologic views and scoring systems. A previous study reported that the occurrence of an acute gastric mucosal lesion was not related to the severity of acute pancreatitis^[6]. However, that study lacked a proven method, such as the APACHE scoring system, for evaluating the severity of acute pancreatitis. The APACHE II score reflects the systemic or physiologic response to inflammation-driven stress during the course of acute pancreatitis^[24], and it was reported to be superior to the Balthazar CTSI for predicting organ failure^[25-27]. On the basis of the highest sensitivity and specificity values that were generated from the ROC curves, the cutoff of an APACHE II score of 6 was selected for evaluating the factors for PUD (AUC, 0.75; 95% CI: 0.64-0.86). The score of 6 was somewhat low to reflect the severity in patients with acute pancreatitis^[10,15], and may be due to the exclusion of critically ill patients.

The potential limitations of our study were the small number of patients ultimately enrolled, and the retrospective design. However, the subjects of this study were limited to patients with acute pancreatitis and who underwent endoscopy under strict criteria that considered the patient's history of medication or surgery. Our clinic has routinely recommended that hospitalized patients are evaluated, and the severity of acute pancreatitis predicted, by using scoring systems for acute pancreatitis such as Ranson's score, the APACHE II score and the CTSI. Another limitation of this study was the exclusion of critically ill patients who did not undergo endoscopy, which affected the exact prevalence of PUD, but this would cause underestimation of the prevalence of PUD because some of these patients had a possibility of having PUD combined with severe acute pancreatitis.

In conclusion, patients with acute pancreatitis have a strong possibility of suffering from PUD. In particular, if patients are diagnosed with severe acute pancreatitis, based on the APACHE II scoring system, then treatment for PUD is strongly recommended.

ACKNOWLEDGMENTS

The authors thank Mrs Yu-Jung Kim for help with the literature and statistical research.

COMMENTS

Background

Acute pancreatitis is caused by an acute inflammatory response due to unregulated activation of pancreatic enzymes, which can lead to extrapancreatic complications because of the persistence of hypovolemia, a decreased intravascular volume and multiorgan dysfunction. The possibility of inflammation due to ischemia in the upper gastrointestinal tract may exist. In fact, patients with acute pancreatitis may complain of various abdominal symptoms such as nausea, vomiting and pain, and these symptoms can sometimes be confused with dyspeptic symptoms; these patients may have complications of acute gastrointestinal mucosal lesions.

Research frontiers

The authors hypothesized that the reduction in intravascular volume and the stress response that diminishes the blood flow will affect the upper gastrointestinal lesion and cause peptic ulcer diseases (PUD). In analysis of PUD, we also tried to identify the status of *Helicobacter pylori* (*H. pylori*) infection through endoscopy.

Innovations and breakthroughs

Patients with acute pancreatitis are liable to suffer from PUD, and PUD is related to severe acute pancreatitis according to the Acute Physiology and Chronic Health Evaluation (APACHE) II score with a cutoff value of 6. Among patients with acute pancreatitis, a low prevalence of *H. pylori* infection was revealed in patients with PUD, especially in patients with a duodenal ulcer.

Applications

In the authors' study, an association between PUD and acute pancreatitis was observed and the use of antiulcer medication may be recommended for patients with acute pancreatitis to relieve symptoms of the suspected ulcer, especially in severe acute pancreatitis. By identifying a definite relationship between acute pancreatitis and PUD, this study provides a challenge to clarify the basic mechanisms of these two diseases. In addition, a large prospective study to confirm these observations is required.

Peer review

This study retrospectively reviewed the clinical records of the patients of acute pancreatitis (AP) focusing on the relationship between AP and gastroduodenal PUD and found a positive relationship between APACHE II score and PUD.

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S- Editor Sun H L- Editor Cant MR E- Editor Lin YP

Antioxidative status of patients with alcoholic liver disease in southeastern Taiwan

Ya-Ling Chen, Li-Ju Chen, Ming-Jong Bair, Mei-Lan Yao, Hsiang-Chi Peng, Sien-Sing Yang, Suh-Ching Yang

Ya-Ling Chen, Suh-Ching Yang, School of Nutrition and Health Sciences, College of Public Health and Nutrition, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, China

Hsiang-Chi Peng, School of Pharmacy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, China

Li-Ju Chen, Department of Nutrition, Mackay Memorial Hospital (Taitung Branch), Chang-Sha street, Taitung 95054, Taiwan, China

Ming-Jong Bair, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital (Taitung Branch), Chang-Sha street, Taitung 95054, Taiwan, China

Mei-Lan Yao, Department of Laboratory, Mackay Memorial Hospital (Taitung Branch), Chang-Sha street, Taitung 95054, Taiwan, China

Sien-Sing Yang, Liver Unit, Cathay General Hospital, 280, Jen-Ai Road, Sec. 4, Taipei 106, Taiwan, China

Author contributions: Chen YL and Chen LJ contributed equally to this work; Chen YL, Chen LJ, Peng HC and Yao ML performed research and analyzed data; Bair MJ and Yang SS performed the diagnosis of alcoholic liver disease; Yang SC designed the research; Yang SS and Yang SC wrote the paper.

Correspondence to: Dr. Suh-Ching Yang, School of Nutrition and Health Sciences, Taipei Medical University, Taipei 110, Taiwan, China. sokei@tmu.edu.tw

Telephone: +886-2-27361661 Fax: +886-2-27373112

Received: July 23, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: February 28, 2011

served as the control group in this study. Venous blood (10 mL) of each subject was drawn into EDTA-containing tubes after 8 h overnight fasting.

RESULTS: Compared to the control group, patients with ALD showed significantly lower erythrocytic catalase (11.1 ± 0.7 U/mg Hb vs 8.0 ± 0.7 U/mg Hb, $P < 0.05$) and superoxide dismutase (9.5 ± 1.6 U/mg Hb vs 3.0 ± 0.2 U/mg Hb, $P < 0.05$) activities. Furthermore, the erythrocytic reduced glutathione/oxidized glutathione ratio was significantly lower in ALD patients than that in the control group (38.1 ± 5.4 vs 15.7 ± 1.9 , $P < 0.05$). The results revealed that patients with ALD experienced more oxidative stress than those in the control group. The non-aboriginal, but not the aboriginal, ALD group had higher erythrocytic glutathione peroxidase (GPX) activity than that in the control group (46.1 ± 7.8 U/g Hb vs 27.9 ± 2.2 U/g Hb, $P < 0.05$). Hepatitis, but not cirrhosis, ALD patients had higher erythrocytic GPX activity than that in the control group (44.3 ± 8.6 U/g Hb vs 27.9 ± 2.2 U/g Hb, $P < 0.05$).

CONCLUSION: Our results indicate that both ethnicity and the severity of ALD may cause different erythrocytic antioxidative enzyme activities especially GPX activity.

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Key words: Alcoholic liver disease; Antioxidative status; Aborigines; Hepatitis; Cirrhosis

Peer reviewer: Takuji Tanaka, MD, PhD, FIAC, Director of The Tohkai, Cytopathology Institute, Cancer Research and Prevention (TCI-CaRP)5-1-2 Minami-Uzura, Gifu 500-8285, Japan

Chen YL, Chen LJ, Bair MJ, Yao ML, Peng HC, Yang SS, Yang SC. Antioxidative status of patients with alcoholic liver disease in southeastern Taiwan. *World J Gastroenterol* 2011; 17(8): 1063-1070 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1063.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1063>

Abstract

AIM: To investigate the antioxidative status of patients with alcoholic liver disease (ALD) in southeastern Taiwan.

METHODS: Our study comprised 27 patients with ALD recruited from Taitung Mackay Memorial Hospital, located in southeastern Taiwan. Patients with ALD included 12 non-aborigines (12 men) and 15 aborigines (11 men and 4 women). According to the severity of ALD, patients with ALD included 10 with hepatitis (9 men and 1 woman) and 17 with cirrhosis (14 men and 3 women). Twenty-two age- and gender-matched healthy adults

INTRODUCTION

Alcohol is one of the major causes of liver disease worldwide. In Taiwan, the prevalence of alcohol dependence has increased 80-fold compared to three decades ago^[1,2]. Except for alcoholic liver disease (ALD), most liver diseases and cirrhosis are due to chronic infection with hepatitis viruses in Taiwan^[3]. Rates of chronic hepatitis B virus and hepatitis C viral infections can reach as high as 15%-20% and 1%-5% respectively^[4]. Evidence shows that chronic hepatitis C viral hepatitis can increase liver damage in alcoholic patients with liver disease^[3], which indicates that a chronic hepatitis virus infection may aggravate the severity of ALD.

It was found that Taiwanese aborigines, who are belong to the Malayo-Polynesia group, are more susceptible to alcohol abuse than ethnic Han Chinese^[5]. In the 1990s a higher prevalence of alcoholism in aborigines was reported (44.2%-55.2%)^[6]. Viral infections and alcohol consumption play important roles in the development of chronic liver diseases in Taiwanese aborigines^[7]. Therefore, ALD is an important issue for aborigines in Taiwan.

Alcohol is mainly metabolized in the liver. Hepatocytes metabolize alcohol in three ways: (1) alcohol dehydrogenase (ADH) which produces acetaldehyde from alcohol in the cytosol; (2) the microsomal ethanol-oxidizing system (MEOS) which catalyzes alcohol to acetaldehyde in the endoplasmic reticulum is highly induced by chronic alcohol consumption; and (3) aldehyde dehydrogenase which catalyzes acetaldehyde to acetate in mitochondria. The ADH pathway reduces nicotinamide adenine dinucleotide (NAD) to its reduced form (NADH) and an imbalance in the NAD/NADH ratio causes a number of metabolic disorders, including inhibition of the Krebs cycle and fatty acid oxidation in ALD^[8-10].

It is well-established that oxidative stress is one of the pathogenic mechanisms of ALD, and oxidative stress is mainly caused by the generation of reactive oxygen species (ROS). ROS associated with alcohol toxicity are generated by the mitochondrial respiratory chain, by ethanol-metabolizing cytochrome P4502E1 (CYP2E1) which is involved in the MEOS of hepatocytes and by NADPH oxidase of Kupffer's cells and liver-infiltrating granulocytes^[11]. Besides hydroxyethyl radicals produced during ethanol oxidation by CYP2E1, nitric oxide (NO) produced by Kupffer's cell NO synthetase and alterations in hepatic iron homeostasis may further result in oxidative damage^[12,13]. Oxidative stress in ALD leads to lipid peroxidation and protein oxidation^[14]. To protect the body from oxidative stress, there are several antioxidant defense mechanisms. The most important antioxidant enzymes involved in these mechanisms are glutathione peroxidase (GPX), glutathione reductase (GRD), superoxide dismutase (SOD) and catalase (CAT)^[15].

The pathological process of ALD can be characterized by different stages of liver damage. The first stage of ALD is hepatic steatosis which is obviously caused by abnormal lipid metabolism^[16-18]. In this stage, steatosis is reversible after alcohol abstinence^[19,20]. The progressive

stage after steatosis is hepatitis. In this stage, steatosis is accompanied by inflammation and cytokine production. The presence of hepatitis indicates initiation of liver cirrhosis, the terminal stage of alcoholic liver disease^[20,21].

Although a few studies discussed ALD in Western countries, the clinical data of patients with ALD in Taiwan, especially data on Taiwanese aborigines, are not well-established. In addition, there are different genetic types of alcohol metabolic enzymes between Western and Asian people^[22]. Therefore it is necessary to establish clinical data on aboriginal patients with ALD in Taiwan. Taitung, situated in southeastern Taiwan, contains the highest proportion of aboriginal residents. There are approximately 240 000 people in Taitung, one-third of whom are aborigines, and the rest are ethnic Han Chinese^[5]. The aim of this study was to compare the antioxidative status between aboriginal and non-aboriginal patients with ALD in the Taitung area. Furthermore, the effect of different severities of ALD, i.e. hepatitis and cirrhosis, on the antioxidative status is also discussed in this study.

MATERIALS AND METHODS

Subjects

This study examined 27 patients with ALD recruited from Taitung Mackay Memorial Hospital. Patients with ALD included 12 non-aboriginal patients (the non-aboriginal ALD group) (12 men) and 15 aboriginal patients (the aboriginal ALD group) (11 men and 4 women). According to the severity of ALD, patients with ALD consisted of 10 patients with alcoholic hepatitis (the hepatitis ALD group) (9 men and 1 woman) and 17 patients with alcoholic cirrhosis (the cirrhosis ALD group) (14 men and 3 women). Twenty-two age- and gender-matched healthy adults served as the control group in this study. The Institutional Review Board for Human studies approved this study. Patient consent was obtained prior to blood collection. Venous blood (10 mL) of each subject was drawn into an EDTA-containing tube after an overnight fasting period of 8 h. Plasma samples were obtained by centrifugation at $1200 \times g$ for 15 min at 4°C. After removing the plasma, erythrocytes were obtained by washing twice with ice-cold physiological saline. Plasma and erythrocyte samples were stored at -80°C until being analyzed.

Biochemical analysis

Plasma aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, γ glutamyltransferase (γ -GT) activity, total cholesterol (TC) concentration, triglyceride (TG) concentration, high-density lipoprotein-cholesterol (HDL-C) concentration, low-density lipoprotein-cholesterol (LDL-C) concentration, albumin concentration, uric acid concentration, and total bilirubin concentration were measured in each patient and control group using standard procedures on an autoanalyzer (SYNCHRON CX System, Hitachi 7170, Tokyo, Japan).

Deionized water (300 μ L) was added to 100 μ L of the erythrocyte sample and mixed well. The mixture was centrifuged at 4°C, and $8000 \times g$ for 10 min. Then the supernatant fractions were used to determine antioxidant enzymes and the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio.

GPX activity

The GPX activity of erythrocytes was determined with a commercial kit (RS 504; Randox Laboratories, Antrim, UK). First, 20 μ L of the diluted sample was added to 1 mL of a mixed substrate (4 mmol/L GSH, 0.5 U/L GRD, and 0.34 mmol/L NADPH dissolved in 50 mmol/L phosphate buffer, at pH 7.2, 4.3 mmol/L EDTA). Then, 40 μ L of cumene hydroperoxide (diluted in deionized water) was added to the mixture. The reaction mixture was incubated at 37°C, and the absorbance at 340 nm was determined every minute for 3 min using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

GRD activity

The GRD activity of erythrocytes was measured with a commercial kit (GR 2368; Randox Laboratories, Antrim, UK). First, 200 μ L of the diluted sample and 400 μ L of 2.4 mmol/L GSSG buffer (dissolved in 125 mmol/L potassium phosphate buffer, at pH 7.5, with 2.5 mmol/L EDTA) were added to 400 μ L of 0.55 mmol/L NADPH (dissolved in deionized water). The absorbance was measured at 340 nm every minute for 5 min using a microplate reader (Molecular Devices).

SOD activity

The SOD activity of erythrocytes was measured with a commercial kit (SD 125; Randox Laboratories, Antrim, UK). First, 50 μ L of the diluted sample and 1.7 mL of the mixed substrate (50 μ mol/L xanthine and 25 μ mol/L 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride, INT) were added to 250 μ L of xanthine oxidase. The reaction mixture was incubated at 37°C, and the absorbance was measured at 340 nm every minute for 3 min using a microplate reader (Molecular Devices).

CAT activity

The CAT activity of erythrocytes was determined according to the method reported by Beers and Sizer^[23]. First, 100 μ L of the diluted sample and 1 mL of 59 mmol/L H₂O₂ (dissolved in 50 mmol/L potassium phosphate buffer, at pH 7.0) were added to 1.9 mL of deionized water. The absorbance was measured at 240 nm every minute for 3 min using a Hitachi U-2000 ultraviolet-visible (UV-VIS) spectrophotometer. One unit of CAT activity was defined as mmol of H₂O₂ degraded/min.

Determination of the GSH/GSSG ratio in erythrocytes and liver tissue

GSH concentration: The concentration of GSH in erythrocytes was determined according to the method of Tietze^[24], using GSH (0-100 μ mol/L) as the standard. The

diluted sample solution or standard (10 μ L) was mixed with 95 μ L of the reagent (2 U/mL glutathione reductase, 200 μ mol/L NADPH, and 2 mmol/L EDTA in 50 mmol/L phosphate buffer, at pH 7.2), followed by the addition of 100 μ L of the reagent (10 mmol/L DTNB in 50 mmol/L phosphate buffer, at pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every minute for 5 min using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices). The concentration was expressed as GSH (μ mol/L) in erythrocytes.

GSSG concentration: The concentration of GSSG in erythrocytes was measured according to the method of Tietze^[24], using GSSG (0-100 μ mol/L) as the standard. The diluted sample solution or standard (70 μ L) was mixed with 4 μ L of 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP). The mixture was allowed to stand at room temperature for 1 h. The reaction mixture (10 μ L) was mixed with 95 μ L of the reagent (2 U/mL glutathione reductase, 200 μ mol/L NADPH, and 2 mmol/L EDTA in 50 mmol/L phosphate buffer, at pH 7.2), followed by the addition of 100 μ L of the reagent (10 mmol/L DTNB in 50 mmol/L phosphate buffer, at pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every minute for 5 min using an ELISA reader (Molecular Devices). The concentration was expressed as GSSG (μ mol/L) in erythrocytes.

The GSH/GSSG ratio: The GSH/GSSG ratio was then calculated by dividing the difference between the total GSH and GSSG concentrations (reduced GSH) by the concentration of GSSG. $\text{GSH/GSSG} = (\text{total GSH} - 2\text{GSSG})/\text{GSSG}$.

Measurement of lipid peroxidation in plasma

Lipid peroxidation was quantitatively measured by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in plasma using the method of Ohkawa *et al.*^[25] with minor modifications. 20 μ L plasma or various levels of TMP (1,1,3,3-tetramethoxypropane; as a standard) were shaken with 800 μ L of 0.22% H₂SO₄ in a 2 mL centrifuge tube. Phosphotungstic acid (100 μ L; 10%) and 200 μ L of 0.67% TBA (in H₂O: glacial acetic acid = 1:1, v/v) were added to the mixture, shaken, and warmed for 60 min in a boiling water bath followed by rapid cooling. Then 600 μ L of an *n*-butyl-alcohol layer was shaken in a separation tube, and the MDA content in the plasma or liver homogenates was determined fluorometrically (with respective excitation and emission wavelengths of 531 and 590 nm) using a Wallace Victor-2 1420 Multilabel Counter (Perkin-Elmer, Waltham, MA, USA).

Statistical analysis

All data are expressed as the means \pm SE. Student's *t* test was used to compare differences of means between the control group and ALD group using EXCEL software

Table 1 Biochemical parameters of control subjects and patients with alcoholic liver disease (mean \pm SE)

	Control	ALD
<i>n</i>	22	27
Male/female	19/3	23/4
Age (yr)	41.2 \pm 2.1	45.3 \pm 2.1
Height (cm)	169.7 \pm 1.2	165.3 \pm 1.3 ^a
Weight (kg)	66.1 \pm 1.8	65.4 \pm 2.3
BMI (kg/m ²)	23.9 \pm 0.6	23.9 \pm 0.8
AST (U/L)	20.7 \pm 0.9	63.6 \pm 8.7 ^a
ALT (U/L)	22.6 \pm 1.7	39.1 \pm 5.2 ^a
AST/ALT	1.0 \pm 0.06	2.86 \pm 0.86 ^a
γ -GT (U/L)	29.2 \pm 3.5	271.1 \pm 81.3 ^a
TC (mg/dL)	185.6 \pm 5.5	153.7 \pm 13.2 ^a
TG (mg/dL)	99.4 \pm 13.2	193.6 \pm 61.2
HDL-C (mg/dL)	50.5 \pm 3.1	36.7 \pm 2.6 ^a
LDL-C (mg/dL)	125.5 \pm 6.8	72 \pm 8.2 ^a
Albumin (gm/dL)	4.75 \pm 0.06	3.33 \pm 0.18 ^a
Uric acid (mg/dL)	6.26 \pm 0.29	6.81 \pm 0.45
Total bilirubin	0.56 \pm 0.04	5.53 \pm 2.21 ^a

^a*P* < 0.05 *vs* the control group (by Student's *t*-test). ALD: Alcoholic liver disease; BMI: Body-mass index; AST: Aspartate aminotransferase; ALT: Alanine transaminase; γ -GT: γ -glutamyltransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol.

Table 2 Erythrocytic antioxidative enzymes activities, erythrocytic reduced glutathione/oxidized glutathione ratio and plasma thiobarbituric acid reactive substances concentration of control subjects and patients with alcoholic liver disease (mean \pm SE)

	Control	ALD
GPX (U/g Hb)	27.9 \pm 2.2	34.4 \pm 4.2
GRD (U/g Hb)	0.55 \pm 0.06	0.62 \pm 0.05
CAT (U/mg Hb)	11.1 \pm 0.7	8.0 \pm 0.7 ^a
SOD (U/mg Hb)	9.5 \pm 1.6	3.0 \pm 0.2 ^a
GSH/GSSG	38.1 \pm 5.4	15.7 \pm 1.9 ^a
TBARS (μ mol/L)	3.5 \pm 0.2	4.1 \pm 0.6

^a*P* < 0.05 *vs* the control group (by Student's *t*-test). ALD: Alcoholic liver disease; GPX: Glutathione peroxidase; GRD: Glutathione reductase; CAT: Catalase; SOD: Superoxide dismutase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; TBARS: Thiobarbituric acid reactive substances.

(Redmond, WA, USA). Statistical significance was assigned at the *P* < 0.05 level. To evaluate differences among the three groups in this study, one-way analysis of variance with Fisher's post hoc test was used. The SAS software (vers. 8.2, SAS Institute., Cary, NC, USA) was used to analyze all data. Differences were considered statistically significant at *P* < 0.05.

RESULTS

The biochemical parameters of the control and ALD group are shown in Table 1. No significant difference was observed with regard to age, weight, body-mass index (BMI), or plasma uric acid concentration between the control and ALD group. The plasma AST activity, ALT activity, AST/ALT ratio, γ -GT activity and total bilirubin

Table 3 Biochemical parameters of control subjects, and non-aboriginal and aboriginal patients with alcoholic liver disease (mean \pm SE)

	Control	Non-aboriginal ALD	Aboriginal ALD
<i>n</i>	22	12	15
Male/female	19/3	12/0	11/4
Age (yr)	41.2 \pm 2.1 ^a	51.3 \pm 2.7 ^b	40.5 \pm 2.4 ^a
Height (cm)	169.7 \pm 1.2 ^a	167.6 \pm 1.6 ^{a,b}	163.6 \pm 1.7 ^b
Weight (kg)	66.1 \pm 1.8 ^a	66.5 \pm 3.7 ^a	64.5 \pm 3.1 ^a
BMI (kg/m ²)	23 \pm 0.6 ^a	23.7 \pm 1.2 ^a	24.1 \pm 1.1 ^a
AST (U/L)	20.7 \pm 0.9 ^a	58.0 \pm 10.7 ^b	68.1 \pm 13.4 ^b
ALT (U/L)	22.6 \pm 1.8 ^a	42.8 \pm 10.0 ^b	36.1 \pm 5.1 ^{a,b}
AST/ALT	1.0 \pm 0.1 ^a	3.0 \pm 1.4 ^a	2.7 \pm 1.1 ^a
γ -GT (U/L)	29.2 \pm 3.5 ^a	335.9 \pm 173.4 ^b	219.3 \pm 53.0 ^{a,b}
TC (mg/dL)	185.6 \pm 5.5 ^a	157.3 \pm 15.8 ^a	150.6 \pm 21.0 ^a
TG (mg/dL)	99.4 \pm 13.2 ^a	136.4 \pm 66.3 ^a	239.4 \pm 109.3 ^a
HDL-C (mg/dL)	50.5 \pm 3.1 ^a	39.8 \pm 4.5 ^b	34.3 \pm 3.1 ^b
LDL-C (mg/dL)	125.5 \pm 6.8 ^a	88.8 \pm 13.4 ^b	58.5 \pm 9.2 ^c
Albumin (gm/dL)	4.75 \pm 0.06 ^a	3.68 \pm 0.23 ^b	2.93 \pm 0.25 ^c
Uric acid (mg/dL)	6.26 \pm 0.29 ^a	6.74 \pm 0.45 ^a	6.89 \pm 0.89 ^a
Total bilirubin	0.56 \pm 0.04 ^a	3.74 \pm 1.43 ^{a,b}	7.31 \pm 4.23 ^b

Values in the same row with different letters (a, b, c) significantly different at *P* < 0.05 (by one-way analysis of variance). ALD: Alcoholic liver disease; BMI: Body-mass index; AST: Aspartate aminotransferase; ALT: Alanine transaminase; γ -GT: γ -glutamyltransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol.

concentrations were significantly higher in the ALD group than those in the control group (*P* < 0.05). The plasma TC, HDL-C, and LDL-C concentrations were significantly lower in the ALD group than those in the control group (*P* < 0.05). Furthermore, patients with ALD had significantly lower plasma albumin concentrations than that in the control subjects (*P* < 0.05). As shown in Table 2, no significant difference in erythrocytic GPX or GRD activities was observed between the control and ALD groups. Patients with ALD showed significantly lower erythrocytic CAT and SOD activities when compared to those in the control group (*P* < 0.05). Furthermore, the erythrocytic GSH/GSSG ratio was significantly lower in patients with ALD than that in the control group (*P* < 0.05). But no significant difference was observed with regard to the plasma TBARS concentration between the control and ALD group.

In order to understand the antioxidant status in aboriginal patients with ALD (aboriginal ALD group) in the Taitung area, patients were divided into an aboriginal ALD, and a non-aboriginal ALD (non-aboriginal ALD group). Biochemical parameters of the control, non-aboriginal ALD, and aboriginal ALD group are given in Table 3. No significant difference was observed in the weight, BMI, AST/ALT, plasma TC, TG or uric acid concentrations among the three groups. Both the non-aboriginal and aboriginal ALD groups had significantly higher plasma AST activity than the control group (*P* < 0.05). The non-aboriginal, but not the aboriginal, ALD group had significantly higher plasma ALT and γ -GT activities than that in the control group (*P* < 0.05). Both the non-aboriginal and aboriginal ALD groups had significantly lower plasma

Table 4 Erythrocytic antioxidative enzymes activities, erythrocytic reduced glutathione/oxidized glutathione ratio and plasma thiobarbituric acid reactive substances concentration of control subjects, and non-aboriginal and aboriginal patients with alcoholic liver disease (mean \pm SE)

	Control	Non-aboriginal ALD	Aboriginal ALD
GPX (U/g Hb)	27.9 \pm 2.2 ^a	46.1 \pm 7.8 ^b	25.0 \pm 0.2 ^a
GRD (U/g Hb)	0.55 \pm 0.06 ^a	0.68 \pm 0.07 ^a	0.58 \pm 0.07 ^a
CAT (U/mg Hb)	11.1 \pm 0.7 ^a	8.5 \pm 0.9 ^b	7.6 \pm 1.1 ^b
SOD (U/mg Hb)	9.5 \pm 1.6 ^a	2.9 \pm 0.3 ^b	3.1 \pm 0.3 ^b
GSH/GSSG	38.0 \pm 5.4 ^a	16.5 \pm 3.8 ^b	15.0 \pm 1.6 ^b
TBARS (μ mol/L)	3.5 \pm 0.2 ^a	3.4 \pm 0.5 ^a	4.6 \pm 1.0 ^a

Values in the same row with different letters (a, b) significantly different at $P < 0.05$. (by one-way analysis of variance). ALD: Alcoholic liver disease; GPX: Glutathione peroxidase; GRD: Glutathione reductase; CAT: Catalase; SOD: Superoxide dismutase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; TBARS: Thiobarbituric acid reactive substances.

HDL-C, LDL-C and albumin concentrations than those in the control group ($P < 0.05$). In addition, the aboriginal ALD group had lower plasma LDL-C and albumin concentrations than those in the non-aborigine ALD group ($P < 0.05$). The aboriginal, but not non-aboriginal, ALD had significantly higher plasma total bilirubin than that in the control group ($P < 0.05$).

As shown in Table 4, the non-aboriginal, but not the aboriginal ALD group had significantly higher erythrocytic GPX activity than the control group ($P < 0.05$). In addition, no significant difference was observed in the erythrocytic GRD activities and plasma TBARS concentration among the three groups. Both the non-aboriginal and aboriginal ALD group had significantly lower erythrocytic CAT activity, erythrocytic SOD activity and erythrocytic GSH/GSSG ratio than those in the control group ($P < 0.05$).

The antioxidant status with different severities of alcoholic liver disease was investigated. Patients with ALD were divided into those with hepatitis ALD (hepatitis ALD) and those with cirrhosis (cirrhosis ALD). In Table 5, no significant difference was observed in the weight, BMI or uric acid concentration among the control, hepatitis ALD, and cirrhosis ALD groups. Both the hepatitis ALD and cirrhosis ALD groups had significantly higher plasma AST activity compared to the control group ($P < 0.05$). The hepatitis, but not the cirrhosis, ALD group had significantly higher plasma ALT activity and AST/ALT ratio than those in the control group ($P < 0.05$). The cirrhosis, but not the hepatitis, ALD group had significantly higher plasma γ -GT activity compared to the control group ($P < 0.05$). The cirrhosis, but not the hepatitis, ALD group had lower plasma HDL-C concentrations than the control group ($P < 0.05$). Both the hepatitis and cirrhosis ALD groups had significantly lower plasma LDL-C concentrations when compared to the control group ($P < 0.05$). In addition, the cirrhosis ALD group had significantly lower plasma albumin concentrations than the hepatitis ALD group ($P < 0.05$). The cirrhosis, but not the hepatitis, ALD group had significantly higher plasma total bilirubin concentrations than the control group ($P < 0.05$).

As shown in Table 6, the hepatitis ALD, but not the cirrhosis ALD group, had significantly higher erythrocytic

Table 5 Biochemical parameters of control subjects, and patients with hepatitis and cirrhosis alcoholic liver disease (mean \pm SE)

	Control	Hepatitis ALD	Cirrhosis ALD
<i>n</i>	22	10	17
Male/female	19/3	9/1	14/3
Age (yr)	41.2 \pm 2.1 ^a	45.4 \pm 4.2 ^a	45.3 \pm 2.2 ^a
Height (cm)	169.7 \pm 1.2 ^a	167.4 \pm 2.3 ^{ab}	164.2 \pm 1.5 ^b
Weight (kg)	66.1 \pm 1.8 ^a	70.4 \pm 3.4 ^a	62.6 \pm 3.0 ^a
BMI (kg/m ²)	23.0 \pm 0.6 ^a	25.2 \pm 1.3 ^a	23.2 \pm 1.0 ^a
AST (U/L)	20.7 \pm 0.9 ^a	66.9 \pm 18.6 ^b	61.7 \pm 9.1 ^b
ALT (U/L)	22.6 \pm 1.8 ^a	46.2 \pm 12.7 ^b	34.9 \pm 3.8 ^{ab}
AST/ALT	1.0 \pm 0.06 ^a	4.49 \pm 2.28 ^b	1.9 \pm 0.22 ^{ab}
γ -GT (U/L)	29.2 \pm 3.5 ^a	207.8 \pm 71.7 ^{ab}	308.4 \pm 123.1 ^b
TC (mg/dL)	185.6 \pm 5.5 ^a	188.9 \pm 26.2 ^a	131.8 \pm 11.5 ^b
TG (mg/dL)	99.4 \pm 13.2 ^a	293.5 \pm 153.5 ^b	134.9 \pm 35.8 ^{ab}
HDL-C (mg/dL)	50.5 \pm 3.1 ^a	45.1 \pm 3.8 ^a	31.8 \pm 3.0 ^b
LDL-C (mg/dL)	125.5 \pm 6.8 ^a	94.4 \pm 15.8 ^b	58.8 \pm 8.0 ^c
Albumin (gm/dL)	4.75 \pm 0.06 ^a	3.87 \pm 0.38 ^b	3.08 \pm 0.17 ^c
Uric acid (mg/dL)	6.26 \pm 0.29 ^a	6.68 \pm 0.71 ^a	6.86 \pm 0.59 ^a
Total bilirubin	0.56 \pm 0.04 ^a	1.22 \pm 0.17 ^a	7.48 \pm 3.06 ^b

Values in the same row with different letters (a, b, c) significantly differ at $P < 0.05$. (by one-way analysis of variance). ALD: Alcoholic liver disease; BMI: Body-mass index; AST: Aspartate aminotransferase; ALT: Alanine transaminase; γ -GT: γ -glutamyltransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol.

Table 6 Erythrocytic antioxidative enzyme activities, erythrocytic reduced glutathione/oxidized glutathione ratio and plasma thiobarbituric acid reactive substances concentration of control subjects, patients with hepatitis and cirrhosis alcoholic liver disease (mean \pm SE)

	Control	Hepatitis ALD	Cirrhosis ALD
GPX (U/g Hb)	27.9 \pm 2.2 ^a	44.3 \pm 8.6 ^b	28.5 \pm 3.8 ^a
GRD (U/g Hb)	0.55 \pm 0.06 ^a	0.74 \pm 0.06 ^a	0.56 \pm 0.07 ^a
CAT (U/mg Hb)	11.1 \pm 0.7 ^a	10.8 \pm 1.2 ^a	6.4 \pm 0.6 ^b
SOD (U/mg Hb)	9.5 \pm 1.6 ^a	3.7 \pm 0.2 ^b	2.6 \pm 0.2 ^b
GSH/GSSG	38.1 \pm 5.4 ^a	18.3 \pm 4.2 ^b	14.2 \pm 1.7 ^b
TBARS (μ mol/L)	3.5 \pm 0.2 ^a	4.2 \pm 1.1 ^a	4.0 \pm 0.7 ^a

Values in the same row with different letters (a, b) significantly different at $P < 0.05$ (by one-way analysis of variance). ALD: Alcoholic liver disease; GPX: Glutathione peroxidase; GRD: Glutathione reductase; CAT: Catalase; SOD: Superoxide dismutase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; TBARS: Thiobarbituric acid reactive substances.

GPX activity than the control group ($P < 0.05$). There was no significant difference in erythrocytic GRD activity and plasma TBARS concentration among the three groups. The cirrhosis, but not the hepatitis, ALD group had significantly lower erythrocytic CAT activity than the control group ($P < 0.05$). Both the hepatitis and cirrhosis ALD groups had significantly lower erythrocytic SOD activities and GSH/GSSG ratio than those in the control group ($P < 0.05$).

DISCUSSION

In most patients with liver injury, the ratio of AST to ALT is 1 or less, whereas in alcoholic hepatitis it is generally

about 2^[26]. An AST to ALT ratio > 1 was reported in patients with alcoholic cirrhosis^[27]. Our observation agreed with the previous study, the AST to ALT ratio was 2.86 in patients with ALD (Table 1) and the AST to ALT ratio in hepatitis ALD was higher than that in cirrhosis ALD (Table 5).

Erythrocytes can easily suffer oxidative damage due to the presence of polyunsaturated fatty acid, heme, iron, and oxygen. The antioxidant enzymes, GPX, GRD, CAT and SOD, located in erythrocytes protect the body from oxidative stress^[28]. In an animal study, decreased activity of Cu, Zn SOD was demonstrated in the liver of rodents after chronic ethanol exposure^[29]. However, both increased and decreased SOD activities in the blood of alcoholics were reported^[30-33], and this may have been due to different durations of alcohol dependence. Free radical scavenging enzymes such as SOD, CAT, and GPX are known to be the first line cellular defense against oxidative damage, disposing of superoxide anion and H₂O₂ before their interaction to form the more harmful hydroxyl radical. In the present study, both CAT and SOD activities decreased significantly in the ALD groups compared with the control group, and it is assumed that excessive superoxide anions might elicit lipoperoxide formation and induce cell damage before being converted to H₂O₂ by SOD^[34]. In the absence of adequate SOD activity, superoxide anion is not converted into H₂O₂, which is the substrate for the H₂O₂ scavenging enzyme CAT^[35]. As a result, there is an inactivation of the H₂O₂ scavenging enzyme CAT, leading to a decrease in its activity^[36].

GPX produces GSSG from GSH, while GRD maintains the cellular level of GSH by reducing oxidized glutathione to its reduced form. Change in activities of these two antioxidant enzymes are not consistent in ALD^[37]. In the present study, erythrocytic GPX and GRD activities showed no difference between patients with ALD and the control group (Table 2). The limited size of the study populations may have contributed to this anomaly.

Glutathione, a tripeptide (γ -glutamylcysteinylglycine), plays an important role in coordinating the body's antioxidant defense processes^[38]. Lower hepatic GSH levels were reported in alcoholic cirrhosis^[38,39]. GSH is also present in erythrocytes^[40]. GSH can be exported from hepatocytes to the sinusoidal blood, where it is rapidly broken down to dipeptides and amino acid by γ -GT and dipeptidases^[40,41]. Erythrocytes can resynthesize GSH after taking up the precursor amino acid^[30]. In agreement with a previous study^[42], the erythrocytic GSH/GSSG ratio, an indicator of the antioxidant status, was significantly lower in the ALD group than the control group in this study (Table 2). The lower erythrocytic GSH concentrations may be due to impaired biosynthesis or decreased release from damaged liver cells^[42]. The TBARS concentration reflects the level of malondialdehyde (MDA) which is the end product of lipid peroxidation. As shown by Peng *et al.*^[15], the MDA concentration not only significantly increased in the ALD group than in the control group, but was also significantly correlated with the duration of alcohol dependence.

However, our present study is not in accordance with a previous study. The plasma TBARS concentration showed a slight increase over that of the control, but it did not reach significance. We believe that the dietary lipid composition may play an important role in lipid peroxidation. Evidence showed that the effect of saturated fatty acids may reduce endotoxemia and lipid peroxidation^[43]. In addition, rats fed diets high in polyunsaturated fatty acids and ethanol could increase the susceptibility of peroxidation^[44]. Therefore, the dietary lipid composition of control subjects and patients with ALD in southeastern Taiwan should be investigated in the future.

Poor health knowledge, a relatively low socioeconomic status, high rates of alcohol abuse, and poor control of chronic diseases are the characteristics of Taiwanese aborigines^[45]. It is believed that both poor health knowledge and relatively low socioeconomic status may lead to malnutrition in Taiwanese aborigines. As shown in this study, the significantly lower albumin concentration, which is an index of malnutrition, was found in the aboriginal ALD group (Table 3). On the other hand, the non-aboriginal, but not the aboriginal, ALD group had higher erythrocytic GPX activities than the control group (Table 4). The higher erythrocytic GPX activity can be explained as an adaption to the oxidative stress of ALD. However, the aboriginal ALD group did not show an adaptive capacity.

As shown in table 5, the cirrhosis ALD group had significantly lower plasma TC, HDL-C, and LDL-C concentrations than those in the hepatitis ALD and control groups. A previous study indicated that patients with alcoholic cirrhosis with Child-Pugh score C showed lower plasma TC concentrations than patients with alcohol abuse without cirrhosis^[46]. This confirms that the severity of liver impairment may affect plasma TC concentrations. In addition, lower plasma HDL-C and LDL-C concentrations with alcoholic cirrhosis were also reported in previous studies^[47,48]. The lower lipoprotein results from impairment lipoprotein synthesis can be explained by decreased protein secretion and increased protein retention in the liver under ethanol exposure^[49].

Changes in erythrocytic antioxidant enzymes differed between the hepatitis and cirrhosis ALD groups compared to the control group (Table 6). The hepatitis, but not cirrhosis, ALD group had higher erythrocytic GPX activity than the control group. The higher GPX activity in hepatitis ALD patients can be explained by adaptation to oxidative stress. In addition, the hepatitis ALD group showed significantly higher erythrocytic CAT activity than the cirrhosis ALD group. The lower erythrocytic CAT activity of cirrhosis ALD patients is consistent with a previous study^[50]. This result reveals that cirrhosis ALD patients had lower erythrocytic enzyme activities than hepatitis ALD patients. Although there were different changes in erythrocytic antioxidant enzymes between the two ALD groups, both the hepatitis and cirrhosis ALD groups showed a lower erythrocytic GSH/GSSG ratio than the control group.

In conclusion, this study indicated that both ethnicity and the severity of alcoholic liver disease can cause different erythrocytic antioxidative enzyme activities especially GPX activity. This finding can not only establish the clinical data of patients in Taiwan but also provide a few clues for the treatment of alcoholic liver disease. Our research also showed no significant difference in plasma TBARS level between control subjects and ALD patients. This different result from studies in Western countries might be attributed to the dietary lipid composition in the Taitung area of southeastern Taiwan.

ACKNOWLEDGMENTS

We thank Taitung Mackay Memorial Hospital.

COMMENTS

Background

Alcohol abuse is one of the major causes of liver disease worldwide. The prevalence of alcohol dependence is increasing in Taiwan. Besides the hepatic viral infection, another high rate prevalence liver disease in Taiwan may increase liver damage in alcoholic liver disease. Thus, more research should be done on establishing the clinical data of alcoholic liver disease in Taiwan area.

Research frontiers

A higher prevalence of alcoholism in aborigines was reported in Taiwan. Besides, both viral infection and alcohol consumption play important roles in the development of chronic liver diseases in Taiwanese aborigines. Therefore, alcoholic liver disease is an important issue for aborigines in Taiwan.

Innovations and breakthroughs

In this study, we indicated that both ethnicity and the severity of alcoholic liver disease may cause different erythrocytic antioxidative enzyme activities, especially erythrocytic glutathione peroxidase (GPX) activity.

Applications

By understanding the different changes of antioxidative enzyme activities between either different ethnicity or severity of alcoholic liver disease, we can not only establish the clinical data of patients in Taiwan but also provide a few clues for treatment of alcoholic liver disease.

Peer review

The manuscript describes the antioxidative condition of 27 alcoholic liver disease (ALD) patients in southeastern Taiwan by biochemical analysis, such as erythrocytic catalase, superoxide dismutase, reduced glutathione/oxidized glutathione ratio and GPX, and plasma low-density lipoprotein-cholesterol concentration. The authors concluded that both ethnicity and the severity of ALD may cause different erythrocytic antioxidative enzyme activities especially GPX activity and the findings are of interest.

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S- Editor Sun H L- Editor O'Neill M E- Editor Lin YP



Hyperthermic intraperitoneal chemotherapy for gastric and colorectal cancer in Mainland China

Tao Suo, Haile Mahteme, Xin-Yu Qin

Tao Suo, Xin-Yu Qin, Department of General Surgery, Zhongshan Hospital, Fudan University, General Surgery Institute of Fudan University, Shanghai 200032, China

Haile Mahteme, Department of Surgical Sciences, Uppsala University, SE75185 Uppsala, Sweden

Author contributions: Suo T and Qin XY designed the study; Suo T performed the search of the literature and analyzed the data; Suo T and Mahteme H wrote the paper; Qin XY revised the paper before submission.

Correspondence to: Xin-Yu Qin, MD, PhD, Department of General Surgery, Zhongshan Hospital, Fudan University, General Surgery Institute of Fudan University, 180 Fenglin Road, Xuhui District, Shanghai 200032, China. qin.xinyu@live.cn
Telephone: +86-21-64041990 Fax: +86-21-64037224

Received: August 12, 2010 Revised: October 11, 2010

Accepted: October 18, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate the current status of peritoneal carcinomatosis (PC) management, as well as the usage of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) in mainland China.

METHODS: A potentially curative therapeutic strategy for selecting patients with PC, known as "Techniques", consists of CRS in combination with HIPEC. A systemic search of published works and clinical trials was performed. Additional papers were retrieved by cross-checking references and obtaining information from Chinese oncologists and relevant conferences. One hundred and one papers and one registered clinical trial on HIPEC were included.

RESULTS: A literature review identified 86 hospitals in 25 out of all 31 areas of mainland China that perform HIPEC. The earliest report included in our survey was published in 1993. Different approaches to HIPEC have been utilized, i.e. palliative, prophylactic, and possibly

curative treatment. Only one center has consistently performed HIPEC according to the "Sugarbaker Protocol", which involves evaluating the extent of PC with peritoneal cancer index and the results of CRS with the completeness of cytoreduction. Positive preliminary results were reported: 7 of 21 patients with PC survived, free of tumors, during an 8-43-mo follow-up period. Hyperthermic strategies that include HIPEC have been practiced for a long time in mainland China, whereas the "Sugarbaker Protocol/Techniques" has been only rarely implemented in China. The Peritoneal Surface Oncology Group International hosts a biannual workshop with the intent to train more specialists in this field and provide support for the construction of quality treatment centers, especially in developing countries like China, whose population is huge and has a dramatically increased incidence of cancer.

CONCLUSION: To popularize Sugarbaker Protocol/Techniques in mainland China in PC management arising from gastric cancer or colorectal cancer will be the responsibility of the upcoming Chinese Peritoneal Surface Oncology Group.

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Key words: Peritoneal carcinomatosis; Hyperthermia; Prophylactic strategy; Sugarbaker Protocol/techniques; Mainland China

Peer reviewer: Dr. Oliver Mann, MD, Senior Attending Physician and Deputy Director, Department of General, Visceral and Thoracic Surgery, University of Hamburg, Martini Str. 52, D-20246 Hamburg, Germany

Suo T, Mahteme H, Qin XY. Hyperthermic intraperitoneal chemotherapy for gastric and colorectal cancer in Mainland China. *World J Gastroenterol* 2011; 17(8): 1071-1075 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1071.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1071>

INTRODUCTION

China has a population of more than 1.3 billion, which makes it the largest developing country in the world with a consequently huge medical burden. The incidence of gastrointestinal cancer in China has been rapidly increasing in recent years^[1]. In 2004-2005 alone, there were 428 380 newly reported cases of gastric cancer (GC) and 339 308 GC-related deaths. Furthermore, in the same period of time, there were 197 873 newly reported cases of colorectal cancer (CRC), including cancers of anal canal, and 101 684 CRC-related deaths^[2]. Although the incidence of GC has decreased for both men and women in some areas of China, the incidence of CRC has annually increased by 4.2% in Shanghai, which is higher than the observed global rate of increase. Notably, a report of 1075 cases of CRC in China has revealed that advanced stage III and VI cases account for 46.8% of all reported cases^[3].

The peritoneum is one of the most commonly affected sites in patients with recurrent GC or CRC. One study performed on a Chinese population found that even early GC has a 1.63% (4/308) rate of peritoneal recurrence^[4]. The natural course of peritoneal carcinomatosis (PC), including those arising from GC and CRC, is only 6-8 mo^[5,6]. In regard to the traditional treatment and follow-up, PC is considered to be a terminal event of systemic metastasis. Over the past two decades, "Sugarbaker Protocol/Techniques", i.e. surgical removal of all macroscopic PC in combination with hyperthermic intraperitoneal chemotherapy (HIPEC), has emerged as a potential curative treatment option for some patients. It becomes increasingly acceptable as a standard method of treatment for certain local peritoneal surface diseases. In recent years, many institutions all over the world have applied this combined modality treatment and have achieved exciting results^[7-14].

The purpose of this survey was to investigate the current status of PC treatment, as well as the frequency of cytoreductive surgery (CRS) and HIPEC utilization in China, as determined by a review of the English and Chinese literature. We sought to identify the number of specialized centers that use this approach in the treatment of GC and CRC. We examined the logic behind the decision to pursue HIPEC and/or CRS and the location of the hospitals that reported the use of the technique. We sought to evaluate the popularity of these strategies and quantified the patients who were involved therein in order to determine the frequency with which this approach was utilized. The design, methodology, and results of those clinical trials are not analyzed and discussed in this report.

MATERIALS AND METHODS

An electronic search was conducted using English and/or Chinese language restrictions and Medline databases (from 1949 to January 2010) on PubMed^[15], Cochrane databases^[16], and other databases for registered clinical trials^[17-19]. We used the following search strategy: (China or Chinese) and (cancer or tumor or carcinoma or malign*)

and (mesothel* or periton*) and (heat* or hyper*) and chemotherapy.

The Chinese National Knowledge Infrastructure (CNKI) database^[20] and the Chongqing Weipu Information Company (CQVIP) database^[21] encompass 95.96% and 83.86% of all significant Chinese journals, respectively. The following search strategy was used for our investigation of the Chinese literature: (associated with abdomen) and (associated with cancer) and (associated with heat or hyperthermia) and (chemotherapy) and (associated with surgery and operation).

In order to supplement the electronic search, additional studies about registered clinical trials in China and intra-peritoneal chemotherapy were retrieved by cross-checking references. Chinese oncologists and relevant conferences provided the information as well.

The reference lists of the obtained articles were also examined so as to identify further relevant citations. The first author performed electronic searches in January 2010. All of the abstracts of citations identified by the search were then scrutinized by the first author to determine their eligibility for this study.

Selection criteria

All reported cases with < 10 subjects, cases that lacked a description of the detailed process, cases without follow-up, and cases with brief reports that lacked abstracts and reviews were excluded. If a series of trials at the same center had been documented, we focused on the most recent results. Papers on the same trial published in different languages were considered to be one publication. As mentioned in the introduction, the quality of these clinical trials and their HIPEC technologies are not evaluated or discussed in this paper.

The included papers and clinical trials were categorized into three groups according to the purpose of treatment (Table 1) as follows.

Perioperative prophylactic strategy: intraoperative and early postoperative HIPEC for patients without visible peritoneal metastasis at the time of primary tumor surgery.

Palliative strategy: HIPEC after palliative operation without an attempt to eliminate visible peritoneal tumors or without surgery.

CRS and HIPEC: use "Sugarbaker Protocol", with an attempt to eliminate PC as a potentially curative strategy, following the HIPEC to control residual diseases. An evaluation of the PC and peritoneal cancer index (PCI), in addition to the extent of the CRS and the completeness of cytoreduction (CCR), was included in this category^[9].

RESULTS

Initially, we obtained 629 records from the CQVIP database, 439 records from the CNKI database, 86 records from Medline, and 11 records from the clinical trial registration database. All 715 abstracts about GC and CRC were skimmed. Ultimately, 101 papers and one registered

Table 1 Hospitals performing HIPEC in different areas of Mainland China

Areas in mainland China	Hospitals	No. of trials	No. of trials (No. of patients)			No. of trials for different types of HIPEC			No. of trials that were performed for reasons other than HIPEC		
			GC	CRC	GC and CRC	iHIPEC	pHIPEC	iHIPEC, pHIPEC	Prophylactic	Palliative	Curative
Beijing	1	1	1 (169)			1			1		
Shanghai ¹	4	4	3 (30, 52, 104)		1 (25)	2		2	4	1	
Tianjin	2	2	1 (41)		1 (32)		2		2		
Chongqing	1	1	1 (54)				1		1		
Guangdong ¹	9	13	5 (63, 61, 25, 32, 44)	5 (44, 35, 20, 53, 358)	3 (30, 72, 157)	5	6	2	12	2	
Guangxi ¹	4	4	3 (29, 30, 35)	1 (68)		4			4	1	
Anhui ¹	5	5	3 (278, 25, 42)	1 (32)	1 (80)	1	4		5	1	
Zhejiang	9	10	8 (58, 135, 43, 40, 31, 32, 146, 45)		2 (76, 81)	4	6		10		
Jiangsu	8	10	8 (31, 38, 45, 46, 100, 25, 29, 32)		2 (58, 49)	3	7		10		
Henan ¹	11	13	9 (35, 34, 43, 50, 234, 49, 29, 32, 30)	2 (52, 87)	2 (48, 29)	5	7	1	11	3	
Hebei ¹	2	4	3 (30, 68, 37)	1 (68)		3	1		4	1	
Shandong ¹	5	7	5 (34, 45, 72, 53, 32)		2 (21, 63)	2	5		5	4	
Fujian	2	2	2 (68, 304)				2		2		
Hubei	4	4	2 (23, 56)		2 (68, 17)	2	1	1	3		1
Hunan	1	1	1 (35)				1		1		
Liaoning ¹	4	5	3 (128, 198, 17)	2 (35, 138)		3	2		4	2	
Jilin	1	2	1 (35)	1 (18)			2		1	1	
Shaanxi	2	2	1 (160)	1 (40)		1	1		2		
Ningxia	1	1	1 (82)			1			1		
Gansu ¹	2	2	2 (25, 50)				1	1	2	1	
Neimeng	2	2	1 (36)		1 (24)	1	1		2		
Sichuan	2	2	2 (33, 71)			1	1		2		
Jiangxi	2	2	1 (96)		1 (30)	2			2		
Yunnan	1	1			1 (25)			1	1		
Xinjiang	1	1	1 (136)			1			1		
Total ¹ (trial number)	86	101	68	14	19	42	51	8	91	17	1

GC: Gastric cancer; CRC: Colorectal cancer; PC: Peritoneal carcinomatosis; HIPEC: Hyperthermic intraperitoneal chemotherapy; iHIPEC: Intraoperative HIPEC; pHIPEC: Postoperative HIPEC. ¹A trial including both prophylactic and palliative strategies was counted twice according different purposes of HIPEC.

clinical trial on HIPEC in China were identified. Among these, nine papers were obtained from Medline with English abstracts and five were published in English^[9,22-29].

Based on the aforementioned criteria, we included 86 hospitals in mainland China whose doctors have extensive experience with HIPEC. The hospitals were located in 25 of the 31 areas of mainland China. In total, these institutions reported 101 clinical trials, and the earliest report was published in 1993. Among the included trials, 68 investigated GC, 14 studied CRC, and 19 examined both. In these trials, as a prophylactic strategy, intraoperative and postoperative HIPEC were used, and some centers used HIPEC for patients who were considered to have no chance of surviving surgery. Forty-two trials involved the use of intraoperative HIPEC, 51 utilized postoperative HIPEC, and eight studies adopted both strategies. Ninety-one trials were performed with prophylactic goals and 17 with palliative goals. Only one trial utilized CRS and HIPEC as two parts of a potentially curative strategy. The doctors in these studies adhered to the "Sugarbaker Protocol" in 21 cases that included 12 patients with GC and 5 patients with CRC (registered trial NCT00454519)^[9]. The authors reported positive preliminary results: 7 of the 21

patients with PC survived and were tumor-free during an 8-43-mo follow-up.

Mitomycin C (MMC), Cisplatin (DDP) and 5-Fu are commonly used alone or in combination with HIPEC to treat GC and CRC. The dosage ranges of these treatments extensively vary across trials. The usage of some other drugs, such as Mitomycin C adsorbed on activated carbon particles (MMC-CH) and Tegafur, has been reported in some trials that examined GC. One trial reported on the combination of IL-2 with HIPEC.

The HIPEC centers located in different areas of mainland China and their respective reports are presented in Table 1.

DISCUSSION

Intraoperative intraperitoneal chemotherapy with heat is regarded as a typical prophylactic strategy for advanced GC in China. Among the 101 trials included in this survey, 91 utilized HIPEC for prophylactic purposes. Two other registered trials sponsored by two university hospitals in Shanghai are currently recruiting participants. Chinese authors have also performed a Cochrane review in order

to assess the efficacy and safety of intraperitoneal chemotherapy for GC. Fewer trials have been performed to examine HIPEC for CRC. Some centers have reported their results after performing HIPEC on patients who were not suited for surgical treatment. This survey reveals that hyperthermic treatment, intraperitoneal chemotherapy and HIPEC are popularly accepted in China as therapies for GC and CRC. The “Sugarbaker Protocol” has been implemented in China, although its application has been limited. In the only trial that examined CRS and HIPEC (21 reported cases), the extent of PC with PCI and the result of CRS with CCR were evaluated. The preliminary results are positive, and these authors have concluded that CRS and HIPEC are relatively safe treatment options for selecting patients with PC originating from the gastrointestinal tract and gynecological malignancies, and result in improved outcomes. On the basis of these results, their registered phase II randomized clinical trial is still recruiting participants.

PC is usually regarded as a disseminated, lethal stage of disease and a situation that necessitates palliative care. Based on the studies that are examined in this survey, we found that most authors still consider PC to be an incurable disease. If doctors do not implement the recent advanced treatment for loco-regional disease, their patients can not benefit from this. CRS followed by HIPEC changes the situation and provides selected patients with a chance for possible long-term survival. This novel approach would represent not only a technological advancement but also a paradigm shift in the conception of treatment. The efforts of the Peritoneal Surface Oncology Group International (PSOGI) and their biannual workshop support such progress. This group has cooperated with numerous individuals from the United States, Europe, Korea and Japan, who all have a common interest in the prevention and treatment of peritoneal surface malignancy. The latest reports from their 2008 meeting provide additional supporting evidence of the efficacy of “Sugarbaker Protocol”^[11-13,30-39].

On one hand, the international community insists that CRS followed by HIPEC is effective for selected patients and will continue to improve with additional research. On the other hand, the “Sugarbaker Protocol” is not widely accepted in China, wherein the general population carries a heavy cancer-related medical burden. For example, only one researcher from China registered for the 7th Uppsala Workshop (2010-03-03 in Sweden). Notably, the most effective strategy will be beneficial for the patients and will be supported by academic researches. By sharing their experiences, these professionals from all over the world can work together to improve the treatment methods available for PC and prolong the patient survival. Another important issue is the dissemination of useful information by PSOGI and the biannual workshop. More specialists need to be trained and funding will be necessary in order to build high-quality centers. The necessary skills should be improved and constructed for specialized institutes^[40], and collaborations with well-established centers that perform

the techniques will be essential for the implementation of this strategy in the developing countries. As expected, 20% of the world's population would benefit from this technique if this approach was established in China.

This report is based on a comprehensive literature review and may also provide some advice to doctors and clinical researchers in China. The professionals that are involved in GC and CRC treatment and research must assimilate new concepts that are supported by strong evidence and rapidly apply the related techniques to patients in need. These doctors must publish the results they obtained from clinical practice in English as well as in Chinese, and furthermore, the results should be presented at conferences and in the medical literature. This hard work and collaboration will facilitate the fight against cancer.

As we mentioned in the introduction, this report did not include all of the clinical trials that relate to HIPEC and have been published in Chinese. The authors selected certain hospitals that met established criteria. These hospitals should be dedicated as HIPEC and CRS centers and potential collaborators with PSOGI in the near future.

Hyperthermic strategies have been practised for a long time in mainland China, whereas the “Sugarbaker Protocol” is only rarely implemented. The PSOGI hosts a biannual workshop with the intent to train more specialists in this field and provide support for the construction of quality treatment centers, especially in the developing countries like China.

COMMENTS

Background

According to the traditional treatment and follow-up, peritoneal carcinomatosis (PC) is considered to be a terminal event of systemic metastasis. Over the past two decades, “Sugarbaker Protocol/Techniques”, i.e. surgical removal of all macroscopic PC in combination with hyperthermic intraperitoneal chemotherapy (HIPEC), has emerged as a potential curative treatment option for some patients. It becomes increasingly acceptable as a standard method of treatment for certain local peritoneal surface diseases by many institutions all over the world, and has achieved exciting results. Hyperthermic strategies have been practiced for a long time in mainland China, whereas the “Sugarbaker Protocol” is only rarely implemented.

Research frontiers

Over the past two decades, “Sugarbaker Protocol/Techniques” has emerged as a potential curative treatment option for some patients. It becomes increasingly acceptable as a standard method of treatment for certain local peritoneal surface diseases. In recent years, many institutions all over the world have applied this combined modality treatment and have achieved exciting results.

Innovations and breakthroughs

This study investigated the current status of PC treatment, as well as the frequency of cytoreductive surgery (CRS) and HIPEC utilization in China, as determined by a review of the English and Chinese literature.

Applications

The Peritoneal Surface Oncology Group International hosts a biannual workshop with the intent to train more specialists in this field and provide support for the construction of quality treatment centers, especially in the developing countries like China. As expected, 20% of the world's population would benefit from this technique if this approach was established in China.

Terminology

PC: most PCs come from gastric, colorectal, appendiceal and ovarian cancers. Peritoneal Surface Malignancy/Oncology: include not only PC, but malignancies other than epithelial cancer, such as sarcomatosis and malignant peritoneal

mesothelioma. Sugarbaker Protocol: surgical removal of all macroscopic PC in combination with HIPEC, has emerged as a potential curative treatment option for some patients.

Peer review

The authors present a very important work on HIPEC procedures in mainland China. Standard operating procedures by the Peritoneal Surface Oncology Group are stated to be necessary. However, it is not quite correct, that HIPEC with or without CRS could ever be done in a curative intention, since the patients treated are all in a metastasized situation and any treatment will be palliative. It would be very interesting if the authors can present an analysis of the many prophylactic HIPEC procedures that seem to have taken place in China. Overall, the presented manuscript gives interesting and so far unknown information.

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S- Editor Sun H L- Editor Ma JY E- Editor Zheng XM

Gut bacteria alteration in obese people and its relationship with gene polymorphism

Hao-Jiang Zuo, Zhi-Mei Xie, Wei-Wei Zhang, Yong-Ru Li, Wei Wang, Xiao-Bei Ding, Xiao-Fang Pei

Hao-Jiang Zuo, Zhi-Mei Xie, Wei-Wei Zhang, Yong-Ru Li, Wei Wang, Xiao-Bei Ding, Xiao-Fang Pei, Department of Medical Technology, West China School of Public Health, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Pei XF and Zuo HJ designed the research; Zuo HJ, Xie ZM, Zhang WW, Li YR, Wang W, Ding XB and Pei XF performed the research; Zuo HJ and Ding XB analyzed the data; Zuo HJ and Pei XF wrote the paper.

Supported by Danone Institute China Diet Nutrition Research and Communication grant (2006)

Correspondence to: Xiao-Fang Pei, MD, Professor of Microbiology, Department of Medical Technology, West China School of Public Health, Sichuan University, Chengdu 610041, Sichuan Province, China. xxpei@sina.com

Telephone: +86-28-85502097 Fax: +86-28-85501502

Received: October 20, 2010 Revised: November 30, 2010

Accepted: December 7, 2010

Published online: February 28, 2011

Abstract

AIM: To investigate the differences in cultivable gut bacteria and peroxisome proliferator-activated receptor $\gamma 2$ (*PPAR- $\gamma 2$*) gene Pro12Ala variation in obese and normal-weight Chinese people.

METHODS: Using culture methods, the amounts of *Escherichia coli*, *Enterococci*, *Bacteroides*, *Lactobacilli*, *Bifidobacteria* and *Clostridium perfringens* (*C. perfringens*) in the feces of 52 obese participants [body mass index (BMI): ≥ 28 kg/m²] and 52 participants of normal-weight (BMI: 18.5-24 kg/m²) were obtained. Study participants completed comprehensive questionnaires and underwent clinical laboratory tests. The polymerase chain reaction-restriction fragment length polymorphism (PCR-PFLP) assay was used to analyze *PPAR- $\gamma 2$* gene Pro12Ala variation.

RESULTS: The obese group exhibited a lower amount of *C. perfringens* (6.54 ± 0.65 vs 6.94 ± 0.57 , $P = 0.001$)

and *Bacteroides* (9.81 ± 0.58 vs 10.06 ± 0.39 , $P = 0.012$) than their normal-weight counterparts. No major differences were observed in Pro12Ala genotype distribution between the two groups; however, obese individuals with a Pro/Ala genotype had a significantly lower level of *Bacteroides* (9.45 ± 0.62 vs 9.93 ± 0.51 , $P = 0.027$) than those with a Pro/Pro genotype. In addition, the obese group demonstrated a higher stool frequency ($U = 975$, $P < 0.001$) and a looser stool ($U = 1062$, $P = 0.015$) than the normal-weight group.

CONCLUSION: Our results indicated interactions among cultivable gut flora, host genetic factors and obese phenotype and this might be helpful for obesity prevention.

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Key words: Obesity; Human gut flora; Culture methods; Gene polymorphism; Peroxisome proliferator-activated receptor $\gamma 2$

Peer reviewer: Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, #301, Nok-Ji Building, Seoul 136-701, South Korea

Zuo HJ, Xie ZM, Zhang WW, Li YR, Wang W, Ding XB, Pei XF. Gut bacteria alteration in obese people and its relationship with gene polymorphism. *World J Gastroenterol* 2011; 17(8): 1076-1081 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1076.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1076>

INTRODUCTION

Obesity and its concomitant consequences are a major cause of metabolic disease in both developed and developing countries^[1]. Its occurrence is attributed to a variety of factors. Recent studies have revealed that gut flora, as an environmental factor, might play an important role in

the development of obesity^[2-6]. However, the majority of these results are based on animal experiments or on studies that involved a small number of human samples. Although molecular methods can detect the majority of uncultivable gut flora, microorganisms present in low numbers but with critical functions are usually omitted; thus, molecular methods provide only a rough profile of gut microbial ecology^[7]. This prompted us to consider using culture methods to reveal differences in cultivable gut bacteria. Moreover, culture methods can demonstrate differences in predominantly cultivable bacteria and provide isolates for further characterization and potential application in animal models, which is important for uncovering the relationship between obesity and gut flora.

Along with gut flora, genetic factors are thought to be important in initiating obesity^[8]. Previous studies have suggested that the nuclear hormone receptor peroxisome proliferator-activated receptor γ 2 (PPAR- γ 2) modulates cellular differentiation and lipid accumulation during adipogenesis^[9-12], which is essential for the development of obesity. Although it is widely accepted that obesity is caused by various environmental and genetic factors, as well as the complex interactions between them, how environmental and genetic factors interact with each other in obesity remains to be elucidated.

Thus, the aim of this study was to analyze the composition of cultivable bacteria in obese individuals and their normal-weight counterparts to obtain cultivable, obesity-related gut bacteria for further study, and to investigate the potential relationship between the PPAR- γ 2 gene Pro12Ala polymorphism and cultivable gut bacteria.

MATERIALS AND METHODS

Participant recruitment and clinical laboratory tests

Participants were randomly recruited from Chengdu, a city located in southwest China. All subjects were Chengdu residents. Participants who were taking antibiotics or microecological modulators, and those who had had a gastrointestinal disease during the preceding month, were excluded from the study. Pregnant or lactating women and individuals with major systemic disorders or a history of malignant tumors were also excluded from the study.

The body height and weight of study participants were measured to determine their body mass index (BMI; kg/m²). According to the definition of obesity and normal-weight recommended by the Chinese guidelines for prevention and management of overweight and obesity in Chinese adults, subjects were divided into obese (BMI ≥ 28 kg/m²) and normal-weight (BMI 18.5-24 kg/m²) groups^[13]. Routine blood, hepatic and renal function tests and electrocardiogram and B-mode ultrasonic abdominal examinations were performed to assess the health status of each participant. Ultimately, 104 volunteers were included in the study: 52 obese and 52 normal-weight subjects. The study was approved by the Ethics Committee of Sichuan University and informed consent was obtained from all participants before data collection.

Table 1 Culture media and incubation conditions

Medium	Time (d)	Culture conditions	Bacterial group
EMB agar ¹	1	Aerobic	<i>Escherichia coli</i>
BEA agar ²	1	Aerobic	<i>Enterococci</i>
LBS agar ²	2	Aerobic with 5% CO ₂	<i>Lactobacilli</i>
BBL agar ¹	2	Anaerobic	<i>Bifidobacteria</i>
SPS agar ¹	2	Anaerobic	<i>Clostridium perfringens</i>
GAM agar ²	2	Anaerobic	<i>Bacteroides</i>

¹Purchased from Beijing Land Bridge Technology Co. Ltd., China;

²Prepared in the laboratory according to methods defined by the Chinese Ministry of Health^[16]. EMB: Eosin methylene blue; BEA: Bile esculin azide; LBS: Lactobacillus selection; BBL: Bifidobacterium culture; SPS: Sulfite-polymyxin-sulfadiazine; GAM: Gifu anaerobic medium.

Questionnaire survey

A comprehensive questionnaire was used to gather basic information as well as information on dietary intake, physical activity and defecation conditions for each participant. In the questionnaire, a 72-h diet recall for each participant was recorded, which was used to assess macronutrient intake from food composition tables for Chinese diets^[14]. For each food, participants selected their serving size, which was represented by standard cups and spoons. Questions about stool form scales and bowel frequency were also included. According to the Bristol stool scale^[15], stool forms are classified into three categories: type 1, emerging stools exhibiting fluffy pieces with ragged edges or soft blobs (transit fast, defecate easily); type 2, emerging stools exhibiting a smooth snake or sausage shape with cracks in the surface (transit normally, defecate normally); and type 3, stools that appear as separate, hard or even nut-like lumps (transit slowly, defecate with difficulty).

Fecal sample preparation, bacterial cultivation and counting

Fecal samples were obtained in the morning and quantitatively cultured for aerobic, facultative and anaerobic bacteria using the methods defined by the Chinese Ministry of Health^[16]. Briefly, freshly voided feces were collected in a sterile box. Fecal samples (10 g) were homogenized and serially diluted in sterile anaerobic solution. Appropriate dilutions were incubated aerobically or anaerobically at 37°C in duplicate using selective media within 2 h after collection. The culture times and conditions are shown in Table 1. The target bacterial colonies on each medium at the corresponding dilution were counted, and the bacteria were subsequently characterized by Gram staining and analytical profile index (API) fermentation tests (bioMérieux, France). Colony counts were expressed as the log of colony forming units per gram of wet feces.

DNA extraction and PPAR- γ 2 genotyping

The Pro12Ala polymorphism of the PPAR- γ 2 gene was characterized using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay according to the previously published methods with minor modifications^[17]. Briefly, peripheral blood was collected into

EDTA-coated tubes. Genomic DNA was isolated using a genomic DNA purification kit (SBS Genetech, China). PCR was performed in a total volume of 50 μ L, containing 30 ng of DNA, 0.4 μ mol/L of each primer (forward: 5'-GCCAATTCAAGCCAGTC-3'; reverse: 5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAA-3'), 1 \times PCR buffer, 2 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTP, and 4 U of *Taq* DNA polymerase (SBS Genetech, China). The PCR cycle conditions consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. Then, the PCR product (267 bp) was incubated with 1 U of *Hpa* II (Fermentas Life Sciences, Canada) for 4 h and digested fragments were separated by electrophoresis in a 3.5% agarose gel and visualized by staining with GoldView I (Solarbio, China). To improve the genotyping quality and validation, all samples were re-genotyped and the results were reproduced with no discrepancies.

Statistical analysis

The populations of six different types of bacteria were log-transformed for further analysis. Quantitative data were expressed as the mean \pm SD. The Student's *t* test was used to compare the amounts of cultivable bacteria in obese and normal-weight groups. Chi-squared tests were performed to analyze differences in qualitative data between the two groups. Abnormal distribution data were analyzed using nonparametric tests. The Hardy-Weinberg equilibrium was tested using the χ^2 test with one degree of freedom. Two-tailed *P* values less than 0.05 were considered statistically significant. The Statistical Program for Social Sciences 13.0 software (SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

Clinical laboratory tests

A total of 104 participants were recruited for the study: 52 obese (18 females) and 52 normal-weight (26 females) individuals. A homogeneity test indicated that the two groups were comparable with respect to age and sex (Table 2). With regard to the results of the clinical laboratory examination, total cholesterol, triglycerides and fasting glucose in the obese group were considerably higher than those in the normal-weight group ($P < 0.01$). Leukocyte counts, granulocytes and intermediate cell counts in the obese group were significantly lower than those of normal-weight subjects ($P < 0.05$). No obvious differences between the obese and normal-weight groups were detected in other parameters (data not shown).

Questionnaire analysis

Physical activity and dietary intake of energy and macronutrients demonstrated no statistical differences between the two groups (data not shown). The results obtained for stool frequency and stool form scales are shown in Figures 1 and 2, respectively. For most people in the obese (44 of 52) and normal-weight (40 of 52) groups,

Table 2 Descriptive characteristics of the study groups (mean \pm SD)

	Obese (<i>n</i> = 52)	Normal-weight (<i>n</i> = 52)
Age (yr)	34.65 \pm 11.91	33.02 \pm 10.37
Sex		
Male	34	26
Female	18	26
Body mass index (kg/m ²)	30.79 \pm 2.80 ^b	20.26 \pm 1.50
Total cholesterol (mmol/L)	4.91 \pm 0.80 ^b	4.26 \pm 0.75
Triglycerides (mmol/L)	2.33 \pm 1.22 ^b	0.95 \pm 0.37
Fasting glucose (mmol/L)	5.42 \pm 0.88 ^b	4.90 \pm 0.45
Leukocyte counts ($\times 10^9$ /L)	6.96 \pm 1.71 ^b	5.85 \pm 1.49
Intermediate cells ($\times 10^9$ /L)	0.48 \pm 0.12 ^a	0.43 \pm 0.10
Granulocytes ($\times 10^9$ /L)	4.44 \pm 1.24 ^b	3.59 \pm 1.14

^a $P < 0.05$, ^b $P \leq 0.01$ vs the normal-weight group.

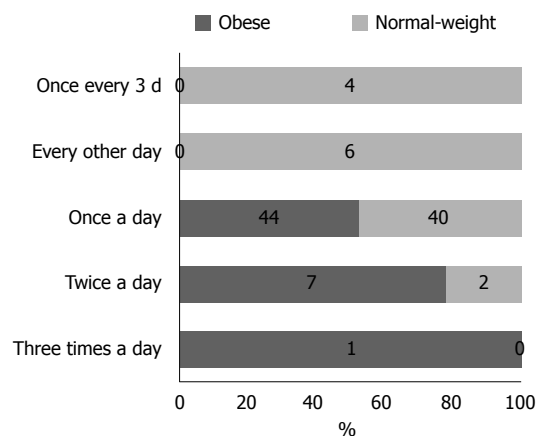


Figure 1 Stool frequency in obese and normal-weight groups. The obese group demonstrated a higher stool frequency ($U = 975$, $P < 0.001$).

stool frequency was once a day. However, stool frequency was higher in the obese group than in the normal-weight group (Figure 1; $U = 975$, $P < 0.001$). Furthermore, a notable difference in stool form scales was observed between the two groups (Figure 2; $U = 1062$, $P = 0.015$). In general, obese participants produced looser stools and defecated more easily than normal-weight controls.

Bacterial cultivation and counting

Quantitative bacterial studies (Table 3) demonstrated that the amount of *Bacteroides* and *Clostridium perfringens* (*C. perfringens*) in feces was significantly lower (*Bacteroides*, 9.81 ± 0.58 vs 10.06 ± 0.39 , $P = 0.012$; *C. perfringens*, 6.54 ± 0.65 vs 6.94 ± 0.57 , $P = 0.001$) in the obese group than in the normal-weight participants. No differences in the concentrations of *Escherichia coli*, *Enterococci*, *Lactobacilli* or *Bifidobacteria* were observed. However, there was a tendency for the amount of *Enterococci* to be higher in the obese group, despite the five other target bacteria demonstrating the reverse trend (Table 3).

Pro12Ala polymorphism in PPAR- γ 2 gene

The *PPAR- γ 2* gene polymorphism was analyzed in 88 subjects. The genotype frequencies were in Hardy-Weinberg

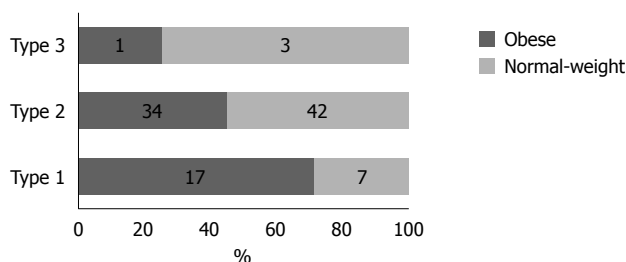


Figure 2 Stool form scale in obese and normal-weight groups. A substantial difference was observed in the stool form scale between the obese and normal-weight groups ($U = 1062$, $P < 0.05$). A greater number of participants in the normal-weight group demonstrated a type 2 (stools exhibiting a smooth snake or sausage shape with cracks in the surface) and type 3 (stools appearing in separate, hard lumps or even in a nut-like form) stool form compared with their obese counterparts (42 vs 34, 3 vs 1).

Table 3 Viable counts of the six gut bacteria in obese and normal-weight groups (mean \pm SD)

Bacterial groups	Obese ($n = 52$)	Normal-weight ($n = 52$)
<i>Bacteroides</i> ^a	9.81 \pm 0.58	10.06 \pm 0.39
<i>Escherichia coli</i>	7.76 \pm 0.92	8.09 \pm 0.81
<i>Enterococci</i>	7.53 \pm 1.05	7.27 \pm 2.07
<i>Lactobacilli</i>	7.98 \pm 1.38	8.26 \pm 0.70
<i>Bifidobacteria</i>	8.75 \pm 1.50	9.17 \pm 0.80
<i>Clostridium perfringens</i> ^b	6.54 \pm 0.65	6.94 \pm 0.57

^a $P < 0.05$, ^b $P < 0.005$ vs the normal-weight group.

Table 4 Genotype distribution and allele frequency of peroxisome proliferator-activated receptor $\gamma 2$ Pro12Ala n (%)

Group	Genotype			Allele	
	Pro/Pro	Pro/Ala	Ala/Ala	Pro	Ala
Obese group ($n = 41$)	33 (80.5)	8 (19.5)	0 (0)	74 (90.2)	8 (9.8)
Normal-weight group ($n = 47$)	40 (85.1)	7 (14.9)	0 (0)	87 (92.6)	7 (7.4)

equilibrium ($\chi^2 = 0.764$, $P = 0.382$). Pro/Pro and Pro/Ala were found in both obese and normal-weight groups; however, Ala/Ala was not detected in either group. No differences were observed in the distribution of Pro12Ala genotypes ($\chi^2 = 0.107$, $P = 0.743$) or allele frequencies ($\chi^2 = 0.300$, $P = 0.584$) between the groups (Table 4). A typical electrophoresis of the PPAR- $\gamma 2$ Pro12Ala polymorphism PCR product digested by *Hpa* II is presented in Figure 3.

Relationship between PPAR- $\gamma 2$ gene polymorphism and cultivable gut bacteria

The genetic effects of the Pro12Ala variant on the six gut bacteria were analyzed in the obese and normal-weight groups. No significant differences between the genotype groups, with respect to the six types of gut bacteria, were observed (data not shown) except in the obese group. Obese individuals with a Pro/Ala genotype had a lower amount of *Bacteroides* than those with a Pro/Pro genotype

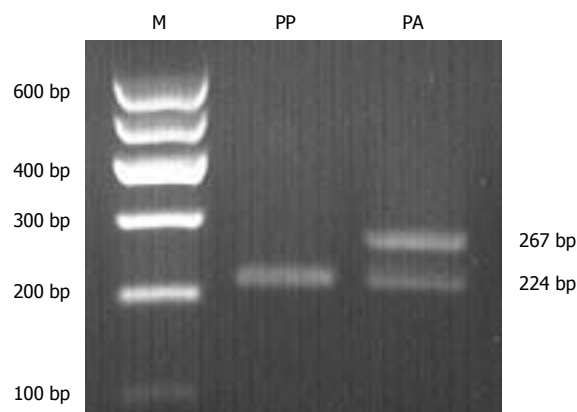


Figure 3 Genotyping analysis of peroxisome proliferator-activated receptor $\gamma 2$ Pro12Ala polymorphism by polymerase chain reaction-restriction fragment length polymorphism. M: Molecular marker; PA: Pro/Ala heterozygote; PP: Pro/Pro homozygote.

(9.45 \pm 0.62 vs 9.93 \pm 0.51, $P = 0.027$). Moreover, in our study, obese Ala allele carriers had a slightly higher BMI value than obese participants without the Ala allele (31.88 \pm 3.26 vs 31.01 \pm 2.37, $P = 0.393$); however, the difference was not statistically significant.

DISCUSSION

Since the first publication on obesity and gut bacteria in 2004^[3], molecular analysis assays have been used to reveal differences in gut bacteria between obese and non-obese individuals as well as in animal models. Although 6 years have passed, data from large-scale human studies using culture methods are lacking, possibly because it is a time-consuming and labor-intensive process. However, culture methods are indispensable for studying the relationship between obesity and gut bacteria.

Our study is the first to use culture methods to analyze differences in fecal bacteria in more than 100 participants (52 obese and 52 normal-weight individuals). Our results demonstrated that the obese people had fewer cultivable *Bacteroides* than normal-weight individuals, which is consistent with previous studies that detected fewer *Bacteroides* in obese rodent models and humans using different molecular detection methods^[4,6,18,19]. Other studies indicated that the abundance of *Bacteroides* could promote the generation of propionate, which limits lipid synthesis from acetate and may contribute to a lean phenotype^[20,21]. Our study supports this by showing that a larger amount of cultivable *Bacteroides* occurs in normal-weight people; thus, altering the amount of this cultivable bacterium in obese individuals may help them lose weight.

Bacteroides are the most dominant group of bacteria in the gut and comprise at least four key species (*B. thetaiotaomicron*, *B. vulgatus*, *B. distasonis* and *B. fragilis*)^[22,23]. *B. thetaiotaomicron* salvages energy by breaking down numerous types of otherwise indigestible polysaccharides and helps shape the metabolic milieu of the intestinal ecosystem^[24-26]; however, symbiotic roles for other members of *Bacteroides* remain unclear. *In vitro* and *in vivo* culture methods, such as

the inoculation of target species of *Bacteroides* into animal models, could facilitate further studies on the role of the different *Bacteroides*. Culture methods can also help isolate and characterize specific cultivable *Bacteroides*, and strains with desirable traits may be applied in animal models to elucidate how their mechanisms of action may be associated with obesity.

In our study, the obese group also demonstrated a lower amount of *C. perfringens* than the normal-weight group. This ubiquitous bacterium is a normal inhabitant of the mammalian colon^[27]. A previous study demonstrated that *Clostridia* produces medium-length fatty acids that increase water absorption, dry up feces, weaken stool mobility and eventually result in constipation^[28]. Another study found that lower numbers of *C. perfringens* might enhance fecal moisture content^[29]. These results may help interpret our findings that the obese group had looser feces and higher stool frequencies (Figures 1 and 2), along with lower levels of *C. perfringens*. However, no study has previously shown that *C. perfringens* interferes with host energy balance, and the role of this bacteria in the development of obesity requires further studies.

Our study also found that the amount of *Enterococci* in the obese group was higher than that in the normal-weight group. *Enterococci* are the most controversial group of gut bacteria because of their beneficial and virulent characteristics^[30], and their role in the development of obesity remains unknown. The abundance of *Enterococci* in the obese group in our study indicates that they may play a role in the development of obesity; however, this has yet to be conclusively demonstrated.

Genetic variations and gut flora could both affect obesity status. Recent evidence^[31] suggests that factors related to host genotype have an important effect on determining the bacterial composition of the gastrointestinal tract. Our study evaluated the possible relationship between polymorphism of the *PPAR-γ2* gene and gut bacteria, and found that the obese Ala allele carriers had a lower amount of *Bacteroides* than their obese counterparts without the Ala allele. This may indicate an association between reduced *Bacteroides* in the gut and the obese Ala allele carriers (Pro/Ala, BMI ≥ 28 kg/m²).

Of the potential thrifty genes, *PPAR-γ2* plays a key role in modulating adipogenic differentiation^[11,12]. During the past decade, the relationship between Pro12Ala variants of the *PPAR-γ2* gene and the obese phenotype has been investigated. However, conclusions from the different studies have been inconsistent. To resolve the apparent discrepancies, a meta-analysis was carried out to evaluate data from 19 136 subjects in 30 independent studies. This analysis demonstrated that Ala allele carriers in a recessive model with a BMI above 27 kg/m² had a significantly higher BMI than noncarriers^[32]. As described above, a lower level of *Bacteroides* might be associated with obesity. Thus, the Pro12Ala variant of the *PPAR-γ2* gene may only have an effect on individuals with a higher BMI because of the collective actions of decreased *Bacteroides* levels in the gut, the Pro12Ala polymorphism and their complex interactions on obesity status.

In this study, we tested a relatively larger sample (52 participants for each group) and decreased numbers of *Bacteroides* and *C. perfringens* were found in the obese group. However, further studies are needed to make a clear conclusion on the relationship between gut bacteria and obesity. Our study also found that obese individuals with a Pro/Ala genotype had a statistically lower level of *Bacteroides* than obese participants with a Pro/Pro genotype. However, the relationship between genotype and gut bacteria requires further elucidation. Furthermore, as this study was only confined to a Chinese population, caution should be taken when extrapolating these results to other races.

In conclusion, this study provides for the first time the data on the differences between six types of gut bacteria in obese and normal-weight Chinese individuals in a large sample (104 participants) using culture methods. We found that the obese group exhibited a lower amount of *Bacteroides* and *C. perfringens*, and a slightly higher amount of *Enterococci* than the normal-weight group. This suggests that obesity may be combated through alteration of specific cultivable gut bacteria. Moreover, our results found that gut bacteria (*Bacteroides*) might be affected by variations in human genetic factors. These results indicate that interactions between gut flora, host genetic factors and the obese phenotype might lead to development of new measures for the prevention, intervention and treatment of obesity.

COMMENTS

Background

The development of obesity is associated with various factors, but the exact etiological mechanisms still remain unclear. Previous studies demonstrated that gut bacteria along with genetic factors might be important in obesity development.

Research frontiers

The relationship between gut bacteria and obesity has been addressed in several journals like Nature and Science in recent years. But the majority of the results are based on animal experiments or on studies that involved a small number of human samples using molecular analysis. Few reports have focused on the relationship between obesity-related genes and gut flora variation. In this study, the authors demonstrated the gut bacteria alteration in obese people using culture methods and its relationship with peroxisome proliferator-activated receptor $\gamma 2$ (*PPAR-γ2*) gene polymorphism.

Innovations and breakthroughs

Recent reports demonstrated less *Bacteroidetes* in obese rodent models and humans using different molecular detection methods, and it was also confirmed in this research by culture method. This study first noticed that the obese individuals with a Pro/Ala genotype had a lower level of *Bacteroides* than obese participants with a Pro/Pro genotype. These results indicate that there may be a relationship between gut bacteria, the *PPAR-γ2* gene and obesity.

Applications

The results of this study indicate that interactions between gut flora, host genetic factors and the obese phenotype might lead to development of new measures for the prevention, intervention and treatment of obesity.

Terminology

The *PPAR-γ2* gene is one of the potential thrifty genes, which encodes the nuclear hormone receptor *PPAR-γ2*. This hormone receptor is selectively expressed in adipose tissue and is strongly up-regulated during adipogenesis, suggesting that it has a specific role in fat-cell differentiation. Thus, the *PPAR-γ2* gene might be related to the development of obesity.

Peer review

The work was well carried out and the manuscript is well written and includes potentially interesting findings.

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S- Editor Tian L L- Editor Ma JY E- Editor Zheng XM

Paclitaxel based vs oxaliplatin based regimens for advanced gastric cancer

Xiao-Dong Li, Hua Shen, Jing-Ting Jiang, Han-Ze Zhang, Xiao Zheng, Yong-Qian Shu, Chang-Ping Wu

Xiao-Dong Li, Jing-Ting Jiang, Xiao Zheng, Chang-Ping Wu, Department of Oncology, the Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu Province, China
 Hua Shen, Yong-Qian Shu, Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Han-Ze Zhang, Department of Epidemiology and Biostatistics, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Author contributions: Wu CP designed the research; Li XD and Shen H collected the data; Jiang JT, Zhang HZ, Zheng X and Shu YQ analyzed the data; Li XD and Jiang JT wrote the paper.

Supported by The National Natural Science Foundation of China, No. 30872176, 30950022 and 30972703; and Grants from Jiangsu Province of China, No. K200403, Department of Public Health and Department of Science and Technology (BS2005616)

Correspondence to: Chang-Ping Wu, MD, Professor, Chief, Department of Oncology, the Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu Province, China. wcpjtt@163.com

Telephone: +86-519-68870978 Fax: +86-519-68870978

Received: October 9, 2010 Revised: November 30, 2010

Accepted: December 7, 2010

Published online: February 28, 2011

Abstract

AIM: To compare the efficacy and safety of paclitaxel combined with fluorouracil plus cisplatin (PCF), and oxaliplatin combined with fluorouracil plus leucovorin (FOLFOX-4) regimens for advanced gastric cancer (AGC).

METHODS: Ninety-four patients with AGC were randomly assigned to receive paclitaxel (50 mg/m² iv) on days 1, 8 and 15, cisplatin (20 mg/m² iv) and fluorouracil (750 mg/m² iv) on days 1-5, or oxaliplatin (85 mg/m² iv) and leucovorin (200 mg/m² iv) on day 1, followed by bolus fluorouracil (400 mg/m² iv) and fluorouracil (600 mg/m² iv) on days 1 and 2. The primary end point was the 1-year survival time.

RESULTS: The overall response rate (ORR) of the pa-

tients was 48.0% and 45.5% to PCF and FOLFOX-4, respectively. The disease control rate (DCR) of PCF and FOLFOX-4 was 82.0% and 81.8%, respectively. The median survival times (MSTs) of the patients were 10.8 and 9.9 mo, respectively, after treatment with PCF and FOLFOX-4. The 1-year survival rate of the patients was 36.0% and 34.1%, respectively, after treatment with PCF and FOLFOX-4. No significant difference was observed in ORR, DCR, MST or 1-year survival rate between the two groups. The most common adverse events were anemia, nausea and vomiting, and grade 3/4 alopecia in PCF treatment group, and anemia, grade 1/2 neurotoxic effect and grade 3/4 neutropenia in FOLFOX-4 treatment group.

CONCLUSION: Patients with AGC have a similar response rate to PCF and FOLFOX-4 regimens with a similar survival rate. The PCF and FOLFOX-4 regimens are efficacious and tolerable as a promising therapy for AGC.

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Key words: Paclitaxel; Oxaliplatin; Advanced gastric cancer

Peer reviewer: Georgina Que, MD, Department of Surgery, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905, United States

Li XD, Shen H, Jiang JT, Zhang HZ, Zheng X, Shu YQ, Wu CP. Paclitaxel based vs oxaliplatin based regimens for advanced gastric cancer. *World J Gastroenterol* 2011; 17(8): 1082-1087 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i8/1082.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1082>

INTRODUCTION

Gastric cancer is the second leading cause of cancer-relat-

ed death worldwide, with the highest incidence in Eastern Asian and European countries^[1]. The incidence of gastric cancer in Jiangsu Province of China is particularly high, and the death rate is much higher than the national average^[2]. Unfortunately, most patients with advanced gastric cancer (AGC) have a miserable outcome. Even after curative gastrectomy, 60% of AGC patients develop local recurrences or distant metastasis^[3-5].

Although the efficacy of palliative chemotherapy is now widely accepted^[6-8], no chemotherapeutic regimen has been established as the consensus standard treatment for AGC. Among various chemotherapy regimens, paclitaxel combined with fluorouracil plus cisplatin (PCF) and oxaliplatin combined with fluorouracil plus leucovorin (FOLFOX-4) regimens are the two commonly used modalities.

It has been demonstrated that paclitaxel, an anticancer agent which binds to microtubules and induces hyperstabilization leading to cell cycle arrest and apoptosis^[9,10], has a promising efficacy against gastric cancer. The response rate of gastric cancer patients to it is about 20%-25%, and the median response time of gastric cancer patients is about 7 mo after treatment with paclitaxel^[11-13]. It was reported that the response rate of patients with gastric cancer to PCF regimen is 33%^[14-17].

Oxaliplatin, a third-generation diaminocyclohexane platinum compound that has a wide range of antitumor activities, appears to have a better safety profile than cisplatin in terms of nausea, vomiting, nephrotoxicity, and ototoxicity^[18,19]. The response rate of AGC patients to FOLFOX-4 regimen is 38%-43% and FOLFOX-4 regimen shows a manageable toxicity profile as the first-line treatment modality for AGC^[20-24].

As is commonly known, there is only one best regimen at one time. No study is available comparing the efficacy and safety of PCF and FOLFOX-4 regimens. Therefore, we designed the present study to observe the therapeutic indexes of the two regimens for AGC.

MATERIALS AND METHODS

Patients

The inclusion criteria for AGC patients were (1) pathologically proved locally advanced (non-resectable) or metastatic gastric cancer; (2) age between 20 and 75 years; (3) measurable or assessable lesions by imaging studies according to the RECIST guidelines^[25]; (4) no prior chemotherapy except for postoperative adjuvant chemotherapy for more than 12 mo before entry into the study; (5) Eastern Cooperative Oncology Group (ECOG) performance status 0-2; (6) adequate bone marrow functions (hemoglobin level ≥ 90 g/L, white blood cell count of $4-10 \times 10^9$ /L, neutrophil count $\geq 2 \times 10^9$ /L, and platelet count $\geq 100 \times 10^9$ /L), hepatic function (total bilirubin $\leq 1.5 \times$ the institutional upper limit of normal value, aspartate aminotransferase/alanine aminotransferase $\leq 2.5 \times$ the institutional upper limit of normal value, and

alkaline phosphatase $\leq 2.5 \times$ the institutional upper limit of normal value), renal function (serum creatinine level ≤ 1.5 mg/dL and creatinine clearance ≥ 50 mL/min); and (7) estimated life expectancy of at least 3 mo and no other malignancies.

The exclusion criteria for patients included (1) pre-existing peripheral toxicity \geq grade 2 of the National Cancer Institute Common Toxicity Criteria (NCI-CTC, Version 3.0); (2) pregnant, and breastfeeding women or women of child-bearing potential without adequate contraception; (3) concurrent or prior malignancy; (4) central nervous system metastases; (5) active infection; (6) other uncontrolled underlying medical conditions that would impair the ability of the patients to receive the planned treatment; (7) inadequate calorie and fluid intake; and (8) concurrent treatment that interfered with the study evaluation.

The study, approved by the ethics committees of all participating medical institutions, was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines. All patients gave their written informed consent before enrollment.

Treatment methods

The patients were divided into PCF group and FOLFOX-4 group. Patients in the PCF group received paclitaxel (50 mg/m^2 iv) for 3 h on days 1, 8 and 15, cisplatin (20 mg/m^2 iv) for 2 h on days 1-5, fluorouracil (750 mg/m^2 iv) for 24 h for 5 d. The treatment was repeated every 28 d for 6 cycles. Patients in the FOLFOX-4 group received oxaliplatin (85 mg/m^2 iv) and leucovorin (200 mg/m^2 iv) for 2 h on day 1, bolus fluorouracil (400 mg/m^2 iv) and fluorouracil (600 mg/m^2 iv) for 22 h on days 1 and 2. The treatment was repeated every 14 d for 12 cycles.

The dose was modified based on the hematologic parameters and the degree of non-hematologic toxicities. Physical examination, chest X-ray, complete blood test and biochemical tests were performed before each chemotherapy cycle. The toxicity was graded based on the NCI-CTC (Version 3.0).

Dose modification

The dose was modified for the PCF group as follows: (1) If the hepatotoxicity was grade 2, the dose of paclitaxel for the following treatment was reduced to 40 mg/m^2 on days 1, 8 and 15. If the hepatotoxicity was grade 3/4, the study was discontinued; (2) If the bone marrow suppression was grade 4, the dose of paclitaxel for the following treatment was reduced to 40 mg/m^2 on days 1, 8 and 15. If the bone marrow suppression was grade 4, the study was discontinued; (3) If the mucositis was grade 3/4, fluorouracil was administered from the next cycle for 3 d; and (4) If the creatinine clearance rate was 30-50 mL/min due to the nephrotoxicity, the dose of cisplatin was reduced by 50%. If the creatinine clearance rate was lower than 30 mL/min, the study was discontinued.

The dose was modified for the FOLFOX-4 group as

follows: (1) If the neurotoxic effect was grade 1/2, the dose of oxaliplatin was reduced by 25%. If the neurotoxic effect was grade 3/4 or persistent, the oxaliplatin was omitted from the regimen until the neurotoxic effect was resolved to grade 1 or better; (2) If the mucositis was grade 3/4, fluorouracil was administered from the next cycle for 3 d; and (3) If grade 3/4 diarrhea, stomatitis or dermatitis occurred, the dose of fluorouracil was reduced by 25%.

Evaluation

The parameters of 12-lead electrocardiogram, computed tomography (CT) scan, and levels of tumor markers (CA19-9, CA72-4, CA24-2 and carcinoembryonic antigen) were obtained from the patients within 7 d after enrollment. Hematology tests, biochemistry tests, and assessment of symptoms and signs were carried out for the patients within 3 d before enrollment and every week during the study period. CT scans were carried out and levels of tumor markers were measured before each cycle. According to the RECIST guidelines^[17], responses concluded complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). To confirm the PR or CR, the levels of tumor markers were measured no less than 4 wk after the objective response was obtained. Responses were assessed by the independent review committee. The overall response rate (ORR) was defined as the sum of CR and PR rates. The disease control rate (DCR) was defined as the sum of CR, PR and SD rates. Toxic effects were evaluated according to the NCI-CTC (Version 3.0). The overall survival time (OST) was defined as the period from the date of treatment to the death of patients. The median survival time (MST) was defined as the half of OST.

Statistical analysis

Statistical analysis was performed with the SPSS software (Version 17.0, SPSS). Chi-square test was used to compare the categorical data. KaplanMeier method was used to calculate the OST. Logrank test was used to compare the OST. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of patients

From January 2003 to December 2007, 94 patients were enrolled in this study. The baseline clinical characteristics of the patients were compared between the two groups (Table 1). No significant difference was observed in any clinical characteristics between the two groups.

Objective response

All the patients were evaluated for their response to PCF and FOLFOX-4 regimens and no patient was excluded from the efficacy analysis because of severe side effects.

Of the patients in PCF group, 1 achieved a CR and 23 a PR, 17 had SD, and 9 PD, with an ORR of 48%. The re-

Table 1 Baseline characteristics of patients enrolled in this study ($n = 73$)

Characteristics	Patients (%)		<i>P</i>
	PCF (<i>n</i> = 50)	FOLFOX-4 (<i>n</i> = 44)	
Gender			
Female	18	13	0.520
Male	32	31	
Age (yr)			
Median	59	58	0.876
Range	20-74	20-75	
Histologic type			
Adenocarcinoma	34	36	0.158
Adenosquamous carcinoma	3	1	
Signet ring cell carcinoma	5	3	
Mucinous carcinoma	7	3	
Neuroendocrine carcinoma	1	1	
No. of metastatic lesion			
0-1	24	21	1
≥ 2	26	23	
Stage			
IIIb	22	17	0.677
IV	28	27	
Prior adjuvant chemotherapy			
No	38	31	0.642
Yes	12	13	

PCF: Paclitaxel combined with fluorouracil plus cisplatin; FOLFOX-4: Oxaliplatin combined with fluorouracil plus leucovorin.

sponse rate of patients who received prior chemotherapy to PCF was 52.6% (20/38) and 33.3% (4/12), respectively, with a DCR of 82.0%.

Of the patients in FOLFOX-4 group, 1 achieved a CR and 19 a PR, and 16 had SD and 8 PD, with an ORR of 45.5%. The response rate of patients who received prior chemotherapy to FOLFOX-4 was 54.8% (17/31) and 23.1% (3/13), respectively, with a DCR of 81.8%. No significance was observed in ORR and DCR between the two groups.

Survival analysis

The MST and the 1-year survival rate of patients in the PCF group was 10.8 mo (95% CI: 8.9-12.7 mo) and 36.0%, respectively.

The MST and the 1-year survival rate of patients in the FOLFOX-4 group was 9.9 mo (95% CI: 8.3-11.4 mo) and 34.1%, respectively.

No significant difference was found in MST and survival rate between the two groups (Figure 1).

Profile of safety and adverse events

No patient was excluded from the efficacy analysis because of severe side effects. Grade 3/4 neutropenia occurred in 8% and 10% of patients in the PCF and FOLFOX-4 groups, respectively. Anemia occurred in 60% and 57% of patients in the PCF and FOLFOX-4 groups, respectively. Nausea and vomiting occurred in 12.0% and 9% of patients in the PCF and FOLFOX-4 groups, respectively.

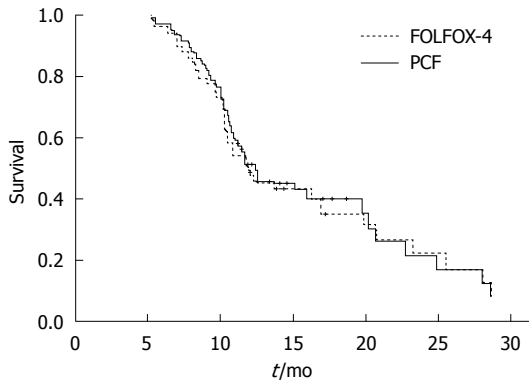


Figure 1 Overall survival curves for patients in PCF and FOLFOX-4 groups. PCF: Paclitaxel combined with fluorouracil plus cisplatin; FOLFOX-4: Oxaliplatin combined with fluorouracil plus leucovorin.

Grade 3/4 alopecia occurred in 10.0% and 0% of patients in the PCF group and FOLFOX-4 group, respectively ($P < 0.05$). Grade 1/2 neurotoxic effect was observed in 6% and 17% of patients in the PCF group and FOLFOX-4 group, respectively ($P < 0.05$).

Diarrhea, hepatic or renal toxicities and oral mucosa ulcer were relatively infrequent and slight.

DISCUSSION

In China, gastric cancer patients are usually diagnosed at a relative advanced stage with metastasis to other organs^[26]. Although a number of treatment modalities for gastric cancer such as surgical resection combined with chemotherapy are available^[27,28], no current regimen can be considered a standard therapy for AGC, thus new therapeutic strategies are required to achieve a better clinical efficacy with an acceptable toxicity profile. PCF and FOLFOX-4 regimens are commonly used as the first-line therapy for AGC. The efficacies of the two regimens against AGC are similar with different adverse events.

Taxane is one of the three milestones of anti-cancer drugs, used in the 1990s. It was reported that the ORR of AGC patients to PCF is 34%-52%^[13]. The survival time of most AGC patients does not exceed 12 mo after PCF therapy with taxane or 5-FU plus cisplatin^[11,12]. Kim *et al*^[14] reported that the MST of AGC patients is 13.2 mo. The results of this study are consistent with the reported findings^[11,12]. Overall, the efficacy of PCF against AGC is stable.

Oxaliplatin has been used in treatment of advanced colorectal carcinoma. It has been shown that the response rate of patients with advanced colorectal carcinoma to oxaliplatin in combination with fluorouracil is 36%-58%^[29-31]. Vita *et al*^[32] revealed that the ORR of patients with advanced colorectal carcinoma to oxaliplatin is 38% with a TTP of 7.1 mo and an OST of 11.2 mo. The results of the current study indicate that a biweekly FOLFOX-4 regimen can significantly improve the symptoms of AGC patients. The decreased ORR observed in our study might be related to the selected gastric cancer patients at IIIb

or IV stage. Considering the smaller sample size and the modified doses in the present study, further study is warranted to confirm the results.

It was reported that the toxic rate of FOLFOX-4 regimen for grade 3/4 neutropenia, and nausea and vomiting is 7.6%-34% and 4%-18.1%, respectively^[13], which is consistent with our results. In the present study, the neurotoxic rate of FOLFOX-4 regimen for diarrhea and oral mucosa ulcer was low, which might be due to the low PS score. The reported neurotoxic rate of PCF regimen for grade 3/4 neutropenia is 22%-86%^[12,33], which is also consistent with our results. Alopecia occurred more frequently in PCF group than in FOLFOX-4 group, and vice versa. Overall, these regimens may not only prolong the survival time but also for improve the life quality of gastric cancer patients.

In summary, both PCF and FOLFOX-4 regimens can be used in treatment of AGC. Further study is warranted to confirm the results of this study.

COMMENTS

Background

Gastric cancer is the second most common cause of cancer-related death globally. Its incidence is the highest in Eastern Asian and European countries. Unfortunately, most gastric cancer patients are at the advanced stage when they are diagnosed. The outcome of patients with advanced gastric cancer (AGC) is poor. Chemotherapy is often used for AGC and its efficacy is now widely accepted. However, no standard combination of chemical drugs has been established. Among the different combinations, paclitaxel combined with fluorouracil plus cisplatin (PCF) and oxaliplatin combined with fluorouracil plus leucovorin (FOLFOX-4) regimens are the two commonly used modalities. There is only one best regimen at one time. No study comparing the efficacy and safety of PCF and FOLFOX-4 regimens is available at present. Therefore, we designed the present phase-2 study to observe the therapeutic indexes of the two regimens for AGC.

Research frontiers

No current regimen can be considered as a standard therapy for AGC. PCF and FOLFOX-4 regimens are the commonly used first-line therapy for AGC. The overall survival rate (ORR) of AGC patients is 34%-52% after PCF therapy. The survival time of most AGC patients after PCF therapy with taxane, or 5-FU plus cisplatin does not exceed 12 mo. It was reported that the response rate of AGC patients to FOLFOX-4 regimen is 36%-58%. Both PCF and FOLFOX-4 regimens can be used in treatment of AGC.

Innovations and breakthroughs

No study comparing the efficacy and safety of PCF and FOLFOX-4 regimens. Therefore, we designed the present phase II study to observe the therapeutic indexes of the two regimens for AGC. The results of this study show that the two regimens are benefit not only for survival but also for quality of life patients after resection of colorectal cancer.

Applications

PCF regimen can be used for those who need to do fine jobs, and FOLFOX-4 regimen can be used for those who care more about their appearance. The current study may help patients to take more consideration about various demands in daily life.

Peer review

The present study provides important data about PCF and FOLFOX-4 regimens for gastric cancer and the article is well written.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Zheng XM

Tulip bundle technique and fibrin glue injection: Unusual treatment of colonic perforation

Filippo Mocciaro, Gabriele Curcio, Ilaria Tarantino, Luca Barresi, Marco Spada, Sergio Li Petri, Mario Traina

Filippo Mocciaro, Gabriele Curcio, Ilaria Tarantino, Luca Barresi, Mario Traina, Department of Gastroenterology, IsMeTT, UPMC, Via tricoli 1, Palermo 90100, Italy
 Marco Spada, Sergio Li Petri, Surgery Unit, IsMeTT, UPMC, Via tricoli 1, Palermo 90100, Italy

Author contributions: Mocciaro F collected data and wrote the article; Curcio G and Tarantino I, Barresi L participated in drafting the article, collected data and reviewed the final version; Spada M and Li Petri S performed the operation; Traina M was the lead investigator, performed the endoscopy, and reviewed the final version.

Correspondence to: Filippo Mocciaro, MD, PhD, Department of Gastroenterology, IsMeTT, Via Tricoli 1, Palermo 90100, Italy. fmocciaro@gmail.com

Telephone: +39-91-900347 Fax: +39-91-2192400

Received: August 22, 2010 Revised: November 11, 2010

Accepted: November 18, 2010

Published online: February 28, 2011

Peer reviewer: AM El-Tawil, MSc, MRCS, PhD, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor, Birmingham, B15 2TH, United Kingdom

Mocciaro F, Curcio G, Tarantino I, Barresi L, Spada M, Li Petri S, Traina M. Tulip bundle technique and fibrin glue injection: Unusual treatment of colonic perforation. *World J Gastroenterol* 2011; 17(8): 1088-1090 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i8/1088.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i8.1088>

INTRODUCTION

Colonoscopy is considered a safe procedure, although complications can occur. The most dreaded of these is iatrogenic perforation. The literature reports perforation rates of 0.03%-0.8% for diagnostic procedures, and a rate of 0.15%-3% for therapeutic procedures^[1]. Mechanisms of perforation are the result of either mechanical disruption of the colonic wall (e.g. thermal injury, forced push into a diverticulum, or stretching of the bowel with loops or the slide-by technique) or excessive air insufflation^[1]. After perforation, prompt abdominal surgery is usually recommended, particularly in the last few years, following the introduction of laparoscopic approaches in clinical practice^[2,3]. Nevertheless, conservative treatment is a feasible and effective option for patients who are clinically stable and without peritonism or life threatening signs. We describe a case of a sigmoid perforation repaired with endoclips and endoloops, and sealed with fibrin glue. The effectiveness of this approach was confirmed on laparoscopic examination.

CASE REPORT

A 63-year-old male with a history of polypectomy for a large polyp of the caecum was admitted to our department for a scheduled colonoscopy. Physical examination, blood chemistry and coagulation tests were normal. After obtaining informed consent, the colonoscopy was per-

Abstract

We report a case of a 63-year-old male who experienced an iatrogenic sigmoid perforation repaired combining three endoscopic techniques. The lesion was large and irregular with three discrete perforations, therefore, we decided to close it by placing one clip per perforation, and then connecting all the clips with two endoloops. Finally we chose to use a fibrin glue injection to obtain a complete sealing. Four days after the colonoscopy the patient underwent a laparoscopic right hemicolectomy due to evidence of a large polyp of the caecum with high grade dysplasia and focal carcinoma *in situ*. Inspection of the sigma showed complete repair of the perforation. This report underlines how a conservative approach, together with a combination of various endoscopic techniques, can resolve complicated iatrogenic perforations of the colon.

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Key words: Colonic perforation; Endoscopic treatment; Fibrin glue injection; Tulip bundle technique

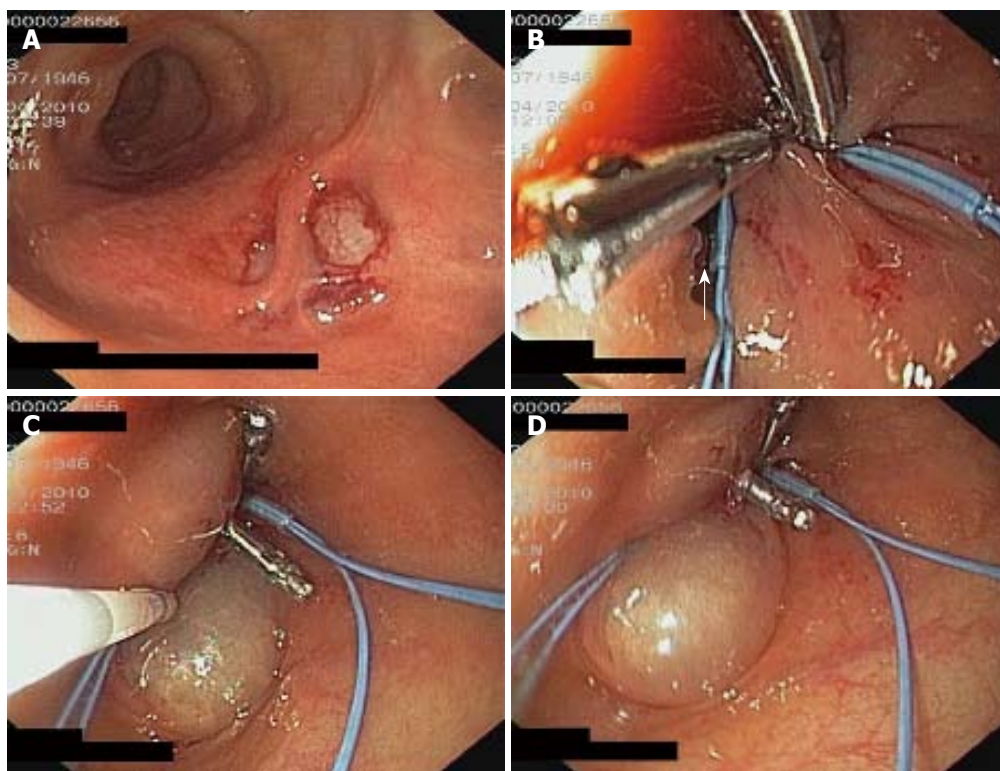


Figure 1 Figure shows the colonic perforation (A), two borders of the perforation did not fit well (arrow) after clips and endoloops placement (B) and completely sealing after fibrin glue injection (C, D).

formed under conscious sedation (propofol). The colon was quite clean, and the colonoscopy proceeded without difficulty, lasting 15 min. Caecum examination, at the same site of the previous polypectomy, showed a large sessile polyp (4 cm in diameter), which was biopsied. No other polyps were detected, although in the sigma, in an environment of diverticula and hyperaemic mucosa, a large and irregular lesion (2.5 cm with 3 discrete perforations) was observed (Figure 1A). The patient's blood pressure, heart rate and O₂ saturation were normal. Due to the form of the lesion, we believed that repair with endoclips alone would have been ineffective, so we decided to close it by placing through-the-scope endoclips over each perforation of the lesion (one clip per perforation), and then connecting all the clips with two endoloops (Figure 1B). Each endoloop was tightened slightly and the endoclips were anchored together securely. However, at the base, two borders of the perforation did not fit well (Figure 1B, arrow), so we chose to use a fibrin glue (Beriplast P®), injected into the submucosa, and obtained complete sealing (Figure 1C and D). Four days after the colonoscopy, the patient underwent a laparoscopic right hemicolectomy due to evidence of high grade dysplasia with focal carcinoma *in situ*, revealed by biopsies of the polyp. Inspection of the sigma showed complete repair of the perforation (Figure 2, arrow). The patient was able to resume oral diet 4 d later and was discharged 7 d later.



Figure 2 Complete repair of the perforation at laparoscopy (arrow).

a diagnostic, therapeutic, and screening tool^[4]. It is safe, with a low rate of perforation (a post-procedure incidence of 0.082% at 7 d in a population-based study of 277 434 colonoscopies^[5]). Risk factors are increasing age, significant comorbidities, obstruction as an indication for colonoscopy, diverticulosis, and invasive interventions during colonoscopy^[3]. Despite the fact that laparoscopic resection is effective in resolving colonic perforation caused by colonoscopy^[2], recent improvements in endoscopic techniques have made it possible to close iatrogenic colonic perforations using mini-invasive procedures. Repair with endoclips has been well described in the literature since 1997^[6-8]. Furthermore, large or difficult intestinal perforations can be treated with a combined placement of endoclips and endoloops, as recently reported by Nakagawa *et al*^[9]. There is little documentation of the use

DISCUSSION

Colonoscopy has played an increasingly important role as

of fibrin sealant for repairing perforations^[10], however, some reports suggest that endoclip placement with fibrin glue sealing is effective in repairing benign lesions, such as fistulas^[11]. In our patient, conservative treatment of the colonic perforation was quite difficult because of both the size and shape of the lesion. An approach using only endoclips may have proved to be ineffective, but endoloop placement made it possible to anchor the clips together. Unfortunately, when closed with the endoloops, the star-like points of the lesion did not fully close the perforation, so we decided to seal the base of the perforation with a submucosal injection of fibrin glue. This conservative approach resulted in full closure of the lesion, as subsequently confirmed by laparoscopic examination.

This report underlines how a conservative approach, together with a combination of various endoscopic techniques, can resolve complicated perforations without the need for surgical intervention.

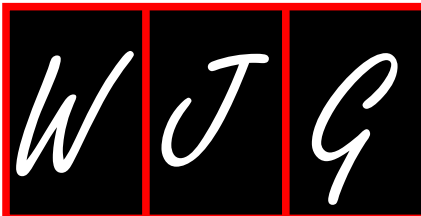
ACKNOWLEDGMENTS

We thank Warren Blumberg for editorial assistance.

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S- Editor Sun H L- Editor Webster JR E- Editor Lin YP



ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Juan-Ramón Larrubia, PhD, Gastroenterology Unit and Liver Research Unit., Guadalajara University Hospital, Donante de Sangre s/n, 19002 Guadalajara, Spain

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Dr. Oliver Mann, MD, Associate Professor, Senior Attending Physician and Deputy Director, Department of General, Visceral and Thoracic Surgery, University of Hamburg, Martini Str. 52, D-20246 Hamburg, Germany

Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

Masahiro Iizuka, MD, PhD, Director, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23, Nakadori, Akita, 010-0001, Japan

Sri P Misra, Professor, Gastroenterology, Moti Lal Nehru Medical Col-

lege, Allahabad 211001, India

Beat Schnüriger, MD, University of Southern California, Keck School of Medicine, Department of Surgery, Division of Acute Care Surgery, (Trauma, Emergency Surgery and Surgical Critical Care), 1200 North State Street, Inpatient Tower (C), 5th Floor, Room C5L100, Los Angeles, California 90033-4525, United States

Boris Kirshtein, MD, Department of Surgery "A", Soroka Medical Center, Ben Gurion University of the Negev, POB 151, Beer Sheva, 84101, Israel

Yuk Him Tam, Dr., Department of Surgery, Prince of Wales Hospital, Shatin, Hong Kong, China

Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Keiji Hirata, MD, Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Tamara M Alempijevic, MD, PhD, Assistant Professor, Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Serbia

Shannon S Glaser, Dr., Department of Internal Medicine, Scott & White Hospital, 702 SW HK Dodgen Loop, Medcial Research Building, Temple 76504, United States.

Yoshiharu Motoo, MD, PhD, FACP, FACC, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Richard A Rippe, Dr., Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States



Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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Name of journal

World Journal of Gastroenterology

Serial publication number

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

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by

Baishideng Publishing Group Co., Limited

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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