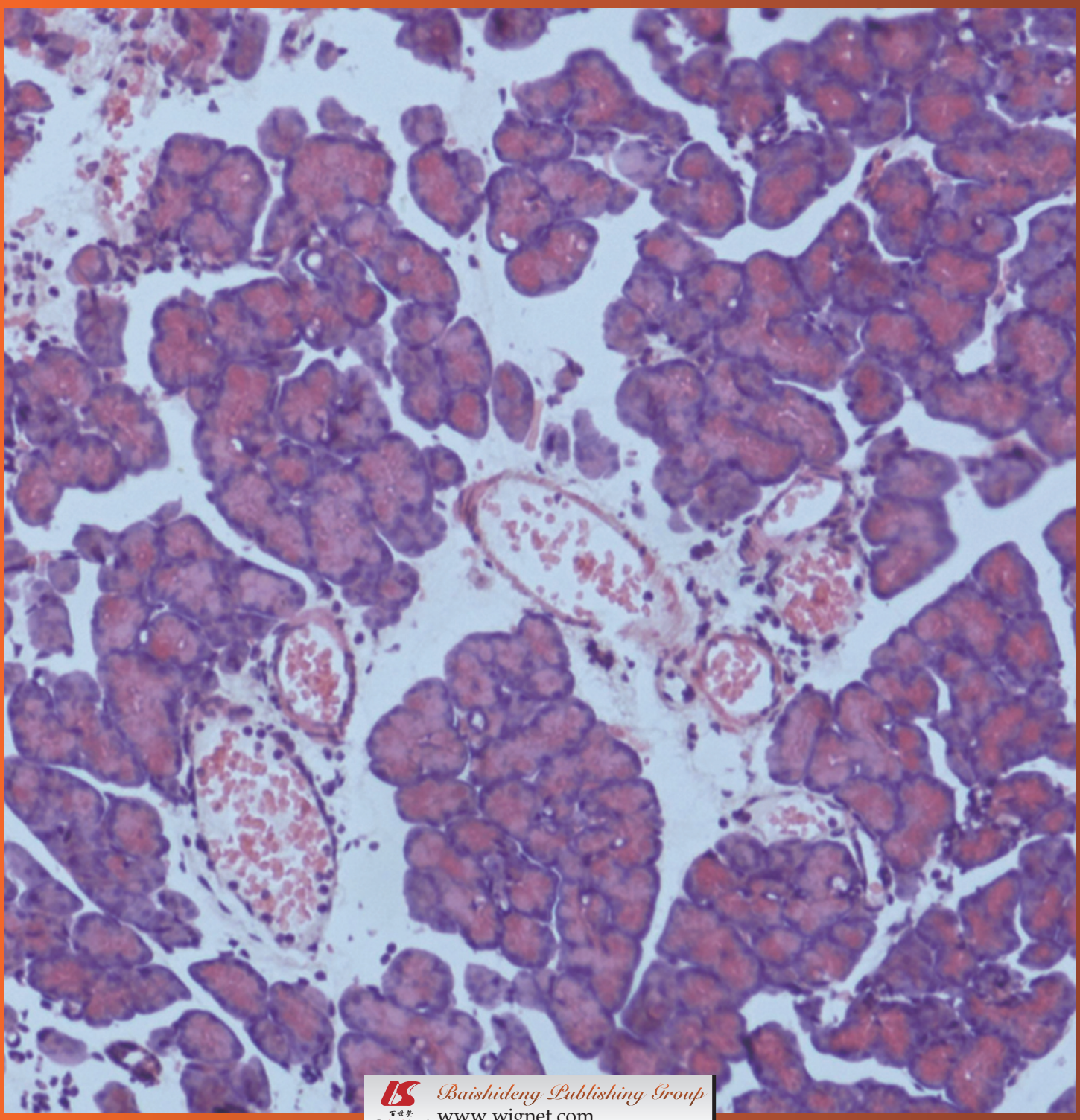
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2011 May 7; 17(17): 2161-2258



Baishideng Publishing Group
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World Journal of Gastroenterology

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

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Jin-Hong Kim, *Suwon*
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Sang Geon Kim, *Seoul*
Seon Hahn Kim, *Seoul*
Sung Kim, *Seoul*
Won Ho Kim, *Seoul*
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Martin D Zielinski, *Rochester*
Michael A Zimmerman, *Colorado*

WJG World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 17 May 7, 2011

EDITORIAL

2161 Wound healing of intestinal epithelial cells

Iizuka M, Konno S

TOPIC HIGHLIGHT

2172 Mallory-Denk Bodies in chronic hepatitis

Basaranoglu M, Turhan N, Sonsuz A, Basaranoglu G

REVIEW

2178 Asymmetric dimethylarginine: A novel biomarker of gastric mucosal injury?

Zhang Z, Zou YY, Li FJ, Hu CP

ORIGINAL ARTICLE

2181 Soluble ST2: A new and promising activity marker in ulcerative colitis

Díaz-Jiménez D, Núñez LE, Beltrán CJ, Candia E, Suazo C, Álvarez-Lobos M, González MJ, Hermoso MA, Quera R

2191 Parallel decline of CD8+CD38+ lymphocytes and viremia in treated hepatitis B patients

Cao W, Qiu ZF, Li TS

BRIEF ARTICLE

2199 Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems

Glanemann M, Knobeloch D, Ehnert S, Culmes M, Seeliger C, Seehofer D, Nussler AK

2206 Value of transient elastography for the prediction of variceal bleeding

Sporea I, Rațiu I, Şirli R, Popescu A, Bota S

2211 Increased intestinal permeability in inflammatory bowel diseases assessed by iohexol test

Gerova VA, Stoynov SG, Katsarov DS, Svinarov DA

2216 Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder

Cha BH, Hwang JH, Lee SH, Kim JE, Cho JY, Kim H, Kim SY

2223 Topical application of glycyrrhizin preparation ameliorates experimentally induced colitis in rats

Kudo T, Okamura S, Zhang Y, Masuo T, Mori M

2229 Hyperbaric oxygenation promotes regeneration of biliary cells and improves cholestasis in rats

Idetsu A, Suehiro T, Okada K, Shimura T, Kuwano H

2236 Limited water infusion decreases pain during minimally sedated colonoscopy

Hsieh YH, Lin HJ, Tseng KC

2241 Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats

Ji RL, Xia SH, Di Y, Xu W

2248 Specific HLA-DQB1 alleles associated with risk for development of hepatocellular carcinoma: A meta-analysis

Xin YN, Lin ZH, Jiang XJ, Zhan SH, Dong QJ, Wang Q, Xuan SY

CASE REPORT

2255 Neoadjuvant sorafenib combined with gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma

Williet N, Dubreuil O, Boussaha T, Trouilloud I, Landi B, Housset M, Botti M, Rougier P, Belghiti J, Taieb J

World Journal of Gastroenterology
Volume 17 Number 17 May 7, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Ji RL, Xia SH, Di Y, Xu W. Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats. World J Gastroenterol 2011; 17(17): 2241-2247 http://www.wjgnet.com/1007-9327/full/v17/i17/.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.
FLYLEAF	I-VII	Editorial Board

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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

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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
http://www.wjgnet.com

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.

PUBLICATION DATE
May 7, 2011

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

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SPECIAL STATEMENT
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2161

World J Gastroenterol 2011 May 7; 17(17): 2161-2171
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Wound healing of intestinal epithelial cells

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Supported by (in part) Health and Labour Sciences Research Grants for research on intractable diseases from Ministry of Health, Labour and Welfare of Japan
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Received: September 11, 2010 Revised: January 15, 2011
Accepted: January 22, 2011
Published online: May 7, 2011

Abstract

The intestinal epithelial cells (IECs) form a selective permeability barrier separating luminal content from underlying tissues. Upon injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the balance of three cellular events; restitution, proliferation, and differentiation of epithelial cells adjacent to the wounded area. Previous studies have shown that various regulatory peptides, including growth factors and cytokines, modulate intestinal epithelial wound healing. Recent studies have revealed that novel factors, which include toll-like receptors (TLRs), regulatory peptides, particular dietary factors, and some gastroprotective agents, also modulate intestinal epithelial wound repair. Among these factors, the activation of TLRs by commensal bacteria is suggested to play an essential role in the maintenance of gut homeostasis. Recent studies suggest that mutations and dysregulation of TLRs could be major contributing factors in the predisposition and perpetuation of inflammatory bowel disease. Additionally, studies have shown that specific signaling pathways are involved in IEC wound repair. In this review, we summarize the function of IECs, the process of intestinal epithelial wound healing, and the functions and mechanisms of the various factors that contribute to gut homeostasis and intestinal epithelial wound healing.

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Key words: Intestinal epithelial cell; Wound healing; Restitution; Growth factors; Toll-like receptor

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Iizuka M, Konno S. Wound healing of intestinal epithelial cells. *World J Gastroenterol* 2011; 17(17): 2161-2171 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2161.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2161

INTRODUCTION

The surface of the gastrointestinal tract is covered with epithelial cells that function under physiological conditions as a barrier preventing undesirable luminal antigens from entering the body[1]. Upon injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area[2]. Previous studies have shown that various regulatory peptides, including growth factors and cytokines, modulate intestinal epithelial wound healing[3]. Recent studies have revealed that novel factors, which include toll-like receptors (TLRs)[4], regulatory peptides[5], particular dietary factors[6,7], and some gastroprotective agents[8-10], also modulate intestinal epithelial wound repair. In addition, it has been shown that the activation of specific signaling pathways is involved in intestinal epithelial wound healing[11,12].

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory intestinal disorders and known as

inflammatory bowel disease (IBD). Although an etiology of both diseases remains unknown, it is suggested that disruption of the intestinal barrier function and repeated intestinal epithelial damage are key features of IBD, as well as other intestinal disorders[1,3]. In this context, it is suggested that the identification of the specific factors that improve wound healing of the intestinal epithelium might contribute to therapeutic strategies in IBD, and many studies using animal colitis models and intestinal epithelial cell (IEC) lines have been conducted.

We summarize the function of intestinal epithelium, the process of intestinal epithelial wound healing, and the functions and mechanisms of the various factors that contribute to intestinal epithelial wound healing and gut homeostasis. We also discuss the association of some of these factors with IBD.

BARRIER FUNCTION OF INTESTINAL EPITHELIAL CELLS

The intestinal epithelial cells (IECs) form a selective permeability barrier separating luminal content from underlying tissues[1,13]. The gastrointestinal epithelial lining consists of a monolayer of columnar cells[13]. This monolayer of IECs is constantly moving at a speed of 5-10 μm/h[14] and is renewed every 2-5 d. The maintenance of this barrier is critical for normal growth, development, and disease prevention[15]. Normally, IECs function as a barrier that prevents undesirable solutes, microorganisms, viruses, and luminal antigens from entering the body[1,16]. Several elements that participate in the barrier function include the epithelial cells themselves along with tight junctions, adherens junctions, and luminal secretions such as mucus or unstirred layers on the apical aspects of the epithelium[1].

Tight junctions function as semi-permeable gates that regulate the passive movement of luminal fluid and solutes through the paracellular pathway, and limit passive diffusion of proteins and lipids between the outer leaflet of the apical and basolateral plasma membrane domains[1,17]. Subjacent to tight junctions, adherens junctions are important in regulating intercellular adhesion[1]. Both tight junctions and adherens junctions are positioned in the apical end of the lateral plasma membrane, and are intimately linked in their regulation and function. Thus, the tight junctions and adherens junctions are collectively referred to as the apical junctional complex (AJC)[1,13,17]. Major transmembrane proteins in the AJC include occludin, claudins, junctional adhesion molecules (JAMs), coxsackie adenovirus receptor (CAR), and E-cadherin[1]. Subjacent to the AJC are spot-like intercellular junctions referred to as desmosomes; the function of which in IECs is poorly understood[13].

Despite the barrier function of IECs, this barrier has to allow some non-pathological gut-derived bacteria access to the immune system, thereby promoting maturation of the immune system and evolution of immune tolerance[1]. As is described later, recent studies have demonstrated that the activation of TLRs by commensal bacteria plays an essential role in inhibiting inflammatory responses and maintaining colonic homeostasis[18].

PROCESS OF WOUND HEALING OF INTESTINAL EPITHELIAL CELLS

The intestinal epithelium can be injured by toxic luminal substances, normal digestion, inflammation, interactions with microbes, oxidative stress, and pharmaceuticals, despite its barrier function[19,20]. After injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area[2]. First, epithelial cells surrounding the wound lose their columnar polarity, take on a flattened morphology, and rapidly migrate into the denuded area to restore barrier integrity. This process has been termed "epithelial restitution"[3,15,21]. Restitution starts within minutes to hours of injury and is independent of proliferation[3,21]. Then, proliferation of the mucosal epithelium to increase the pool of enterocytes available to resurface the defect generally begins hours or days after the injury[21]. Finally, maturation and differentiation of epithelial cells is needed to maintain the mucosal barrier function[3]. Rat IECs (IEC-6) at 24 h after wound formation are shown in Figure 1A. Most of the epithelial cells found in the wounded area across the wound border are thought to have migrated in the process of restitution. The process of repair of IEC-6 cells wounded by mechanical cell denudation is shown in Figure 1B. These wound assays were performed in our laboratory.

During the restitution of IECs, extensive reorganization of the actin cytoskeleton is necessary[22]. The organization and remodeling of the actin cytoskeleton is suggested to be controlled by the Rho family of small GTPases, which includes Rho, Rac and Cdc42[23]. These proteins have been implicated in the formation of stress fibers, lamellipodia (cytoskeletal protein actin projection on the mobile edge of the cell), and filopodia (slender cytoplasmic projections, similar to lamellipodia, which extend from the leading edge of migrating cells)[23]. In detail, Rho regulates stress fibers and focal adhesion assembly; Rac regulates the formation of lamellipodia protrusions and membrane ruffles; and Cdc42 triggers filopodial extensions at the cell periphery[24,25]. The wound margin of IEC-6 cells is shown in Figure 1C. Lamellipodia- and filopodia-like epithelial cells are found at the leading edge of the wounded IEC-6 cells (Figure 1C). Other investigations regarding the mechanism of cell migration have also been reported[26-28].

Chemokines can be subdivided into distinct inflammatory or homeostatic subsets, with the latter being minimally regulated by pro-inflammatory cytokine stimulation[29]. It has been shown in leukocytes that Rho-GTPase regulates homeostatic chemokine receptor CXCR4-mediated chemotaxis or metastasis to the sites of cognate

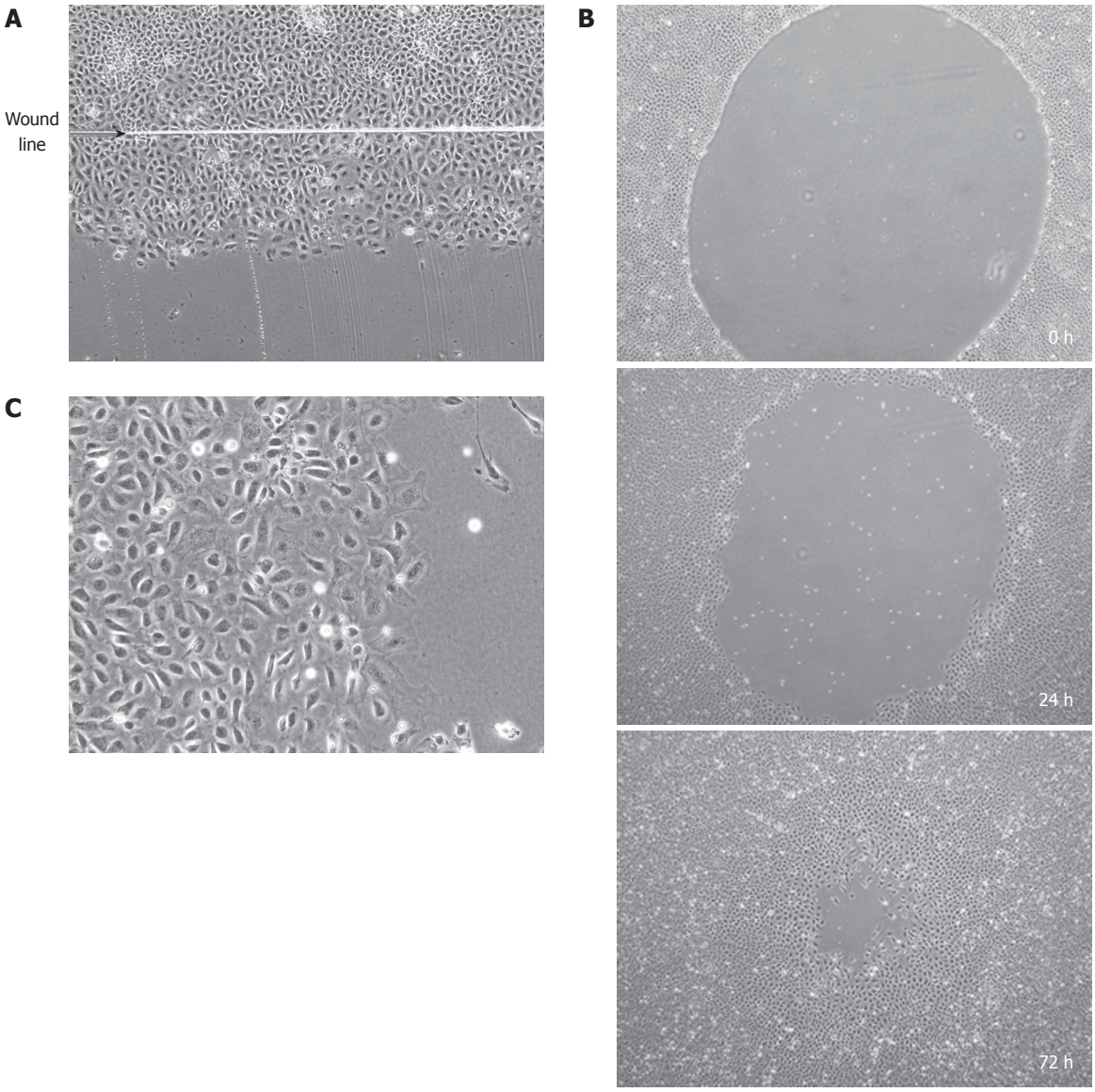

Figure 1 Wounded rat intestinal epithelial cell-6 cells. A: Confluent monolayers of intestinal epithelial cell (IEC)-6 cells were wounded with a razor blade. Wounded IEC-6 cells 24 h after wound formation. Wound line is shown with an arrow; B: IEC-6 cells wounded by mechanical cell denudation (× 40). Wounded cells at 0, 24 and 72 h after wound formation are shown; C: Wound margin of IEC-6 cells (× 200).

ligand CXCL12 production[30]. Moyer *et al*[2] have demonstrated that CXCL12 increases Rho-GTP and F-actin localization to the leading edge of wounded IEC-6 and T84 monolayers. Since Rho-GTP is known to be required for membrane protrusions in intestinal cell lines[28], it has been postulated that CXCR4-mediated activation and localization of Rho at the leading edge of restitutive epithelial cells may facilitate the formation of lamellae that are required for wound healing[2]. It has also been suggested that CXCR4 and CXCL12 function as an autocrine and paracrine mucosal signaling network that regulates the competency of the epithelial barrier to withstand injury and mediate repair following damage[2].

Defensins are highly conserved key molecules that participate in host defense through the direct killing of microbes[31]. Like the homeostatic chemokine receptor CXCR4, the chemokine receptor CCR6 is expressed by immature dendritic cells and circulating T cells, which directs their trafficking to sites of inflammation following binding by the chemokine ligand CCL20[19,32]. Vongsa *et al*[19] have demonstrated that human β-defensins and CCL20 stimulated accumulation of F-actin, phosphorylation of the myosin light chain and RhoA, and restitutive migration of IEC-6 cells. These findings suggest that chemokines and β-defensins are protective host defense molecules that function not only to recruit immune cells and kill microbes, but also to increase the efficiency of wound healing in the gut. Annexin 2 is a calcium-dependent phospholipid-binding protein that also plays a role in regulating the actin cytoskeleton, and has been implicated in cell migration[33,34]. It has been shown that annexin 2 regulates IEC migration and wound closure through Rho-dependent signaling pathways and related actin cytoskeletal remodeling[33]. We have summarized various

Table 1 Various factors promoting or inhibiting wound healing of intestinal epithelium and mucosa

Categories	Factors	Promoting effect	Inhibitory effect	No effect	Models
(Chemokines)	CXCL12	+			Cells[2]
	CCL20, defensins	+			Cells[19]
Annexin 2		+			Cells[33]
(Growth factors)	TGF-α	+			Cells[21,44]
	TGF-β	+			Cells[40]
	EGF, HB-EGF	+			Cells[11,21]
	HGF	+			Cells[36]
	FGF	+			Cells[35]
	KGF	+			Cells[35], rat[37]
	IGF-Ⅰ, -Ⅱ	+			Mice[38], cells[39]
(Cytokines)	IL-1β	+			Cells[21]
	IL-2	+			Cells[41]
	IFN-γ	+[21]	+[5]		Cells[21], mice and cells[5]
	PDGF, IL-6, TNF-α			+	Cells[21]
Trefoil peptide		+			Cells[42]
Prostaglandin, COX-1, COX-2		+			Mice[51-54]
(Toll-like receptors)	TLR2	+			Mice and cells[92-94,97]
	TLR3	+[95]	+[96]		Mice[95], cells[96]
	TLR4	+[97-99]	+[100]		Mice[97-99] and cells[100]
	TLR5	+			Mice and cells[101,102]
	TLR9	+			Mice[103-105]
(Dietary factors)	Glutamine	+			Rats[6], cells[68]
	Histidine	+			Mice[7]
	Vit D	+			Mice and cells[72]
(Gastroprotective agents)	Rebamipide	+			Rats[8,77]
	Ecabet sodium	+			Cells[9] and rats[79]
	Sucralfate	+			Cells[10]
(Other factors)	Epimorphin	+			Cells[60]
	Muc3	+			Mice and cells[62]
	HIF	+			Mice[63]
	GM-CSF	+			Mice[64]

COX: Cyclooxygenase; TGF: Transforming growth factor; EGF: Epidermal growth factor; HB-EGF: Heparin-binding epidermal growth factor; HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; KGF: Keratinocyte growth factor; IGF: Insulin-like growth factor; IL: Interleukin; IFN: Interferon; PDGF: Platelet-derived growth factor; TLR: Toll-like receptor; HIF: Hypoxia-inducible factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; TNF: Tumor necrosis factor.

factors that can have specific effects on IEC or intestinal mucosal wound healing in Table 1.

REGULATORY PEPTIDES AND SIGNALING PATHWAYS IN INTESTINAL WOUND HEALING

Previous studies have shown that various growth factors [including transforming growth factor (TGF)-α and TGF-β, epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), insulin-like growth factor (IGF)-Ⅰ and IGF-Ⅱ], the cytokine interleukin (IL)-1β and IL-2, and trefoil peptides enhance restitution or proliferation of IECs[3,21,35-42]. In contrast, platelet-derived growth factor (PDGF), IL-6, and tumor necrosis factor (TNF)-α have no effect on cell migration[21]. With regard to interferon (IFN)-γ, a previous study has shown that IFN-γ enhanced epithelial cell restitution by 3.8-fold[21]. However, a recent study has shown that IFN-γ inhibits enterocyte migration by preventing inter-enterocyte gap junction communication[5]. Among the regulatory peptides, TGF-β is known to be a central factor that intrinsically contributes to the restitution of wounded IECs, although it inhibits IEC proliferation[21]. It is noteworthy that cytokines (IL-1β, IL-2), EGF, FGF, HGF and TGF-α promote epithelial cell restitution through enhanced production of bioactive TGF-β, namely, the TGF-β-dependent pathway[21,35,36,41]. On the other hand, it has been shown that both trefoil peptides, which are secreted onto the surface of the gastrointestinal tract, and galectin-2 and -4, which ameliorate colitis in several models of intestinal inflammation, promote IEC restitution through a TGF-β independent pathway[42,43]. In contrast, TGF-α mediates its stimulatory effects on the proliferation and restitution of IECs through different mechanisms; as described above, it enhances IEC restitution through a TGF-β-dependent pathway but promotes IEC proliferation through the activation of extracellular signal-regulated kinase (ERK)1/ERK2 mitogen-activated protein kinase (MAPK)[44].

It has been shown that the activation of specific signaling pathways is involved in intestinal epithelial wound repair. El-Assal *et al*[11] have demonstrated that heparin-binding epidermal growth factor-like growth factor (HB-EGF) enhances restitution of the intestine *in vivo* and *in vitro* in a phosphatidylinositol 3-kinase (PI3K)/Akt- and MAPK/ERK kinase (MEK)/ERK1/2-dependent fashion. Using mouse epithelial cell lines, Dise *et al*[15] have

shown that the PI3K and Src signaling cascades cooperate with Rac and promote IEC migration in response to EGF. Other studies have shown that the activation of the ERK1/2 MAPK or PI3/Akt pathway plays an important role in the regulation of intestinal epithelial proliferation, survival, and wound healing[12,45-47]. Recent studies have suggested that nuclear factor (NF)-κB has not only pro-inflammatory but also has a tissue-protective function in IECs[48]. In addition, Pickert *et al*[49] have reported using conditional knockout mice with an IEC-specific deletion of signal transducer and activator of transcription (STAT) 3 activity that intestinal epithelial STAT3 activation regulates immune homeostasis in the gut by promoting IL-22 dependent mucosal wound healing. Trem2 is a cell surface receptor that is specifically induced in macrophages by IL-4/IL-13. Seno *et al*[50] have shown that Trem2 signaling promotes efficient wound healing of colonic mucosal injuries by inhibiting cytokines that can enhance M1 macrophage activation, and by promoting cytokines that can promote M2 macrophage activation.

Previous studies have shown that prostaglandin (PG) E2 plays a major role in the regeneration of the epithelial crypts and in the prevention of decreased epithelial cells proliferation after radiation- or dextran sodium sulfate (DSS)-induced intestinal injury[51,52]. Mucosal PGs are synthesized from arachidonate by cyclooxygenase (COX)-1 or COX-2[52]. Thus, it has also been shown that COX-1 and COX-2 share a crucial role in the defense of the intestinal mucosa[53,54]. In this context, Brun *et al*[55] have shown that the neuropeptide neurotensin (NT) significantly increases COX-2 mRNA levels and stimulates PGE2 release in the colonic cell line HT29. They also have shown that NT significantly enhances the migration of HT-29 cells into the denuded area of a wound model. It has also been shown that PGE2 reduces radiation-induced apoptosis in the intestine through transactivation of the EGFR, enhanced activation of Akt, and reduced Bax translocation from the cytoplasm to the mitochondria[56].

Epithelial-mesenchymal interactions are necessary for proper gut morphogenesis[57]. These interactions also play important roles in intestinal epithelial wound healing. Epimorphin is expressed on the surface of mesenchymal cells in various organs, including intestinal mucosa, and is suggested to play a key role in the morphogenesis of epithelial cells[58,59]. We found that epimorphin also has a novel function to promote restitution of IECs under oxidative stress conditions through the activation of the EGF receptor and MEK/ERK, PI3K/Akt signals[60]. HGF, FGF and KGF are also secreted by mesenchymal cells[57]. Göke *et al*[61] have shown that fibroblasts, which are derived from primitive mesenchyme, promote intestinal cell proliferation in addition to affecting secretory responses, differentiation, and morphogenesis, and that this function is predominantly mediated by the paracrine action of HGF.

Recent studies using experimental colitis models have shown that the Muc3 mucin cysteine-rich domain[62], hypoxia-inducible factor (HIF)[63], and granulocyte-macrophage colony stimulating factor (GM-CSF)[64] improve wound healing of the colonic mucosa. We summarize the various factors and signaling pathways that contribute to intestinal epithelial and mucosal wound healing in Figure 2.

DIETARY FACTORS AND INTESTINAL WOUND HEALING

Several studies have shown that enteral nutrition with an elemental diet is efficacious in the treatment of CD, especially for maintaining clinical remission or reducing clinical and endoscopic recurrence after resection[65-67]. Although some of this effectiveness may be due to the low antigenic load, low fat content, and modulation of the commensal bacterial flow, the precise mechanisms remain unclear. In this context, the effectiveness of the constituent amino acids of the elemental diet for intestinal epithelial and mucosal wound healing has been reported.

Glutamine, the most abundant free amino acid in the bloodstream, is a non-essential amino acid that is essential for gut homeostasis and is an essential respiratory substrate for cells in the small intestinal mucosa[68,69]. Enteral glutamine is thought to stimulate intestinal mucosal protein synthesis and protect against apoptosis[68]. Larson *et al*[68] have shown that glutamine supplementation stimulates IEC growth and prevents apoptosis, and that activation of ERK is an important contributor to glutamine-mediated intestinal cell survival. Sukhotnik *et al*[6] have evaluated the preventive effects of oral glutamine supplementation in an intestinal ischemia-reperfusion injury in a rat. It was found that pretreatment with oral glutamine prevents mucosal injury and improves intestinal recovery following ischemia-reperfusion injury through the stimulation of cell proliferation rather than the inhibition of cell apoptosis.

On the other hand, Andou *et al*[7] have assessed the role of histidine, an essential amino acid, in controlling colitis by using an IL-10-deficient cell transfer model. In this study, it was shown that dietary histidine reduced histological damage of the colon and TNF-α mRNA expression by inhibiting NF-κB activation, following the down-regulation of pro-inflammatory cytokine production by macrophages. Son *et al*[70] also have reported that histidine significantly inhibits both hydrogen peroxide- and TNF-α-induced IL-8 secretion and mRNA expression in intestinal epithelial-like cell lines. This study has also shown that histidine abolishes the NF-κB-dependent activation of the IL-8 promoter induced by TNF-α, suggesting that histidine has the potential to attenuate intestinal inflammation. These reports investigating the function of glutamine and histidine have suggested the efficacy of an elemental diet for improvement of intestinal mucosal wound healing in patients with CD.

Previous studies have suggested a link between vitamin D deficiency and IBD risk[71]. Kong *et al*[72] have investigated the role of the vitamin D receptor (VDR) in mucosal barrier homeostasis by using the DSS-induced colitis model. In this study $VDR^{+/+}$ mice were mostly resistant

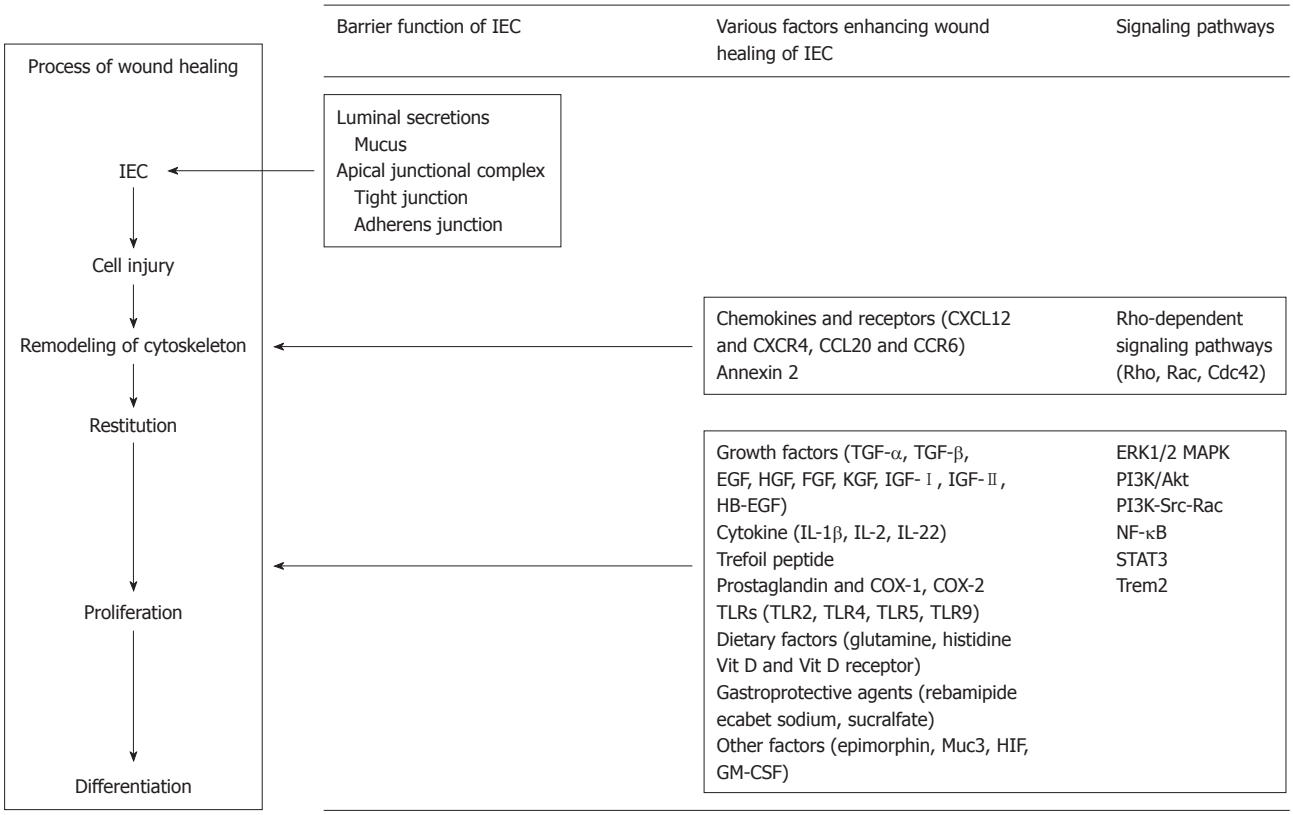

Figure 2 Various factors and signaling pathways contributing to the process of wound healing of intestinal epithelial cells and intestinal mucosa. IEC: Intestinal epithelial cell; TGF: Transforming growth factor; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; KGF: Keratinocyte growth factor; IGF: Insulin-like growth factor; HB-EGF: Heparin-binding epidermal growth factor; IL: Interleukin; COX: Cyclooxygenase; TLR: Toll-like receptor; HIF: Hypoxia-inducible factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; NF: Nuclear factor; STAT: Signal transducer and activator of transcription.

to 2.5% DSS, but $VDR^{-/-}$ mice developed severe colitis, leading to death. They also found severe disruption in the epithelial junctions in $VDR^{-/-}$ mice after DSS treatment. In cell cultures, 1,25-dihydroxy-vitamin D_3 [1,25$(OH)_2D_3$] markedly enhanced tight junctions and stimulated epithelial cell migration *in vitro*. These observations suggest that VDR plays a critical role in mucosal barrier homeostasis by preserving the integrity of junction complexes and the healing capacity of the colonic epithelium.

GASTROPROTECTIVE AGENTS IN INTESTINAL EPITHELIAL WOUND HEALING

Recent studies have shown that some gastroprotective agents, including ecabet sodium (ES), rebamipid, and sucralfate, have therapeutic effects on IBD and other types of colitis[73-76]. Previous studies have shown that rebamipide enema is effective for treatment of experimental DSS-induced colitis in rats[8,77]. Okayama *et al*[77] have suggested that the protective effect of rebamipide may be attributable to both the radical scavenging action and the increase in the production of mucus in the colon. Watanabe *et al*[78] have performed wound assays using gastric epithelial cells and have shown that rebamipide prevents delay of wound repair induced by hydrogen peroxide. On the other hand, Sasaki *et al*[9] have investigated the therapeutic mechanism of ES for intestinal mucosal injury by using the rat IEC-6 cell line. The investigation clarified that ES prevents the delay of wound repair in IEC-6 cells induced by hydrogen peroxide, probably through the activation of ERK 1/2 MAPK and the induction of COX-2. Takagi *et al*[79] have shown that intracolonic administration of ES accelerates TNBS-induced ulcer healing *in vivo*. Shindo *et al*[10] have investigated the therapeutic mechanism of sucralfate for intestinal mucosal injury. It has been demonstrated that sucralfate also prevents the delay of wound repair in IEC-6 cells induced by hydrogen peroxide through the induction of COX-2 and an anti-apoptotic mechanism. The effects of sucralfate may be initiated by the activation of the NF-κB pathway. These studies have suggested the possibility that some gastroprotective agents could be used for the treatment of IBD.

ROLE OF THE INNATE IMMUNE SYSTEM IN COLONIC HOMEOSTASIS AND INTESTINAL WOUND HEALING

The commensal bacteria population is comprised of at

least 400 species, with a load of as many as 10^{12} bacteria per gram of intestinal content[80]. As described previously, IECs are the first line of defense against commensal or pathogenic luminal microflora. Recognition of enteric bacteria is mediated by several mechanisms, the most important of which rely on host receptors specific for conserved bacterial structures not found in the host[81]. The two major host receptors currently recognized in humans are TLRs and the nucleotide-binding oligomerization domain (NOD)-containing proteins[81]. In this context, it is notable that mutations in NOD2, a cytoplasmic innate immune-recognition receptor, are associated with susceptibility to CD[82,83]. TLRs are innate immune-recognition receptors that bind a spectrum of pathogen-associated molecular patterns (PAMPs) present in pathogenic and commensal bacteria and viruses, as well as some endogenous proteins[84-86]. TLR family members were first noted to be expressed by immune cells such as monocytes and dendritic cells[84-86], then, it was shown that IECs also express TLRs[4]. With regard to the association of TLRs with IBD, several studies have shown the alteration of TLR expression and the specific antibody to the TLR ligand in patients with IBD. First, Cario *et al*[87] reported the upregulation of TLR4 in IECs in UC and CD, and the downregulation of TLR3 in CD. A recent study has shown the association between the TLR2 polymorphism R753Q and the severe UC phenotype[88]. Such associations have also been found between the TLR4 polymorphism at Asp299Gly and the development of CD and UC, and the TLR4 polymorphism at Thr399Ile and UC[89,90]. On the other hand, the presence of high titers of flagellin-specific antibodies in the serum of CD patients has been reported[91].

The following *in vivo* and *in vitro* studies have provided evidence that each TLR contributes to intestinal homeostasis and intestinal mucosal repair. TLR2 recognizes lipoproteins derived from a variety of bacteria, peptidoglycan, lipoteichoic acid from many Gram-positive bacteria, and lipopolysaccharide (LPS) from *Leptospira* and *Porphyromonas gingivalis*[4]. Cario *et al*[92] have shown that TLR2 stimulation effectively preserves the tight junction-associated barrier assembly in IECs against stress-induced damage, and suppresses mucosal inflammation and apoptosis of IECs *in vivo*. The same group has shown that TLR2 controls gap junction intercellular communication and commensal-mediated intestinal epithelial wound repair by modulating intestinal epithelial connexin-43[93]. Furthermore, they have shown that TLR2 controls terminal goblet cell differentiation by selectively regulating trefoil factor 3 expression in the intestine and confers anti-apoptotic protection to the intestinal mucosa[94].

TLR3 recognizes double-stranded RNA, which is a molecular pattern associated with viral infection[86]. A recent study has shown that administration of TLR3 ligand polyinosinic acid: cytidylic acid protects against DSS-induced colitis[95]. In contrast, Sato *et al*[96] have performed wound assays using IEC-6 cells and have shown that TLR3 ligand rotavirus double-stranded RNA induces apoptosis and diminishes wound repair of IECs *in vitro*.

TLR4 recognizes LPS that is an integral component of the outer membranes of Gram-negative bacteria[4]. Using DSS-induced colitis mice, Rakoff-Nahoum *et al*[97] have demonstrated that commensal bacteria are recognized by TLRs (TLR2 and TLR4) under normal steady-state conditions, and that this interaction plays a crucial role in the maintenance of intestinal epithelial homeostasis. They have shown that activation of TLRs by either the TLR2 or TLR4 ligand is critical for the protection against gut injury and associated mortality. This group has also shown that TLR-mediated signaling plays a critical role in intestinal inflammation in the context of deficiency in the anti-inflammatory cytokine IL-10, but not in the context of insufficient activity of regulatory T cells[98]. Another study using a murine colitis model also has shown that TLR4 is important in intestinal response to injury and in limiting bacterial translocation[99]. On the other hand, Cetin *et al*[100] have reported that LPS inhibits IEC-6 cell migration through a RhoA-dependent increase in focal adhesions and enhanced cell adhesiveness.

Flagellin, the primary structural component of bacterial flagella, is recognized by TLR5 present on the basolateral surface of IECs[101]. A recent study has shown that, in salmonella infections, flagellin plays a dominant role in the activation of not only innate immunity but also anti-apoptotic processes in epithelial cells[102]. The same group also has shown that pretreatment of epithelial cells with flagellin can protect cells from a subsequent bacterium-mediated apoptotic challenge through the activation of NF-κB and PI3K/Akt signaling[101]. These studies suggest that the TLR5 ligand flagellin has a fundamental cytoprotective effect in inflammatory stress.

TLR9, an intracellular protein in immune cells, is expressed on the surfaces of IECs, both on the apical and basolateral membrane[18]. TLR9 recognizes CpG motifs, derived from bacterial DNA[4]. Recent studies have demonstrated that TLR-9 signaling in IECs contributes to colonic homeostasis[18,103-105]. Katakura *et al*[103] have shown that TLR9 agonist suppresses the severity of experimental colitis by inducing type I IFN (IFN-α/β). The same group also has shown that administration of probiotics (non-viable irradiated or viable probiotics) ameliorates the severity of DSS-induced colitis[105]. They have suggested that TLR9 signaling is essential in mediating the anti-inflammatory effect of probiotics, and that live microorganisms are not required to attenuate experimental colitis. They have shown that while basolateral TLR9 signaling is fully capable of inducing an NF-κB-mediated pro-inflammatory response, apical TLR9 signaling does not induce an inflammatory response due to a defect in NF-κB activation[18,104]. They have suggested that stimulation of apical TLR9 compromises the inflammatory cascade induced basolaterally by several other TLR ligands, and thus, apical exposure to luminal microbial DNA restrains

intestinal inflammation and supports colonic homeostasis *via* the activation of TLR9.

In conclusion, most of these studies investigating the function of TLRs strongly suggest the possibility that the activation of TLRs by commensal bacteria plays an essential role in maintaining colonic homeostasis and protection of IECs from gut injury. These studies also support the therapeutic efficacy of some antibiotics[106-108] or probiotics[109] in IBD patients. Thus, modulations of host-commensal interactions *via* TLRs might be targeted for optimal therapeutic strategies in IBD.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2172

World J Gastroenterol 2011 May 7; 17(17): 2172-2177
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Mallory-Denk Bodies in chronic hepatitis

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Received: September 11, 2010 Revised: December 9, 2010
Accepted: December 16, 2010
Published online: May 7, 2011

Abstract

Mallory-Denk Bodies (MDB) are important as investigators, suggesting MDB as an indicator of the histologic severity of chronic hepatitis, causes of which include hepatitis C, primary biliary cirrhosis (PBC), and nonalcoholic fatty liver disease (NAFLD). Matteoni *et al* scored MDB in patients with NAFLD as none, rare and many, and reported that MDB plays a prominent role in this classification scheme in an earlier classification system. In this study, we evaluated 258 patients with chronic hepatitis due to metabolic, autoimmune and viral etiologies. Liver biopsy samples were evaluated with hematoxylin and eosin, periodic acid-Schiff-diastase, Gordon and Sweet's reticulin, Masson's trichrome, and iron stains. Both staging and grading were performed. Additionally, MDB were evaluated and discussed for each disease. We examined patients with nonalcoholic steatohepatitis (NASH; 50 patients), alcoholic hepatitis (10 patients), PBC (50 patients), Wilson disease (WD; 20 patients), hepatitis B (50 patients), hepatitis C (50 patients) and hepatocellular carcinoma (HCC; 30 patients). Frequency of MDB was as follows; NASH: 10 patients with mild in 60% and moderate in 40% and observed in every stage of the disease and frequently seen in zone 3. PBC: 11 patients with mild in 10%, moderate in 70%, and cirrhosis in 20%, and frequently seen in zone 1. WD: 16 patients with moderate and severe in 60% and cirrhosis in 40% and frequently seen in zone 1. Hep B: 3 patients with mild in 66% and severe in 34%. Hep C: 7 patients with mild in 40% and moderate in 60% and observed in every stage. HCC: 3 patients with hep B in 2 patients. We found that there is no relationship between MDB and any form of chronic hepatitis regarding histologic severity such as alcoholic steatohepatitis and NAFLD and variable zone distribution by etiology.

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Key words: Non-alcoholic fatty liver disease; Mallory-Denk Bodies; Hepatitis B and C; Hepatocellular carcinoma; Primary biliary cirrhosis; Wilson disease

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Basaranoglu M, Turhan N, Sonsuz A, Basaranoglu G. Mallory-Denk Bodies in chronic hepatitis. *World J Gastroenterol* 2011; 17(17): 2172-2177 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2172.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2172

HISTORICAL BACKGROUND OF MALLORY-DENK BODIES IN HEPATOLOGY

Frank Burr Mallory first reported Mallory-Denk Bodies (MDB) in patients with alcoholic cirrhosis in 1911[1]. Then, this pearl of pathology was reported in various hepatic diseases such as the hepatitis B and C virus, Wilson's

disease, chronic cholestatic injuries, nonalcoholic fatty liver disease (NAFLD), drug injuries, focal nodular hyperplasia, and hepatocellular carcinoma (HCC).

THE PATHOGENESIS OF MDB FORMATION IN CHRONIC HEPATITIS: WHAT WE KNOW

MDB are an intracellular deposition of misfolded protein aggregated into ubiquitin (Ub)-rich cytoplasmic inclusions in ballooned hepatocytes[2]. MDB formation, which consists of abnormally phosphorylated, ubiquitinated, and cross-linked keratins and non-keratin components, are not entirely interchangeable since not all ballooned hepatocytes contain MDB. Both ballooning of hepatocytes and MDB are the two hallmarks of ongoing inflammation (Figure 1). To understand the pathogenesis of MDB, we first have to know the development of ballooning of hepatocytes.

Ballooning or swelling of hepatocytes is induced by oxidative stress and its products such as oxyradicals. The swelling of hepatocytes could be explained by water accumulation in the cytoplasm as a response to accumulated stress proteins such as heat shock proteins (HSPs) or fat. HSPs are the precursor of MDB and indicate hepatocytes injury[2,3]. Currently, we do not know exactly whether ballooning are adaptive (physiological, reversible) or degenerative changes (pathological, most likely irreversible) of hepatocytes against the changed environment.

Swelling of hepatocytes represents volume increase (hydration) of the hepatocytes. It occurs against different stressors, particularly oxidative stress. It is reported that mild volume changes (up to 10% increase on the volume of the hepatocytes) without the biochemical evidence of free radicals are physiologic and adaptive. Hepatocyte damage may not be observed. However, high grade swelling (such as at least 30% increase on the volume of the hepatocytes) could be degenerative and can cause hepatocyte apoptosis, necrosis, and even death.

Most of the previous studies came from animal models. A hepatocyte includes two big compartments, namely wet (water) and cellular dry solids (fat, protein, nucleic acids, anions and cations and other solutes) which are in balance. Any intracellular solute (such as fat or abnormal proteins) accumulation into the cytoplasm may cause water movement, and water volume increase within the hepatocyte. This may change hepatocyte membrane transport system activation (such as ion channels) and may increase intracellular metabolism (increase both protein and glycogen synthesis and inhibit both proteolysis and glycogenolysis). The only purpose of these mechanisms is to maintain the functioning of both hepatocytes, and the liver as an organ. The volume ratio between these two compartments should be maintained at the same level under normal conditions and also against stressors.

It is experimentally shown in rats that the initial response of hepatocytes against iron, which is a well-known strong oxidative agent, was the accumulation of increased stress proteins, such as HSPs, within the cytoplasm. Increased stress proteins, along with the other elements of the increased metabolic process, cause macromolecular crowding and activate volume regulator mechanisms. To maintain the ratio between wet (water) and increased dry compartments (such as HSPs), volume regulator mechanisms increase the hepatocyte hydration with increasing water content of the cytoplasm (cloudy swelling). The real mission of this regulatory system is to preserve the intracellular environment and hepatocyte functioning. This is an adaptation mechanism of hepatocytes against oxidative stress (up to 10% increase on the volume of the hepatocytes). However, too much increased water within the cytoplasm of hepatocytes may cause degenerative changes and disturbances in both normal hepatocyte morphology and functioning. Then, hepatocyte apoptosis, necrosis and death may occur.

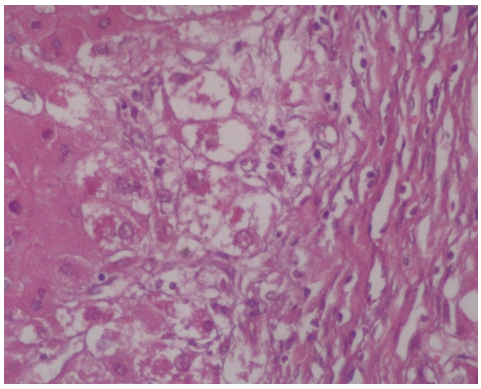

Figure 1 Zone 1 distribution of Mallory-Denk Bodies in primary biliary cirrhosis (× 400). This distribution was observed in both early and later stages of primary biliary cirrhosis, such as cirrhosis.

Additionally, we can discuss the toxic fatty liver animal model. Investigators used rats treated with CCl-4, which is a well-known steatogenic oxidant agent. CCl-4 is a cause of high grade hepatocyte swelling. This chemical poison was injected into the rats daily (one injection per day). Then the livers of the rats were examined for three compartments (water, fat, and fat-free dry solids) at the entry, after 1 injection, and after 6 injections. Investigators observed that both fat and water were increased significantly while fat-free dry solids were not changed or only slightly changed. The highest grade increase of water was seen after the sixth injection (25% water increase after 1 injection, and 48% water increase after 6 injections) which was related to the increased hepatocyte hydration. This means that there were many well-established ballooned hepatocytes in the liver of CCl-4 injected rats.

However, these protection mechanisms are not infinite. A strong oxidative stress may cause high amplitude ballooning of hepatocyte due to both increased hydration and dry solids. One of the increased cellular dry solids is stress protein, such as HSPs. HSPs, as examples of misfolded proteins, are the indicators of cellular dysfunction. Under normal conditions, these potentially harmful proteins are targeted by covalent attachment of multi-Ub

Table 1 Analysis of our 258 patients for Mallory-Denk Bodies and staging of the disease *n* (%)

	No. of pts	MDB (+) pts	Staging[1] in pts with MDB	Comment
NASH	50	10 (20)	Mild in 60% and moderate in 40%	MDB observed in every stage of NASH, predominantly in zone 3
Alcoholic (ASH)	10	5 (50)	Mild in 40% and moderate in 60%	MDB observed in every stage, predominantly in zone 3
PBC	50	11 (22)	Mild in 10%, moderate in 70%, and cirrhosis in 20%	Frequently seen in later stages with predominantly in zone 1
WD	20	16 (80)	Moderate and severe in 60% and cirrhosis in 40%	Frequently seen in later stages with predominantly in zone 1
Hep B	48	3 (6)	Mild in 66% and severe in 34%	Mild case with steatosis
Hep C	50	7 (14)	Mild in 40% and moderate in 60%	MDB observed in every stage
HCC	30	3 (10)		Two cases with Hep B
Total	258	55 (21)		

[1]Mild: Stage 1; Moderate: Stage 2-3; Severe: Stage 4, and cirrhosis. MDB: Mallory-Denk Bodies; ASH: Alcoholic steatohepatitis; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis; WD: Wilson disease; HCC: Hepatocellular carcinoma; pts: Patients.

chains. Then, a protective mechanism, the Ub-proteasome pathway eliminates these products. When this control system fails, under the strong oxidative stress, abnormal cytokeratins (CKs) may become accumulated along with HSPs (70, 90, and 25), Ub, tissue transglutaminase, proteasome subunits, tubulin, and p62. Young, tiny and then well-formed MDB were well established in ballooned hepatocytes. This mechanism has not been fully understood yet. There are two possible ways for this pathway to fail: (1) production of these misfolded proteins exceeding the capacity of this protective system or (2) inhibition of the pathway. Well-established MDB might be a degenerative rather than adaptive response of hepatocytes against stronger stressors. Then, satellite cell activation occurs. Sustained liver injury leads to fibrosis and cirrhosis.

In conclusion, there is no contrast of MDB pathogenesis in hep B, hep C, PBC, NAFLD and the others.

DIFFERENCES IN THE FREQUENCY AND ZONAL DISTRIBUTION OF MDB IN PATIENTS WITH CHRONIC HEPATITIS

MDB are typical features of alcoholic steatohepatitis (ASH) and NAFLD. NAFLD exhibits slightly less prominent MDB than ASH[4]. MDBs can also be detected after intestinal bypass surgery for morbid obesity, in chronic cholestasis, PBS, Wilson disease (WD) and other types of copper toxicosis, various metabolic disturbances, and hepatocellular neoplasms. In idiopathic copper toxicosis and HCC, MDBs may coincide with another type of cytoplasmic inclusions.

MDB is not present in the majority of individuals with hep C, or even in hep C patients with NAFLD (the presence of MDB in patients with hep C is 7.1%). However, hepatocyte ballooning with MDB is much more likely to lead to advanced fibrosis in these patients.

MDB in periportal liver cells have been reported in patients with Wilson's disease. MDBs are found in mice by feeding them the hepatotoxic substances griseofulvin and 3,5-diethoxycarbonyl- 1,4-dihydrocollidine.

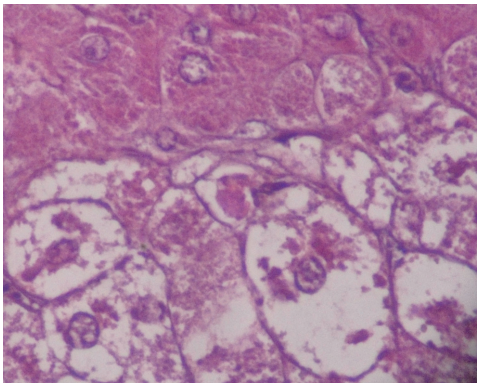

Figure 2 Mallory-Denk Bodies, located perinuclear, has been shown as a larger picture in a patient with primary biliary cirrhosis (× 1000).

MDBs have not been observed in acute cholestasis, acute viral hepatitis, acute toxic or drug-induced liver diseases.

OUR RESULTS: FREQUENCY OF MDB IN DIFFERENT FORMS OF CHRONIC HEPATITIC DISEASES

Two hundred and fifty-eight patients with hepatitis have been examined at our pathology department. Outpatient percutaneous percussion guided needle liver biopsies were performed using Menghini soft tissue biopsy needles (1.4 or 1.6 mm, adult size, Hepafix, Braun, Melsungen, Germany). Liver biopsy samples were evaluated with hematoxylin and eosin, periodic acid-Schiff-diastase, Gordon and Sweet's reticulin, Masson's trichrome, and iron stains by a single pathologist, unaware of the clinical and biochemical data. We examined patients with nonalcoholic steatohepatitis (NASH; 50 patients), alcoholic hepatitis (10 patients), primary biliary cirrhosis (PBC; 50 patients), WD (20 patients), hepatitis B (50 patients) and hepatitis C (50 patients), and HCC (30 patients) for grading and staging and MDB. Results were shown at Table 1. Furthermore, the characteristics of MDB in each disease have been

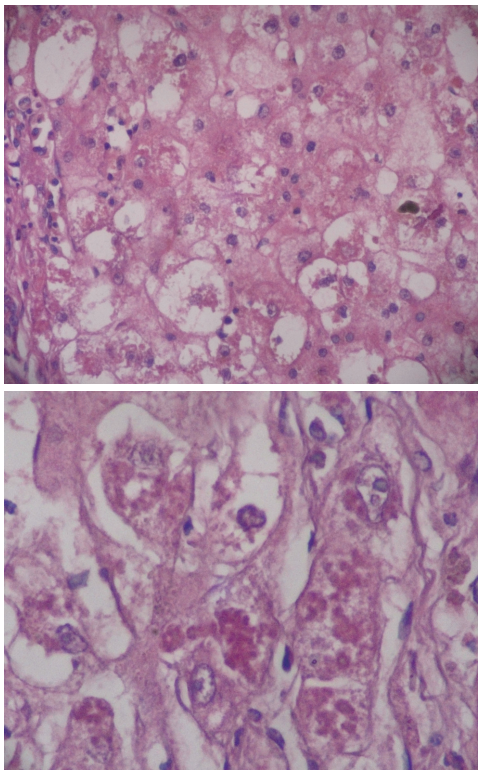

Figure 3 Mallory-Denk Bodies, located both perinuclear and intracytoplasmic, was observed in zone 1 in early stages of Wilson disease, but in every zone at the later stages, such as cirrhosis (× 400, 1000, respectively). Hydrophobic degeneration was more exaggerated. The diameter of Mallory-Denk Bodies was not homogenous in patients with Wilson disease.

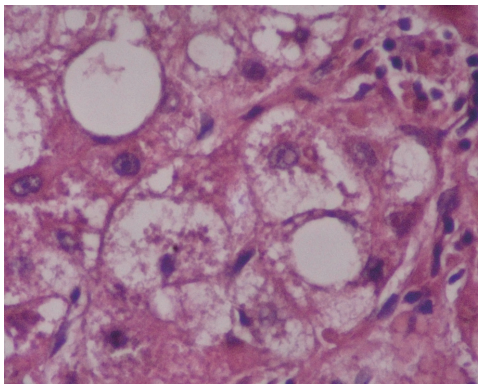

Figure 4 Perinuclear location of Mallory-Denk Bodies in patients with hepatocellular carcinoma depends on the cause (× 1000). In this case, hepatitis B is responsible from Mallory-Denk Bodies.

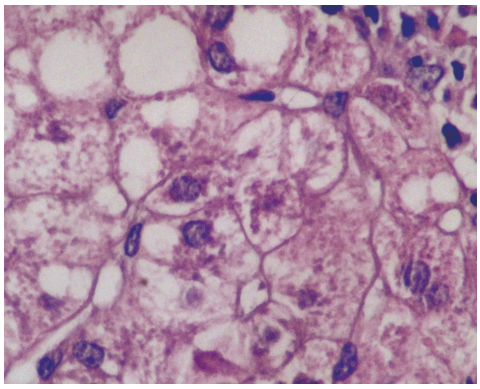

Figure 5 Tiny and uniform Mallory-Denk Bodies was seen in patients with nonalcoholic steatohepatitis (× 1000).

shown in Figures 1-5. MDB showed zone 1 distribution in PBC and its distribution, located perinuclear, observed in both early and later stages, such as cirrhosis. MDB, located both perinuclear and intracytoplasmic, was observed in zone 1 in early stages of WD, but in every zone at the later stages, such as cirrhosis. Hydrophobic degeneration was more exaggerated in WD and the diameter of MDB was not homogenous in patients with WD. Perinuclear localized MDB in patients with HCC depends on hepatitis B, which is responsible for MDB. Lastly, tiny and uniform distribution of MDB was seen in patients with NASH. Although patients with PBC and WD showed MDB frequently in later stages, we found that there is no relationship between MDB and any form of chronic hepatitis regarding histologic severity, such as in ASH and NAFLD. Moreover, zone distribution of MDB is very variable by etiology, such as zone 1 in PBC and WD as zone 3 in ASH and NAFLD. Of the 3 hepatitis B patients with MDB, 2 showed steatosis with mild fibrosis. Most probably, MDB in these cases was caused by steatosis due to metabolic reasons.

MDB IN NAFLD

In an earlier classification system, Matteoni *et al*[5] scored MDB in patients with NAFLD as none, rare and many. MDB plays a prominent role in this classification scheme as well as follows; Type 1: Fatty liver alone, Type 2: Fat and inflammation, Type 3: Fat and ballooned hepatocytes, and Type 4: Fat and ballooned hepatocytes and either MDB or fibrosis.

The Kleiner system is widely accepted and, as such, has superseded the previous classification system (Matteoni *et al*'s system)[6]. Although this system includes several parameters, there are four main histologic features as steatosis, inflammatory infiltration, ballooning degeneration of hepatocytes, and fibrosis are more important than others. Also, significantly, presence or otherwise of MDB is of much less consequence in the Kleiner system.

Frequency of MDB is very variable, reported from 7% to 90% in adult patients with NAFLD[7-18]. Furthermore, it was not demonstrated or uncommon in pediatric patients with NAFLD[17,18].

DIAGNOSTIC CRITERIA OF MDB

Technical difficulties on the showing of MDB may be the most important reason for the differences. To better understand this issue and its contradictions, we must be aware of some basic knowledge about MDB. MDB are intracellular depositions in ballooned hepatocytes which reflects a peculiar morphological manifestation of liver cell injury[19,20].

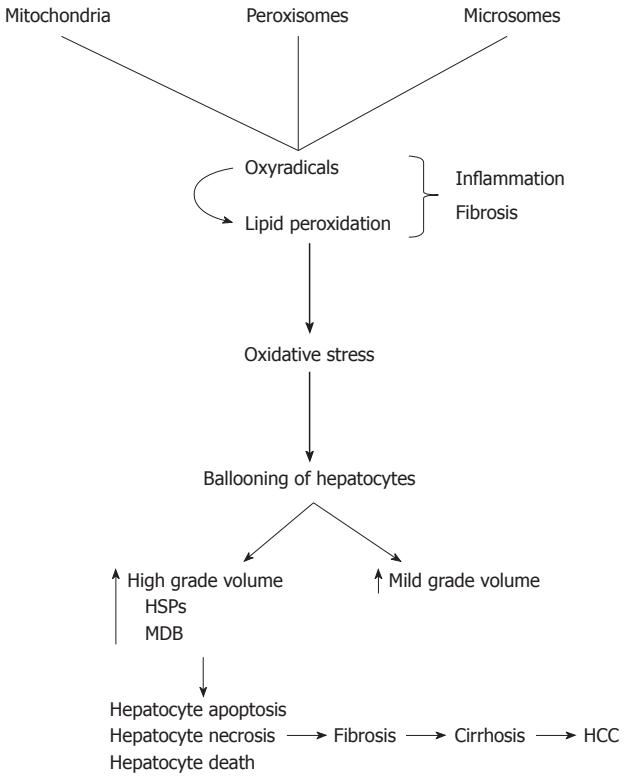

Figure 6 Shows development of Mallory-Denk Bodies in ballooned hepatocytes. MDB: Mallory-Denk Bodies; HSPs: Heat shock proteins; HCC: Hepatocellular carcinoma.

Three stages are demonstrated according to the development rank of MDB in ballooned hepatocytes: (1) misfolded proteins such as CKs, Ub, protein p62, and high and low molecular weight HSPs; (2) pre-MDB (young/tiny form) positive ballooned hepatocytes; and (3) mature or well-established MDB positive ballooned hepatocytes.

Misfolded proteins present the earliest stage of MDB and induced in response to a variety of cellular injuries. They are indicators of cellular dysfunction. Normally, these potentially harmful proteins are targeted by covalent attachment of multi-Ub chains. Then, a protective mechanism, which is the Ub-proteasome pathway, eliminates these products. When this control system fails, the CKs become accumulated in MDB along with HSPs, Ub, tissue transglutaminase, and p62. Then, mature MDB in ballooned hepatocytes is well established. There are two possible ways for this pathway to fail: (1) production of these misfolded proteins exceeding the capacity of this protective system (overexpression); and (2) inhibition of this pathway.

Pathogenesis of MDB is thought to include lipid peroxidation, oxidative stress, free radicals, bile retention, defective protein synthesis and copper accumulation (Figure 6). There are many modalities to detect MDB such as immunostaining using cytokeratin and Ub, and special staining methods for other misfolded proteins. The other one, the historically important hematoxylin and eosin, is not suitable for detecting misfolded proteins or small/tiny MDB. Almost all previous studies interested in NAFLD used hematoxylin and eosin staining for showing both mature and young forms of MDB. This is a cost effective and an easily available method. Furthermore, there is more evidence and experience about its safety in the medical literature. On the other hand, the immunostaining method is relatively new and expensive. Immunostaining demonstrates not only mature form of MDB but also both its young forms and misfolded proteins such as CKs, Ub, p62 protein and HSPs in ballooned hepatocytes. The question is which one is the best to use in general practice and clinical trials? We have to look at the current medical literature to give the correct answer for this question. However, there is no growing body of evidence on this issue. A study from Denmark used immunostaining for the cell stress protein Ub in 148 fine needle liver biopsies, which included 88 biopsies from patients with clinically diagnosed or suspected alcoholic liver disease and 60 selected biopsies from non-alcoholics[20]. They showed that MDB in ballooned hepatocytes by both hematoxylin and eosin and immunostaining (Ub) in all of 33 biopsies with alcoholic hepatitis. Of the 55 biopsies from alcoholic patients without alcoholic hepatitis (without MDB or pre-MDB in hematoxylin and eosin stained sections), Ub (+) cells were found in eight (14.5%) by immunostaining. Finally, they studied 60 selected biopsies from non-alcoholic patients, and demonstrated a few Ub (+) cells in two out of ten patients with PBC, but none in biopsies with hepatitis. According to the study results, as Ub/immunostaining is a highly sensitive and specific tool in the detection of MDB and MDB precursors in alcohol-using patients, its use in other forms of hepatitis is limited.

p62 is another misfolded protein and encoded by an immediate-early response gene that rapidly responds to a variety of extracellular signals, particularly oxidative stress. Then, protein p26 binds non-covalently to Ub.

These results may also represent a sampling error of liver biopsy. The method of liver biopsy we chose (such as percutaneous or ultrasound guided or CT guided or laparoscopic), the type of biopsy needle, the average length of the biopsies performed, and how many samples are obtained or number of biopsies performed would be important.

POSSIBILITIES IN THESE DIFFERENCES AND MANAGEMENT

Both environmental factors and genetic differences between the study groups may play a role in these differences[21]. These remarkable differences may be induced by different levels of alcohol consumption and an incomplete patient history of alcohol consumption in these studies. Ignored alcoholic liver disease is a significant cause of MDB in some. Incomplete examination of Wilson's disease, such as just measuring serum ceruloplasmin levels or with no examination of hepatitis C virus infection, may play a role. This is especially relevant with Wilson's disease and hepatitis C because both microvesicular and macrovesicular fatty changes and glycogen nuclei are shown in both diseases.

ALCOHOLIC LIVER DISEASE

MDB is found in 70% to 75% of patients with alcoholic liver disease[22,23]. Men and women who drink more than 80 g and 40 g of ethanol/d, respectively were accepted at substantial risk for the development of liver disease in the previous studies. Recent studies showed that the risk of liver disease begins at 30 g of ethanol/d[24]. This finding has led to a general recommendation that the maximal safe level of ethanol consumption is 20 g/d or two "drinks" per day for men and 10 g/d for women. However, it should be kept in mind that disease severity does not correspond to classic dose dependency.

Although one of the major criteria for the diagnosis of NAFLD is no excessive alcohol consumption in all previous case series, there has been no certain agreement on this issue, and a wide range of alcohol consumption was generally allowed. Thus, these remarkable differences on the frequency of MDB might be explained with the generously allowed alcohol consumption in previous series. It must also be considered that patients sometimes underreport ethanol intake.

CONCLUSION

Frequency (MDB) is reported as very variable. Both environmental factors and genetic differences between the study groups, and technical difficulties for showing of MDB and its early stage components may play a role in these remarkable differences.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2178

World J Gastroenterol 2011 May 7; 17(17): 2178-2180
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Asymmetric dimethylarginine: A novel biomarker of gastric mucosal injury?

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Received: September 14, 2010 Revised: December 13, 2010
Accepted: December 20, 2010
Published online: May 7, 2011

Abstract

Nitric oxide (NO), a multifunctional endogenous gas molecule, is metabolized from L-arginine by enzymatic reaction in the presence of nitric oxide synthase. NO, an important gas signaling molecule, is a gastric mucosa protective factor that contributes significantly to maintain normal gastric mucosa integrity. NO increases gastric mucosa blood flow, regulates the secretion of mucus and bicarbonate, and inhibits the secretion of gastric juice. Asymmetric dimethylarginine (ADMA) has been identified as the major endogenous inhibitor of nitric oxide synthase. The function of ADMA is to decrease NO production *via* inhibiting nitric oxide synthase activity. Besides inhibiting NO synthesis, ADMA also directly induces oxidative stress and cell apoptosis, and participates in inflammation reaction. Its systemic accumulation was observed in conjunction with several cardiovascular and metabolic diseases. ADMA also mediates gastric ulcer injury induced by ethanol, stress, *helicobacter pylori* and indomethacin. The mechanism of ADMA directly producing adverse effect in gastric mucosa is incompletely understood. It is widely accepted that NO bioavailability decrease is the majority reason. Promotion of apoptosis and aggravation of inflammation may be other important mechanisms of ADMA-induced gastric injury. ADMA might be a novel clinical and experimental biomarker related to gastric mucosa disorder. Although therapeutic tool targeting to ADMA is available in multiple cardiovascular diseases, it is unknown in gastrointestinal disease. The strategy to inhibit ADMA is beneficial to gastric ulcer induced by ethanol in rats. Thus, ADMA might be a candidate of therapeutic target in gastric mucosa damage.

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Key words: Asymmetric dimethylarginine; Mucosal injury; Nitric oxide

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Zhang Z, Zou YY, Li FJ, Hu CP. Asymmetric dimethylarginine: A novel biomarker of gastric mucosal injury? *World J Gastroenterol* 2011; 17(17): 2178-2180 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2178.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2178

INTRODUCTION

Nitric oxide (NO), a multifunctional endogenous gas molecule, is metabolized from L-arginine by enzymatic reaction in the presence of NO synthase (NOS)[1]. It is accepted as the most critical messenger and functional executant in a variety of biologic activities. Numerous researches have reported the beneficial effects of NO, including endothelium protection, inhibition of local inflammation and cell pathoproliferation[2]. NO bioavailability is most directly regulated by the abundance and/or activity of NOS. NOS can be competed by substrates, endogenous L-arginine and asymmetric dimethylarginine (ADMA)[1]. The function of ADMA is to decrease NO production *via* inhibiting the NOS activity. ADMA is derived from the catabolism of

proteins containing methylated arginine residues[3]. Arginine methylation of cellular proteins is catalyzed by protein arginine methyltransferases[3]. ADMA is hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH)[3].

Growing evidence has shown that excessive accumulation of ADMA can decrease NO bioavailability in cells/tissues, induce domain dysfunctions or produce detrimental effects in multiple systems[4]. Besides inhibiting NO synthesis, ADMA can directly induce oxidative stress and cell apoptosis, and participate in the inflammation reactions[5,6]. It is well documented that ADMA is involved in a wide range of cardiovascular disorders. ADMA impairs endothelial functions, thus leading to hypertension, atherosclerosis, coronary heart disease, diabetes mellitus, pulmonary hypertension and renal failure[7,8]. Although it is well established that ADMA is an independent risk factor of cardiovascular diseases, the pathological role of ADMA in other diseases is still unknown, including gastric diseases.

NO AND GASTRIC MUCOSAL PROTECTION

Gastric mucosal diseases, including gastric ulcers, affect 25%-30% of the world's population. The mechanism of gastric mucosal damage and protection is yet unclear. NO, an important gas signaling molecule, is a gastric mucosal protective factor that contributes significantly to maintaining normal gastric mucosal integrity[9]. Gastric mucosa is composed of gastric epithelial cells and glands. Gastric epithelial cells can secrete multiple active substances, including NO. There is substantial evidence that NO may be an important defendant factor of gastric mucosa[10]. In the animal models of gastric mucosal injury induced by ethanol, endothelin, ischemia/reperfusion, cold-stress, water immersion restraint, indomethacin and aspirin, NO exerts protective effects on gastric mucosal integrity[11-13]. The mechanisms underlying the protective effects of NO could be explained as follows: (1) NO increases gastric mucosal blood flow; (2) NO regulates the secretion of mucus and bicarbonate, which compose the first level of gastric tissue defense; and (3) NO inhibits the secretion of gastric juice[14,15].

ADMA AND GASTRIC MUCOSAL DAMAGE

Helicobacter pylori (*H. pylori*) infection is a definitive cause of gastroduodenal ulcer. Epidemiological analysis has shown that 70%-80% gastric ulcers and 95%-100% duodenal ulcers are attributed to *H. pylori* infection. In a clinical investigation of *H. pylori*-positive and -negative volunteers, *H. pylori* infection was found to increase the tissue contents of ADMA[16]. More importantly, a laboratory research has shown that proteolysis of *H. pylori* extract also results in a substantial accumulation of ADMA, indicating that *H. pylori* infection must be taken into account as a cause of increased ADMA levels[17]. Strong associations have also been found between ADMA and gastric mucosal dysfunction. Exogenous administration with ADMA inhibits the

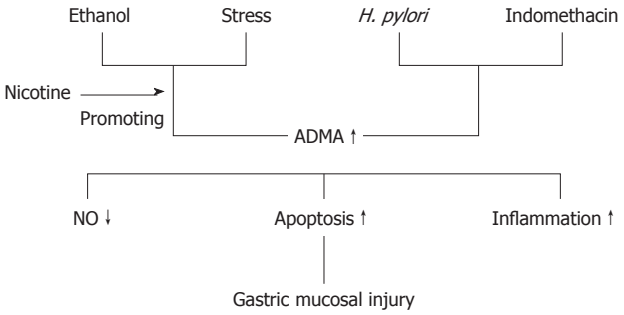

Figure 1 The hypothesized role of asymmetric dimethylarginine in gastric mucosal injury and the underlying mechanisms. ADMA: Asymmetric dimethylarginine; NO: Nitric oxide.

mucosal alkaline response to acid exposure, which is regarded as one of the important beneficial factors of gastric mucosa complaint. And an increase in ADMA mediates the water extract of *H. pylori*-induced acid-base imbalance in duodenal tissues[17]. In our *in vitro* experiment, we found that intervention of *H. pylori* stimulated ADMA release from cultured gastric epithelial cells[18]. We also observed that *H. pylori* infection markedly exacerbated ADMA-produced gastric tissue injury in rats[19].

Besides *H. pylori* invasion, gastric mucosal function is also disturbed by other factors, such as ethanol, indomethacin and cold-stress. Our recent studies have explored the role of ADMA in gastric mucosal injury induced by other causes in addition to *H. pylori* infection. The results have shown that ADMA levels are increased in gastric juice in three separate gastric mucosal models, including ethanol, indomethacin and cold-stress initiated mucosal injury, accompanied with a decrease in NO level and DDAH activity[19]. Pretreatment with L-arginine, an antagonist of ADMA, markedly reduces the degree of gastric injury and elevates NO bioavailability. Administration of exogenous ADMA significantly exacerbates gastric injury, concomitantly with a decrease in plasma level of NO. These findings indicate that ADMA may participate in the gastric mucosal injury process induced by various deleterious factors. Multiple exogenous NOS inhibitors, such as L-NMA, L-NAME and L-NMMA intensify gastric mucosal injury by decreasing gastric mucosal blood flow, while NO donor (nitroprusside sodium) or substrate (L-arginine) reverses such effects[13,20].

Cigarette smoking is one of the risk factors provoking gastroduodenal ulceration[21]. It increases both the incidence and relapse rate of peptic ulcer diseases and delays ulcer healing. Besides reducing bioavailability of NO, nicotine, a major component of tobacco, aggravates gastric mucosal injury due to some factors such as ethanol or stress[22]. Recently, we have found that in the rats treated with ethanol, the ulcer index and ADMA level were increased and the NO level was decreased, and these effects of ethanol were augmented by pre-treatment with nicotine[23]. Nicotine alone did not show significant impact on ulcer index, ADMA level and NO level. *In vitro* incubation of epithelial cells with ethanol induced cell injury and increased ADMA level in the cultural medium, an effect that was amplified in the presence of nicotine. Therefore, the

accelerated effect of nicotine on gastric mucosal dysfunction is associated with endogenous ADMA.

The mechanism of ADMA directly producing adverse effect in gastric mucosa is incompletely understood. It is widely accepted that NO bioavailability decrease is the major reason. Apoptosis reaction produced by ADMA has been observed in different types of cells, such as endothelial cell and smooth muscle cell[6,24]. Cell apoptosis has also been observed in cultured gastric epithelial cells when exposed to an exorbitant concentration of ADMA[23]. ADMA could directly induce the production of inflammatory cytokines tumor necrosis factor (TNF)-α and soluble intercellular adhesion molecule-1 *via* activation of p38 MAPK and ERK1/2 pathways in cultured endothelial cells[25]. We deduce that inflammatory reaction may be another important factor for ADMA-induced gastric injury because TNF-α level, the marker of inflammation, is significantly elevated when cells are damaged by ADMA in gastric mucosal epithelial cells. The hypothesized role of ADMA in gastric mucosal injury and the underlying mechanisms are summarized in Figure 1.

PERSPECTIVES AND CLINICAL IMPLICATIONS

ADMA might be a novel clinical and experimental biomarker related to gastric mucosal disorder. The mechanisms of impairment involve NO generation inhibition, inflammatory reaction and apoptosis promotion. Although the therapeutic tool targeting ADMA is available in multiple cardiovascular diseases, it is unknown in gastrointestinal diseases. The strategy to inhibit ADMA is beneficial to the treatment of gastric ulcer induced by ethanol in rats[26]. Thus, ADMA might be a candidate of therapeutic target in gastric mucosal damage.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2181

World J Gastroenterol 2011 May 7; 17(17): 2181-2190
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Soluble ST2: A new and promising activity marker in ulcerative colitis

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Supported by FONDECYT grant 1070954 and DA-CLC 2803
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Received: July 22, 2010 Revised: October 15, 2010
Accepted: October 22, 2010
Published online: May 7, 2011

Abstract

AIM: To correlate circulating soluble ST2 (sST2) levels with the severity of ulcerative colitis (UC) and serum levels of pro-inflammatory cytokines, and to demonstrate the predictive power of sST2 levels for differentiation between active and inactive UC.

METHODS: We recruited 153 patients: 82 with UC, 26 with Crohn's disease (CD) and 43 disease controls [non-inflammatory bowel disease (IBD)]. Subjects were excluded if they had diagnosis of asthma, autoimmune diseases or hypertension. The serum levels of sST2 and pro-inflammatory cytokines [pg/mL; median (25th-75th)] as well as clinical features, endoscopic and histological features, were subjected to analyses. The sST2 performance for discrimination between active and inactive UC, non-IBD and healthy controls (HC) was determined with regard to sensitivity and specificity, and Spearman's rank correlation coefficient (r). To validate the method, the area under the curve (AUC) of receiver-operator characteristic (ROC) was determined (AUC, 95% CI) and the total ST2 content of the colonic mucosa in UC patients was correlated with circulating levels of sST2.

RESULTS: The serum sST2 value was significantly higher in patients with active [235.80 (90.65-367.90) pg/mL] rather than inactive UC [33.19 (20.04-65.32) pg/mL], based on clinical, endoscopic and histopathological characteristics, as well as compared with non-IBD and HC ($P < 0.001$). The median level of sST2 in CD patients was 54.17 (35.02-122.0) pg/mL, significantly higher than that of the HC group only ($P < 0.01$). The cutoff was set at 74.87 pg/mL to compare active with inactive UC in a multicenter cohort of patients. Values of sensitivity, specificity, and ability to correctly classify UC, according to activity, were 83.33%, 83.33% and 83.33%, respectively. The AUC of the ROC curve to assess the ability of this molecule to discriminate between active *vs* inactive UC was 0.92 (0.86-0.97, $P < 0.0001$). The serum levels of sST2 in patients with UC significantly correlated with endoscopic and histo-

pathological scores (r = 0.76 and r = 0.67, P < 0.0001, respectively), and with the pro-inflammatory cytokine, tumor necrosis factor-α (r = 0.69 and r = 0.61, respectively, P < 0.0001). Interestingly, we found a direct correlation between total intestinal ST2 content and serum levels of sST2, adjusted to endoscopic activity score in patients with mild (r = 0.44, P = 0.004), moderate (r = 0.59, P = 0.002) and severe disease (r = 0.82, P = 0.002). Only patients with inactive UC showed no significant correlation (r = 0.45, P = 0.267).

CONCLUSION: sST2 levels correlated with disease severity and inflammatory cytokines, are able to differentiate active from inactive UC and might have a role as a biomarker.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Soluble ST2; Biomarkers

Peer reviewers: Dr. Christoph Reichel, Priv.-Doz., Head of the Gastroenterological Rehabilitation Center Bad Brückenau, Clinic Hartwald, German Pension Insurance Federal Office, Schlüchterner Str. 4, 97769 Bad Brückenau, Germany; Dr. Takayuki Yamamoto, Department of Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, 510-0016 Yokkaichi, Japan

Díaz-Jiménez D, Núñez LE, Beltrán CJ, Candia E, Suazo C, Álvarez-Lobos M, González MJ, Hermoso MA, Quera R. Soluble ST2: A new and promising activity marker in ulcerative colitis. *World J Gastroenterol* 2011; 17(17): 2181-2190 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2181.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2181

INTRODUCTION

Inflammatory bowel diseases (IBDs) belong to the group of chronic diseases that cause intestinal inflammation. Ulcerative colitis (UC) and Crohn's disease (CD) are the two most important diseases in this group. Their characteristics are mainly episodes of active inflammation or remission. In order to provide a differential diagnosis of these diseases, it is necessary to know the clinical, endoscopic, histological, radiologic and serologic characteristics, as well as their course throughout time[1].

Currently, classifications of IBD are based on epidemiologic (age, gender, race), clinical (activity rate, localization and phenotype) and genetic [single nucleotide polymorphism (SNP)][2,3] parameters, and the presence of biological markers[4,5]. However, due to the high percentage of non-classifiable IBD (10%-15%) and the difficulty of a differential diagnosis, it has become necessary to search for new markers for these diseases.

One ideal characteristic of an IBD biomarker is the specificity; however, it also has to be easy to detect, tests must be minimally invasive, low-cost, quick to perform and replicable across laboratories[6,7]. In addition, the biomarker should be able to identify individuals at risk of developing the disease, detect the activity, monitor the effect of the treatment and, finally, have a prognostic value for the reactivation of the disease[7,8]. Current biomarkers for IBD include serological levels of specific antibodies (ASCA, ANCA, anti-OmpC, anti-Cbir, anti-glycans)[9-12], serum (CRP and cytokines)[13-15] and fecal proteins (calprotectin and lactoferrin)[5,7,16-20]. Nevertheless, the majority of these markers show a low sensitivity and/or specificity, and they cannot reflect the real intestinal damage.

In this context, sST2 protein has recently been identified as a new and reliable biomarker of heart failure[21-26]. High serum levels of sST2 have been described in patients with chronic inflammatory diseases, such as autoimmune diseases[27,28] and asthma[29].

ST2 belongs to the interleukin (IL)-1R super-family, is coded in human chromosome 2 and is expressed as two splice variants: one membrane bound, ST2L, which is a receptor of IL-33; and a soluble protein, sST2[30-32].

Recently, in our laboratory, we have described for the first time increased levels of sST2 in serum and total ST2 in the colonic mucosa in IBD patients, and also its distribution in epithelial and infiltrating cells from colonic mucosa. In addition, we showed that serum sST2 levels significantly correlate with total ST2 levels in colonic mucosa[33]. Supporting our results, other groups also have shown evidence that the ST2/IL-33 system could be participating in the development of IBD[34-36].

To date, there are no studies that correlate levels of sST2 with severity of the UC.

The aims of the present study were to determine in another cohort of UC patients whether serum sST2 and intestinal total ST2 levels correlate with the severity of the disease, based on endoscopic and histological activity rates, and with serum levels of pro-inflammatory cytokines.

MATERIALS AND METHODS

Participants were recruited from the Gastroenterology Departments at "*Clínica Las Condes*", "*Hospital Clínico de la Universidad de Chile*" and "*Hospital Clínico de la Pontificia Universidad Católica de Chile*", respectively. Patients were diagnosed based on standard clinical, endoscopic and histological criteria. The study was approved by the Ethics Committee/Ethics Review Board of each participating center, and all patients signed an informed consent prior to their participation in this study.

During the study process, between January 2008 and December 2009, 153 patients were subjected to colonoscopy. Procedures were carried out by gastroenterologists with more than 5 years of experience in colonoscopy (co-authors RQ, MA-L), and findings were classified according to the clinical criteria of the Montreal Classification. Inclusion criteria for the study were: IBD diagnosed patients, > 18 years, blood specimens collected just before colonoscopy, biopsies taken and informed consent.

Exclusion criteria were: non-classifiable inflammatory disease, indeterminate colitis, infectious ileocolitis, asthma, history of autoimmune diseases, celiac disease and hypertension.

Patients were grouped based on endoscopic and histological criteria: Group UC (n = 84) and CD (n = 26), and non-IBD controls (irritable bowel syndrome, colorectal cancer, family history of colorectal cancer, diverticular disease and chronic diarrhea; n = 43). In addition, a group of healthy subjects (n = 40, between 18 and 45 years old) were included to determine reference levels of sST2.

A 5 mL blood specimen was obtained from each patient, and 3 to 4 biopsies were immediately frozen in liquid nitrogen and stored at -80℃ until analysis. From the healthy subjects, only a blood sample was obtained for analysis.

In the case of UC, endoscopic activity was determined in the most swollen area using the endoscopic Mayo Score[37]. In the case of CD, clinical activity was determined according to the Harvey-Bradshaw Index (HBI)[38], and for endoscopic activity, we used the Simple Endoscopic Score for Crohn's Disease (SES-CD)[39]. Histopathological score was used for the evaluation of intestinal inflammation in both diseases. Each biopsy was graded on a scale of 0-3 (0 = normal; 1 = mild; 2 = moderate; 3 = severe and included those patients with active ulceration) according to Gomes *et al*[40].

Quantification of serum sST2 and total intestinal ST2 levels

Levels of sST2 and total intestinal ST2, in serum and protein extract of colonic mucosa, respectively, were determined using a commercial enzyme-linked inmunosorbent assay (ELISA) kit for human ST2 (DuoSet, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Serum samples obtained from 5 mL of blood were subjected to a treatment with protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein extracts were obtained from each sample by homogenization using a lysis buffer supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Basel, Switzerland) and subsequent disruption by sonication. Levels of total intestinal ST2 were adjusted to the total protein concentration determined by Bradford protein assay. All samples were analyzed in duplicate and each determination was expressed in pg/mL. The detection limit of the technique, provided by the kit's manufacturer, is 20 pg/mL.

Measurement of serum inflammatory cytokines

Serum levels of IL-33 (Apotech, Geneva, Switzerland), IL-6 (Human IL-6 ELISA Ready-Set-Go, eBioscience, San Diego, CA, USA), and tumor necrosis factor (TNF)-α (Human TNF-α ELISA Ready-Set-Go, eBioscience) were measured using an ELISA kit, according to the manufacturer's instructions. The detection limits provided by the kit manufacturers are 5 pg/mL, 2 pg/mL and 4 pg/mL for IL-33, IL-6 and TNF-α, respectively. Samples were prepared as previously described for ST2 determination.

Statistical analysis

Data were analyzed using the statistical software GraphPad Prism4 (La Jolla, CA) and are presented as average ± SD for variables with a normal distribution, and as a median (25th-75th) in the case of non-parametric distributions. Differences and significances among analyzed groups were established by multiple comparisons using the non-parametric Kruskal-Wallis tests. Further comparisons of individual groups *vs* control were performed by using Bonferroni-Dunn statistics and a 5% significance level. Sensitivity and specificity for levels of sST2, and their respective confidence intervals of 95%, were calculated according to endoscopic activity and compared to a non-inflammatory condition. The best cut-off value for sST2 that discriminated between inactive UC and active UC patients and the different levels of endoscopic activity was determined by area under the curve (AUC). Uni and bivariate analyses were carried out to determine the risk factors associated with each one of the following demographic and clinical parameters: gender, age, extent of the disease and medication at endoscopy. The associations between serum levels of sST2 and total intestinal ST2, and serum cytokines, were analyzed using Spearman's rank correlation coefficient (r). For each statistical test that was used, values of $P \leqslant 0.05$ were considered significant.

RESULTS

Main characteristics of IBD patients

During the study period, a total of 153 patients were recruited. Of these, 84 (54.9%) corresponded to UC, 26 (16.9%) to CD and the other 43 (28.1%) to non-IBD controls. Table 1 summarizes the main characteristics of IBD patients following the Montreal classification and also indicates gender and age distribution among groups, as well as medication at endoscopy. At the time of the procedure, 40 UC (47.6%) and 12 CD patients (46.1%) were active according to endoscopic Mayo and SES-CD criteria, respectively.

Determination of reference levels and cut-off value for sST2 in patients with IBD

The reference level of sST2 in serum, determined in the healthy subject group (HC), was 32.40 (19.00-49.00) pg/mL; in the case of the non-IBD, CD and UC groups, levels were 46.33 (26.00-74.66) pg/mL, 54.17 (35.02-122.0) pg/mL and 67.59 (30.78-199.1) pg/mL, respectively, with significant differences ($P < 0.001$) between UC *vs* HC and CD *vs* HC (Figure 1A). Due to the low number of patients with each of the CD phenotypes, such as inflammatory, penetrating and stenosing, we decided to focus on the analysis of sST2 levels restricted to the group of UC patients. Analysis of the levels of sST2 in the serum of the UC group, according to the Mayo Score of endoscopic activity (active ⩾ 2) resulted in concentrations of

Table 1 Clinical characteristics of the patient groups *n* (%)

	IBD						Controls	
	UC				CD		Non-IBD	HC
No. of patients	84				26		43	40
Female (%)	48 (57.1)				13 (50)		23 (53.4)	18 (45)
Age (mean ± SD, yr)	38 ± 12.6				42.6 ± 15.5		49.8 ± 18.3	30.8 ± 5.4
Location of disease								
Ulcerative proctitis, E1	16 (19)							
Left-sided colitis, E2	16 (19)							
Extensive colitis, E3	52 (62)							
Small bowel, L1					4 (15.5)			
Colon, L2					15 (57.6)			
Ileocolonic, L3					7 (26.9)			
Disease behavior								
Inflammatory					25 (96.1)			
Stenosing					1 (3.9)			
Penetrating					0			
Index disease	0	1	2	3	⩽ 8	⩾ 9		
Endoscopic Mayo score	8 (9.5)	40 (47.6)	25 (29.7)	11(13.2)				
Histopathological score	8 (9.5)	35 (41.6)	32 (38.1)	9 (10.7)				
Harvey-Bradshaw Index					22 (84.6)	4 (15.4)		
SES-CD					24 (92.3)	2 (7.7)		
Medication at endoscopy								
No medication	14 (16.6)				5 (19.2)			
Topical 5-ASA	16 (19)				2 (7.7)			
Systemic 5-ASA	24 (28.5)				7 (26.9)			
Systemic steroids	10 (11.9)				3 (11.6)			
5-ASA + steroids	13 (15.5)				0			
5-ASA + azathioprine	7 (8.5)				4 (15.4)			
Azathioprine	0				5 (19.2)			

Data regarding location of disease, disease behavior, index disease and medication at endoscopy are represented as number of patients (%). UC: Ulcerative colitis; CD: Crohn's disease; HC: Healthy subjects; SES-CD: Simplified endoscopic activity score for Crohn's disease; 5-ASA: 5-aminosalicylic acid derivatives; IBD: Inflammatory bowel diseases.

Table 2 Sensitivity, specificity and cut-off values for sST2 in ulcerative colitis patients compared to other groups

	Inactive UC				Active UC			
	Cut-off value (pg/mL)	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Cut-off value (pg/mL)	Sensitivity (%)	Specificity (%)	AUC (95% CI)
HC	33.08	54.29	52.08	0.58 (0.46 -0.71)	64.25	94.29	91.67	0.98 (0.95-1.00)[b]
Non IBD	42.67	60.98	64.58	0.58 (0.45-0.70)	76.29	80.49	83.33	0.90 (0.84-0.97)[b]
iUC	-	-	-	-	74.87	83.33	83.33	0.92 (0.86-0.97)[b]

[b]$P < 0.0001$. iUC: Inactive ulcerative colitis; HC: Healthy subjects; IBD: Inflammatory bowel disease; AUC: Area under the curve.

33.19 (20.04-65.32) pg/mL for inactive UC and 235.8 (90.65-367.9) pg/mL for active UC, with significant differences between active UC *vs* HC, non-IBD and inactive UC ($P < 0.0001$) (Figure 1B).

Figure 1C shows the ROC curve for sST2 levels in the active UC group in relation to the inactive UC group of patients. The optimal cut-off value estimated for sST2 that allows for the discrimination of active UC compared to inactive UC was 74.87 pg/mL. The AUC of the ROC values, cut-off, sensitivity and specificity are summarized in Table 2.

Levels of sST2, IL-33, TNF-α and IL-6 in serum and their correlation with disease activity

The range of serum sST2 concentrations for the different UC sub-groups, according to endoscopic Mayo score, is shown in Figure 2 and Table 3. Significant differences were observed among groups with moderate (Score 2) and severe activity (Score 3) comparing to inactive (Score 0) and mild activity (Score 1) sub-groups ($P < 0.001$) (Figure 2A). Regarding the histopathological compromise of the mucosa, the serum sST2 levels were significantly higher in the severe (Score 3) and moderate (Score 2) inflammation groups compared to both normal (Score 0) and mild (Score 1) sub-groups ($P < 0.001$ and $P < 0.01$, respectively) (Figure 2B). Endoscopic and histopathological scores directly correlated with serum sST2, with $r = 0.76$ and $r = 0.67$, respectively (Table 3).

Levels of serum TNF-α in the different UC sub-groups were directly proportional to endoscopic and histopathological scores. When comparing serum sST2 and cytokine levels, only TNF-α significantly correlated, both

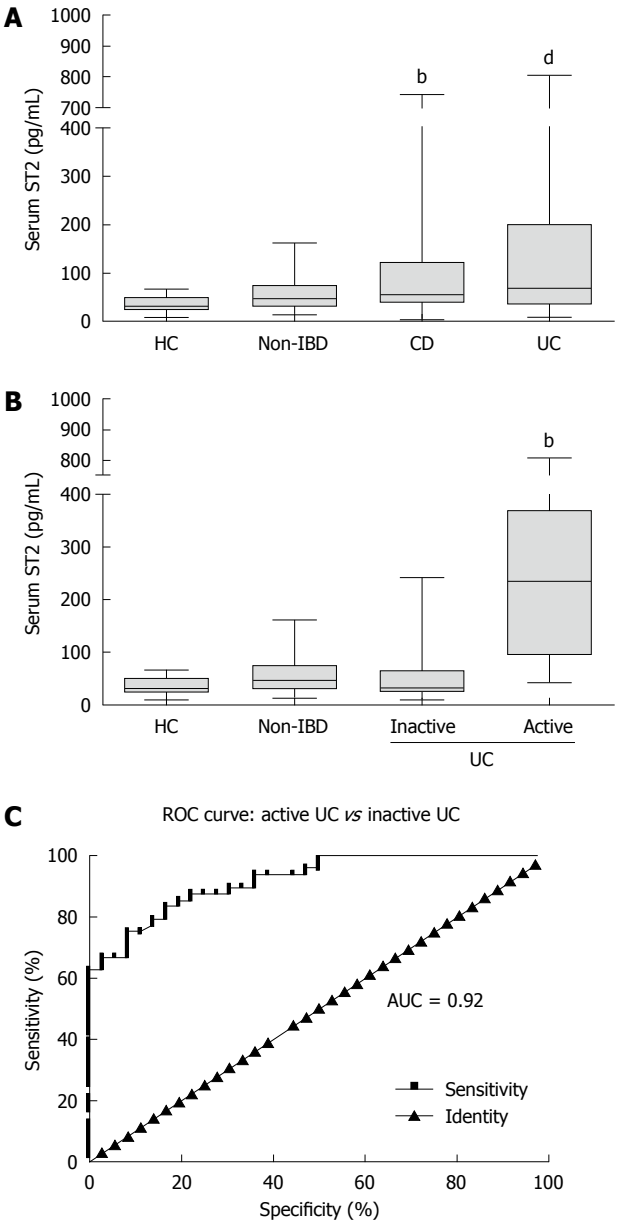

Figure 1 Distribution of serum levels of sST2 in the groups of studied patients. Box-plot showing the distribution of serum sST2 levels in healthy patients (HC) compared to non-inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) patients (A) and comparison of HC with non-IBD and inactive and active UC patients (B). Data are represented as median and percentiles (25th-75th) and significant differences in serum sST2 levels are shown between UC and CD patients *vs* HC (A) and between active UC and other groups (B). Receiver operating characteristics (ROC) curves for sST2 cut-off point determination (C). ROC curves illustrate the specificity and sensitivity in serum sST2 level determination to differentiate active from inactive UC. A: $^{b}P < 0.01$, CD *vs* HC; $^{d}P < 0.001$, UC *vs* HC; B: $^{b}P < 0.001$, active UC *vs* HC, non-IBD and inactive UC.

with endoscopic ($r = 0.69$, $P < 0.0001$) and histopathological scores ($r = 0.61$, $P < 0.0001$) (Table 3).

Uni and bivariate analyses of serum levels of sST2, with reference to demographic and clinical parameters such as age, gender, localization of the disease and medication at the endoscopy for each one of the analyzed groups, are shown in Table 4. In addition to the activity score, significant differences were observed for localization ($P = 0.0061$) and medication ($P = 0.0067$) of the UC group (Table 4). These results show the same trend observed in Figure 2A and B, based on endoscopic and histopathological scores, regarding gender ($P < 0.0001$), localization ($P < 0.0001$) and medication with 5-aminosalicylic acid (5-ASA) ($P = 0.0005$) (data not shown). These findings demonstrate that sST2 values exclusively depend on the severity of the disease.

Table 3 Correlation data of endoscopic and histopathological scores with serum sST2, interleukin-33, interleukin-6, tumor necrosis factor-α levels and total intestinal ST2

Disease activity index	Endoscopic score	Histopathological score
sST2 (pg/mL)		
r	0.7624	0.6762
P-value	< 0.0001	< 0.0001
IL-33 (pg/mL)		
r	0.2869	0.1687
P-value	0.0146	0.1657
IL-6 (pg/mL)		
r	0.2315	0.0888
P-value	0.0676	0.2855
TNF-α (pg/mL)		
r	0.6961	0.6112
P-value	< 0.0001	< 0.0001
Intestinal ST2 (pg/mL per mg protein)		
r	0.6267	0.6034
P-value	< 0.0001	< 0.0001

IL: Interleukin; TNF: Tumor necrosis factor; *r*: Spearman's rank correlation coefficient.

Levels of total intestinal ST2 correlate with disease activity scores and serum levels of sST2 in UC patients

In order to determine if the findings observed at the systemic level reflect the local damage, total intestinal ST2 was measured in colonic mucosa. Similarly to the serum sST2 levels, total intestinal ST2 levels in UC are closely distributed to activity endoscopic ($P < 0.0001$) (Figure 2C) and histopathological score ($P < 0.0001$) (Figure 2D). Significant differences were observed between moderate and severe activity sub-groups compared to inactive and mild (Figure 2C and D). Similarly, total intestinal ST2 levels significantly correlated with endoscopic ($r = 0.62$, $P < 0.0001$) and histopathological scores ($r = 0.60$, $P < 0.0001$) (Table 3), as seen for serum sST2 levels (Figure 2A and B).

Furthermore, serum sST2 levels and total intestinal ST2 directly correlate, according to endoscopic Mayo activity score, in the severe ($r = 0.82$, $P = 0.0027$), moderate ($r = 0.59$, $P = 0.0020$) and mild ($r = 0.44$, $P = 0.0045$) sub-groups (Figure 3).

DISCUSSION

Our group first reported that the ST2/IL-33 system, described in other inflammatory diseases, could be involved in the pathogenesis of IBD, because levels of ST2 and IL-33 in IBD patients were higher than in healthy sub-

Table 4 Baseline serum sST2 levels according to clinical characteristics of patients

	Serum sST2 (pg/mL), median (Percentiles 25th-75th)		
	UC	Non-IBD	HC
Gender			
Female	55.5 (29.3-150.1)	43.9 (22.1-73.1)	29.4 (17.0-40.3)
Male	99.7 (34.0-216.4)	59.7 (35.6-74.9)	36.2 (27.0-53.2)
Age (yr)			
18-24	190.8 (52.6-489.6)	63.2 (31.7-74.9)	29.0 (17.0-45.0)
25-34	66.2 (30.4-313.3)	46.3 (18.5-116.6)	32.4 (16.9-40.8)
35-44	125.4 (37.0-182.9)	48.1 (32.2-67.8)	44.3 (27.9-59.6)
45-54	48.2 (24.9-116.4)	43.6 (26.0-72.4)	51.0 (35.7-65.0)
⩾ 55	51.3 (19.9-90.5)	53.0 (25.9-78.8)	0
Location of disease			
Ulcerative proctitis, E1	56.5 (22.4-141.6)		
Left-sided colitis, E2	35.8 (19.4-66.2)		
Extensive colitis, E3	110.2 (34.0-345.8)[a]		
Medication at endoscopy			
No medication	36.4 (19.6-105.6)		
Topical 5-ASA	39.7 (20.4-75.7)		
Systemic 5-ASA	59.0 (23.6-141.7)		
Systemic steroids	242.4 (124.9-349.1)[a]		
5-ASA + steroids	125.4 (50.0-417.9)		
5-ASA + azathioprine	41.1 (68.9-109.0)		
Azathioprine	0		

[a]$P \leqslant 0.05$ *vs* other groups in the analysis. UC: Ulcerative colitis; HC: Healthy subjects; IBD: Inflammatory bowel disease; 5-ASA: 5-aminosalicylic acid derivatives.

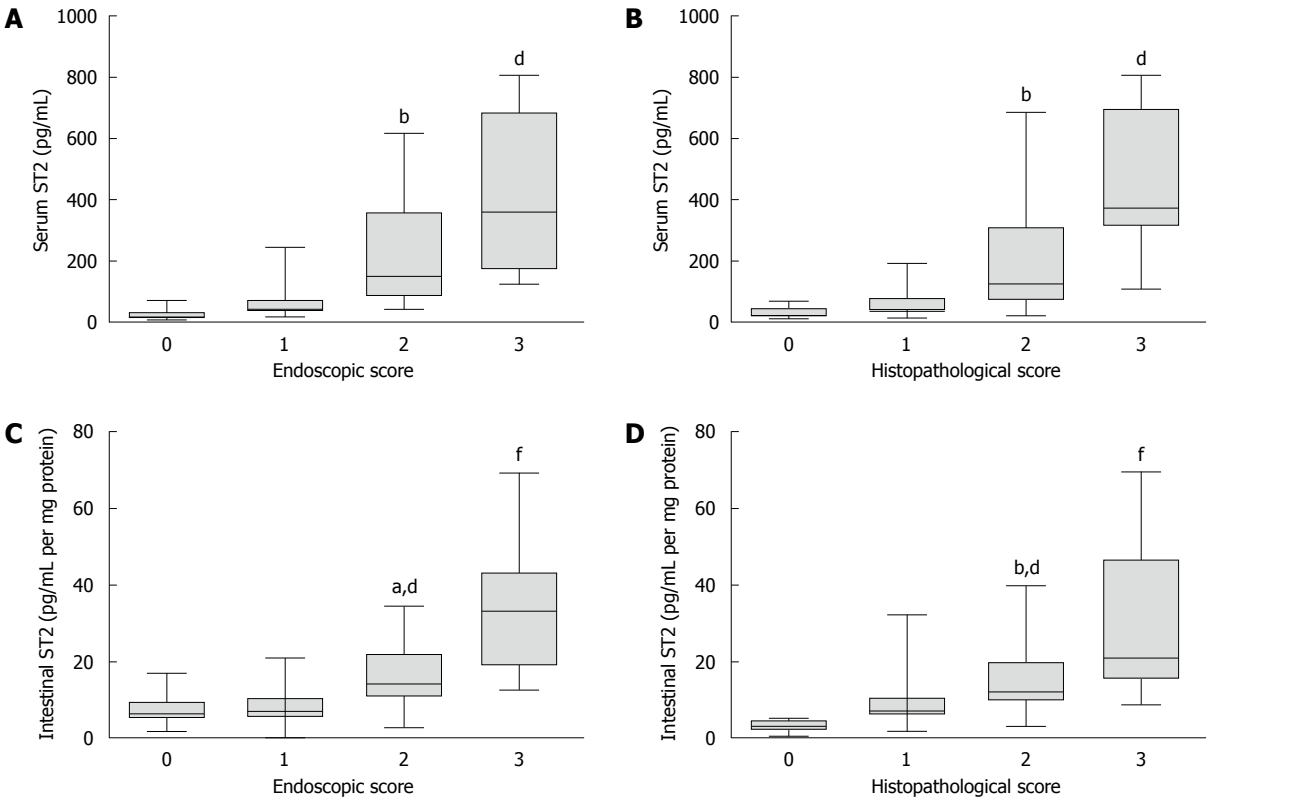

Figure 2 Analysis of serum sST2 and total intestinal ST2 levels in ulcerative colitis patients according to endoscopic and histopathological activity. Distribution of serum sST2 and total intestinal ST2 levels in ulcerative colitis (UC) patients according to the 4 rank Endoscopic Mayo Activity Score (A and C) (Activity: 0 = inactive; 1 = mild; 2 = moderate; 3 = severe) and histopathological score (B and D) (Degree of inflammation: 0 = normal; 1 = mild; 2 = moderate; 3 = severe with active ulceration). Data are represented as median and percentiles (25th-75th). Serum sST2 levels are significantly different among UC patient sub-groups of moderate and severe activity in relation to inactive and mild activity, independent of the score used. Total intestinal ST2 levels directly correlate with endoscopic activity (C) and degree of mucosal inflammation (D). A: [b]$P < 0.001$, 2 *vs* 0 and 1; [d]$P < 0.001$, 3 *vs* 0 and 1; B: [b]$P < 0.01$, 2 *vs* 0 and 1; [d]$P < 0.001$, 3 *vs* 0 and 1; C: [a]$P < 0.05$, 2 *vs* 1; [d]$P < 0.01$, 2 *vs* 0; [f]$P < 0.001$, 3 *vs* 0 and 1; D: [b]$P < 0.01$, 2 *vs* 1; [d]$P < 0.001$, 2 *vs* 0; [f]$P < 0.001$, 3 *vs* 0 and 1.

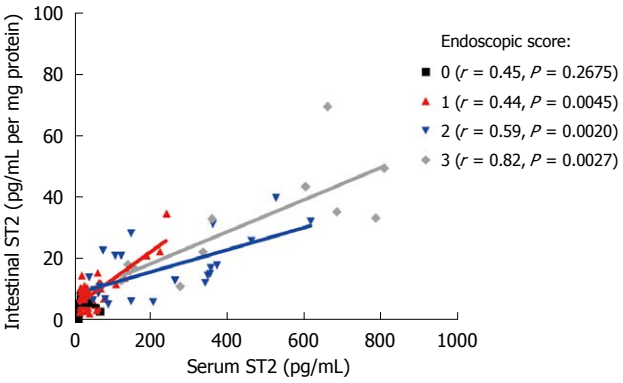

Figure 3 Graphic representation of the direct correlation between serum sST2 and total intestinal ST2 levels according to endoscopic activity scores. Activity: 0 = inactive; 1 = mild; 2 = moderate; 3 = severe. The trend lines for each analyzed group are shown. *r*: Spearman's rank correlation coefficient.

jects[33]. Recently, Pastorelli *et al*[34] also reported an increase in ST2/IL-33 system components in patients with IBD, both in the colonic mucosa as well as in serum. However, they reported that the circulating IL-33 levels in IBD patients were higher than our results and than in other diseases, even higher that those shown in sepsis[34]. Another two articles in the field[35,36] also confirmed elevated IL-33 expression in the mucosa of active IBD patients, with the limitation that these observations were conducted mainly at mRNA level, but ST2 levels were not studied.

The present study demonstrates, for the first time, a direct association between serum levels of sST2 protein and the degree of endoscopic and histopathological activity in UC.

Currently, a large number of serological, fecal or miscellaneous molecules have been proposed as indirect markers of IBD severity: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and even antineutrophil cytoplasmic antibodies (ANCA) and anti-*Saccaromyces cerevisiae* antibodies (ASCA). However, lately they have become less useful for the diagnosis and prognosis of the diseases. This is mainly due to a low sensitivity and specificity to intestinal inflammation[41] and these markers do not allow accurate differentiation between IBD and other intestinal diseases, nor do they discriminate activity status. Some molecules detected in serum are rather systemic markers that in general do not show inflammatory bowel processes[12,42]. The use of a serum inflammation marker that reflects the intestinal damage would be helpful for the management and prognosis of IBD patients. Fecal molecules, such as calprotectin and lactoferrin, represent an inflammatory neutrophilic process of the intestinal mucosa[16]. However, these are also non-specific markers of inflammation, which are increased in organic intestinal diseases such as diverticular disease[43,44] polyposis[45] and colorectal cancer[46,47].

Serum levels of sST2 allow for a highly valuable discrimination between UC patients and healthy subjects; however, this efficacy is reduced when trying to differentiate UC from organic intestinal diseases presenting any degree of inflammation. Alternatively, sST2 would allow, as happens with fecal calprotectin, the differentiation between UC and functional diseases, such as irritable bowel syndrome, chronic diarrhea and abdominal pain[5,19,48]. Many studies have shown that calprotectin significantly correlates with endoscopic and histological activity scores in CD and UC patients[49-51]. Calprotectin level decreases during clinical remission, which could be related to endoscopic mucosal healing[42,49,52], and consequently is considered a predictor of IBD reactivation. Serum sST2 levels allow for the differentiation between active and inactive UC with a high sensitivity and specificity. The cut-off value determined (74.87 pg/mL) permits the differentiation between active and inactive UC patients, as well as healthy subjects.

Similarly to fecal calprotectin, serum sST2 levels from UC patients significantly correlated with endoscopic (r = 0.76), as well as histopathological score (r = 0.67). Serum IL-33 level, another of the cytokines evaluated, did not show a direct relationship with disease activity; this might be due to the low levels detected compared to sST2, despite being the specific ligand of ST2. Serum sST2 levels in UC patients correlate with activity scores comparable with TNF-α, a commonly used serum inflammation marker. These characteristics result in the proposition of sST2 as an appropriate marker of inflammatory activity degree in UC. However, correlation of serum sST2 levels has to be achieved with other activity biomarkers previously associated with IBD, such as CRP or calprotectin.

In the case of CD patients, the analysis of serum sST2 values showed similar tendencies to those in UC, in relation to control patients (Figure 1A). The low incidence of CD in Chile[53], in addition to the exclusion criteria used in our study, account for the low number of CD patients included. Future studies will allow us to determine the association of sST2 with the inflammatory, stenosing and penetrating phenotypes of CD so as to support the concept that sST2 may also be applicable as a biomarker in CD.

Recently, ST2 has been described as a biomarker for heart failure, as serum levels correlate with hemodynamic variables, cardiac damage (BNP and pro-BNP) and inflammatory markers (CRP)[22,23,54,55]. In those studies, serum sST2 levels increase after myocardial infarction[21,56]; hence patients with a history of cardiopathies and hypertension were excluded.

In addition, some biochemical properties of sST2 support its characteristic as a reliable biomarker in UC, mainly based on its stability[57] and limited dependence on epidemiological and clinical factors, such as age, gender and diet[58].

In our study, serum sST2 levels in healthy subjects were similar to those described previously [32.4 (19-49) pg/mL *vs* 49 (4-89) pg/mL][54]. In addition, serum sST2 levels were higher in males than in females, and slightly increased between 18 and 24 years in age, as previously described[58]. However, when considering serum sST2 levels together with endoscopic activity, adjusted by gender, the distribution remained the same; therefore, we conclude that sST2

levels do not depend on these factors.

Therapeutic strategies for IBD patients are determined according to severity and localization of the affected area. UC patients with pancolitis presented higher serum sST2 levels in relation to proctitis or left-sided colitis. UC patients receiving systemic corticoids showed an increased sST2 level when compared to other treatments. Due to the low number of patients, we were not able to determine whether corticoids affect the sST2 concentration and its correlation with activity scores. However, in UC patients, ST2 levels did not show an association with mesalazine (5-ASA) treatment and in those patients sST2 levels follow activity degree of the disease. One of the most important qualities of a biomarker is that it has to be used in clinical practice and not be affected by drug therapy[12]. One of the main limitations of calprotectin as an IBD marker is the influence of non-steroidal anti-inflammatory drugs on its level, as previously shown[59-61]. In our study, 66.3% of IBD patients were receiving 5-ASA treatment, so measurement of calprotectin in those patients may be inconclusive.

Total ST2 levels in the colonic mucosa of UC patients significantly correlated with endoscopic and histopathological activity scores. In addition to the fact that total intestinal ST2 levels are directly associated with serum sST2 levels, these findings verify it as a new and promising UC activity biomarker. The relation between serum sST2 and inflammatory bowel activity would allow, in the future, the avoidance of a colonoscopic procedure in patients that do not require it. Association studies between ST2 and other biomarkers, such as calprotectin, may confirm its use.

It is possible that sST2 not only acts as a marker of UC activity; functions attributed to sST2 account for a role as an immunomodulator in inflammatory processes. At the cellular level, sST2 has been described as an inhibitor of IL-33/ST2L signaling[62], which causes polarization of naive T cells into Th2, and further, the production of IL-5 and IL-13 that are associated with UC[63,64]. On the other hand, ST2L activation with IL-33 stimulates TNF-α, IL-6 and IL-8 secretion in mast cells[65,66] and, together with IgE, stimulates degranulation[67]. The increase of sST2 during periods of inflammation may be involved in the control of the immune response associated with IBDs such as UC.

In summary, we demonstrated that serum sST2 levels allow for the effective differentiation between the endoscopic activity degrees of UC. Determining whether serum sST2 levels could have any prognostic value for UC (and possibly for CD), whether sST2 levels could monitor the treatment impact on endoscopic mucosal healing, and whether they could predict the risk of complications in IBD course or need of surgery, are some of the questions that should be answered by further studies.

ACKNOWLEDGMENTS

We acknowledge the help of C Heine, F López-Kostner and C Figueroa in sample collection and thank D Waissbluth for helping in collection of data.

COMMENTS

Background

Inflammatory bowel diseases (IBDs) belong to the group of chronic diseases that cause intestinal inflammation. Ulcerative colitis (UC) and Crohn's disease are the two most important diseases in this group. Their characteristics are mainly episodes of active inflammation or remission. Currently, classifications of UC are based on epidemiologic, clinical and genetic parameters, and the presence of biological markers. Therapeutic strategies for UC patients are determined according to severity and localization of the affected area.

Research frontiers

To date, there are no studies that correlate levels of soluble ST2 (sST2) with severity of UC. It is possible that sST2 not only acts as a marker of UC activity; functions attributed to sST2 account for a role as immunomodulator in inflammatory processes. At the cellular level, sST2 has been described as an inhibitor of interleukin (IL)-33/ST2L signaling. The increase of sST2 during periods of inflammation may be involved in the control of the immune response associated with IBD.

Innovations and breakthroughs

Soluble ST2 protein has been identified as a new and reliable biomarker of heart failure. High serum levels of sST2 have been described in patients with chronic inflammatory diseases, such as autoimmune diseases and asthma. Recently, in their laboratory, the authors have described for the first time increased levels of sST2 in the serum and total ST2 in the colonic mucosa of UC patients. In this study, we show that serum sST2 levels significantly correlate with total ST2 levels in the colonic mucosa. Supporting our results, other groups also have shown evidence that the ST2/IL-33 system participates in the development of IBD.

Applications

The relation between serum sST2 and inflammatory bowel activity would allow, in the future, avoidance of colonoscopy procedures in patients that do not require them. In addition, some biochemical properties of sST2, such as its stability, support its characteristic as a reliable biomarker in UC. If sST2 levels decreased during clinical remission, these could be related to endoscopic mucosal healing, and therefore be considered a predictor of UC reactivation.

Terminology

ST2 belongs to the IL-1R super-family, is coded in human chromosome 2 and is expressed as two splice variants: one membrane bound, ST2L, which is a receptor of IL-33; and a soluble protein, sST2.

Peer review

The authors examined the expression of components of the ST2/IL-33 system in serum and colonic mucosa in UC patients and correlated levels of sST2 with severity of the disease. The study revealed that sST2 levels are able to differentiate active from inactive UC and correlate with endoscopic and histopathological activity scores. In addition to the fact that total intestinal ST2 levels are directly associated with serum sST2 levels, these findings verify that circulating sST2 levels may play an important role as a new and promising biological marker in UC.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2191

World J Gastroenterol 2011 May 7; 17(17): 2191-2198
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Parallel decline of CD8+CD38+ lymphocytes and viremia in treated hepatitis B patients

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Received: August 20, 2010 Revised: September 25, 2010
Accepted: October 2, 2010
Published online: May 7, 2011

Abstract

AIM: To assess the peripheral T lymphocyte subsets in chronic hepatitis B virus (HBV) infection, and their dynamics in response to adefovir dipivoxil monotherapy.

METHODS: Proportions and absolute counts of peripheral natural killer cells, B cells, CD8+, CD4+, CD8+CD38+, CD8+CD28+ and CD4+CD28+ T cells were determined using three-color flow cytometry in chronic hepatitis B patients (n = 35), HBV carriers (n = 25) and healthy controls (n = 35). Adefovir dipivoxil was initiated in 17 chronic hepatitis B patients who were regularly followed for 72 wk, during which period the T cell subsets and serum viral load were measured at each follow-up point.

RESULTS: The peripheral CD4+ T cell counts and CD8+ T cell counts decreased in chronic HBV infection. In chronic hepatitis B patients, proportions of CD8+CD38+ T cells were 62.0% ± 14.7%, much higher than those of HBV carriers and healthy controls. In the 13 hepatitis B patients who were treated and responded to adefovir dipivoxil, proportions of CD8+CD38+ T cells decreased from 53.9% ± 18.4% pre-therapy to 20.1% ± 11.3% by week 72 (P < 0.001), concomitant with viral load decline (HBV DNA fell from 7.31 to 3 log copies/mL). CD8+ T cell counts also underwent an average increase of 218 cells/μL by the end of 72-wk treatment. In those who failed the therapy, the CD8+CD38+ T cell population had more fluctuations.

CONCLUSION: CD8+ T cells abnormally activated in chronic HBV infection can be partially reversed by antiviral therapy. HBV-associated immune activation may be a crucial part of the pathogenesis and a promising target of treatment.

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Key words: Hepatitis B virus; Chronic hepatitis B; CD8+CD38+; T cell subsets

Peer reviewer: Shinji Shimoda, MD, PhD, Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan

Cao W, Qiu ZF, Li TS. Parallel decline of CD8+CD38+ lymphocytes and viremia in treated hepatitis B patients. *World J Gastroenterol* 2011; 17(17): 2191-2198 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2191.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2191

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health concern. Despite the availability of effective vaccines since the 1980s, approximately one third of the world's population has serological evidence of past or present infection with HBV[1]. In China, over 8% of the

whole population is chronically infected. Although these patients can remain asymptomatic for years, chronic hepatitis B (CHB) is a progressive disease that can result in cirrhosis or hepatocellular carcinoma, and related death.

Hepatitis B virus is a noncytopathic virus. Clinical outcomes of HBV infection largely depend on the quality and strength of the host's immune response. Studies have revealed that T cellular immune responses are essential for disease pathogenesis[2,3], and have identified CD8+ T lymphocytes as the main cellular subset responsible for viral control[4,5]. Compared with acute self-limiting infection, lack of vigorous and multispecific T cell response in chronic HBV infection has been observed, which leads to the failure of viral clearance and the progression of disease[6,7]. The composition of peripheral T cell subpopulations, on the other hand, serves as a valuable index for evaluating T immune status in chronic HBV infection. Impaired balance of peripheral T subpopulations has been reported at various stages of chronic HBV infections, associated with HBV replication levels, and can be partially restored after antiviral therapy[8-11]. However, results from previous studies are controversial as regards the exact changes taking place during chronic HBV infection, and few have gone so far as to investigate the dynamics of more specific T cell subpopulations (e.g., CD38+/CD28+ activated T cell subpopulation or other functional subpopulations), or their changes upon antiviral treatment.

CD38, a marker of T cell activation, has shown elevated level of expression with many acute or chronic infections[12,13]. With some infections, a correction of CD38 expression level could also be observed soon after effective treatment, e.g. human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) infections[14]. Whether it is the same case with chronic HBV infection deserves more exploration. Adefovir dipivoxil (ADV), a synthetic nucleotide analogue of adenosine monophosphate, has been proven to provide biochemical, virological and histological improvement at the 10 mg oral dose daily[15-17]. Its potency at inhibiting HBV replication raises the possibility that ADV may help to improve and restore the T cell profile, including CD38+ expression. The present investigation intended to give an initial assessment of the peripheral T lymphocyte subpopulations at different clinical stages of chronic HBV infection, and evaluate dynamics of these subpopulations with ADV monotherapy in treatment-naive chronic hepatitis B patients, especially those of CD8+CD38+ T lymphocytes.

MATERIALS AND METHODS

Patients

A total of 60 patients were enrolled from March to November 2007 at the Outpatient Hepatitis Clinic of Peking Union Medical College Hospital (PUMCH), Beijing, China. Among them, 35 patients displayed clinical, biochemical and virological evidence of HBe-positive chronic hepatitis B [hepatitis B surface antigen (HBsAg) positive, hepatitis B e antigen (HBeAg) positive, anti-HBe negative, anti-HBc positive], with fluctuating levels of alanine aminotransferase (ALT) (ranging from 82 to 252 U/L) and HBV DNA (ranging from 7.8×10^5 to 4.3×10^8 copies/mL). The other 25 patients were assigned as HBV carriers with positive HBsAg, various HBV DNA levels and normal ALTs. Standards of patient selection were based on 2009 AASLD (American Association for the Study of Liver Diseases) guidelines on chronic hepatitis B [18]. Another 35 healthy subjects were also included as controls. All individuals were negative for anti-hepatitis C virus and HIV-1, and for other markers of viral or autoimmune hepatitis. The study protocol was approved by the local Ethical Committee, and all subjects provided written informed consent. Full baseline characteristics and virological data are summarized in Table 1.

Table 1 Baseline characteristics of the patients (n = 95) n (%)

Characteristics	Hepatitis B patients (n = 35)	HBV carriers (n = 25)	Healthy controls (n = 35)
Mean age (yr)	34.4 ± 13.5	31.2 ± 13.3	34.0 ± 9.1
Sex (male/female)	29/6	13/12	21/14
Ethnic background	Han 35 (100)	Han 25 (100)	Han 35 (100)
Serum ALT level (IU/L)	281 ± 59.1	25.3 ± 11.5	21.4 ± 10.2
HBV DNA level (log copies/mL)	7.68 ± 0.98	8.43 ± 0.89	< 3
Positive for HBeAg	35 (100)	25 (100)	0 (0)
Prior medication for HBV	0 (0)	0 (0)	0 (0)

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

Among the 35 chronic hepatitis B patients, 17 were then assigned to adefovir dipivoxil monotherapy at the oral dose of 10 mg/d (Deyang Huakang Pharmaceutical Co., Ltd., Sichuan, China), based on their clinical manifestations and patient agreement. All treated patients were followed for at least 48 wk after adefovir dipivoxil initiation; 14 of them were followed for 72 wk, and still remain on the follow-up list. Virological, biochemical and serological assessments, as well as T cell subset measurement of these patients, were carried out before treatment and at each follow-up point.

Virological assessments

Serum HBV DNA load was assessed with real-time fluorescent quantitative polymerase chain reaction method (Real-Time-PCR) using a Lightcycler PCR system (FQD-33A, Bioer) with a lowest limit of detection of approximately 10^3 viral copies/mL. The experimental procedures were performed in strict accordance with the reagent kit (Shenzheng PG Biotech Co., Ltd.) package insert. The primer was provided in the kit, the reaction volume was 40 μL, and the reaction condition was 37℃ for 5 min, 94℃ for 1 min then 40 cycles as 95℃ for 5 s and 60℃ for 30 s. HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb) were measured by ELISA (enzyme-linked immunosorbent assay) method (Anthos 2010, Austria). The experimental methods followed the guidelines written in the reagent kit (Sino-American Biotech Co., Ltd.).

Table 2 Counts and proportions of patient peripheral lymphocyte subsets

T subsets	CHB patients (n = 35)	HBV carriers (n = 25)	Healthy controls (n = 35)
White blood cells × 10^9/L	4.96 ± 1.18[b]	5.60 ± 0.96[b]	6.53 ± 1.42
Lymphocytes × 10^9/L (%)	1.60 ± 0.51[b] (32.7 ± 8.7)	1.72 ± 0.44[b] (31.2 ± 8.8)	2.13 ± 0.49 (33.1 ± 6.6)
Natural killer cells /μL (%)	197 ± 102[a] (12.2 ± 5.1)	272 ± 189 (15.1 ± 8.4)	310 ± 181 (14.6 ± 7.8)
B cells/μL (%)	228 ± 148 (14.3 ± 6.3)	220 ± 113 (12.6 ± 4.6)	242 ± 95 (11.4 ± 3.8)
CD3+ T cells/μL (%)	1145 ± 380[b] (71.5 ± 7.1)	1190 ± 278[b] (70.4 ± 9.6)	1516 ± 382 (71.4 ± 7.6)
CD3+CD4+ T cells/μL (%)	573 ± 194[b] (36.3 ± 6.9)	673 ± 148[b] (40.1 ± 6.9)	816 ± 259 (38.2 ± 7.0)
CD3+CD8+ T cells/μL (%)	489 ± 213[a] (30.1 ± 8.0)	445 ± 157[b] (25.9 ± 6.1)	609 ± 177 (28.9 ± 5.7)
CD4+/CD8+	1.35 ± 0.62	1.64 ± 0.50	1.39 ± 0.44
CD8+CD38+ T cells/μL (%)	301 ± 152 (62.0 ± 14.7[b,d])	285 ± 121 (51.8 ± 18.5)	274 ± 81 (46.3 ± 11.9)
CD4+CD28+ T cells/μL (%[1])	532 ± 191[b] (96.0)	622 ± 149[a] (95.7)	744 ± 221 (94.7)
CD8+CD28+ T cells/μL (%)	235 ± 125[a] (51.1 ± 15.5)	245 ± 100[a] (55.7 ± 16.0)	306 ± 94 (51.3 ± 11.7)

[1]Medians were used as the CD4+CD28+ T cell proportions were non-normal variables according to Kolmogorov-Smirnov Normality Test. [a]$P < 0.05$, [b]$P < 0.01$ *vs* normal healthy controls; [d]$P < 0.01$ *vs* hepatitis B virus (HBV) carriers. CHB: Chronic hepatitis B.

Peripheral blood T lymphocyte subset measurement

Peripheral blood T cell subset measurements were routinely performed during each follow-up review. Percentages of T cell subsets were determined on 100 μL ethylenediamine tetra-acetic acid (EDTA) blood sample, using a three-color direct immunofluorescence method (Beckman-Coulter, USA, EPICS2XL). In sample Tube 1, CD3+ T cells were autogated and analyzed for CD4 and CD8 cell expression using CD4/CD8/CD3 cell triple staining. In Tube 2, CD16/CD19/CD3 cell triple staining was used to identify B cells and natural killer (NK) cells. In Tubes 3, 4 and 5, CD38/CD8/CD3, CD28/CD8/CD3 and CD28/CD4/CD3 cell triple antibody cocktails were used, respectively, for analysis of CD38 and CD28 expression. Absolute counts of lymphocyte subpopulations were calculated according to complete blood cell counts on the same day.

Statistical analysis

SPSS version 11.0 was used. Baseline variables were assessed for all 95 subjects. For those who were included in the adefovir dipivoxil treatment and follow-up, virological and immunological parameters till the end of 72 wk were collected. Normal variables were summarized as means and standard deviations, and non-normal variables as medians and interquartile range (IQR) according to Kolmogorov-Smirnov Normality Test. Normal data were compared by Student t test or one-way ANOVA adjusted for multiple comparison, as appropriate. Multiple comparison of non-normal data was carried out by Kruskal Wallis test. All tests were two-sided, and a P-value $\leqslant 0.05$ was considered significant. Associations between variables were assessed using Spearman's rank correlations. Tested results of serum HBV DNA which were below the lower limit of detection (less than 1000 copies per milliliter) were all analyzed as being 1000 copies per milliliter for the ease of analysis.

RESULTS

T lymphocyte subsets in chronic HBV infection

As shown in Table 2, the T lymphocyte counts in chronic hepatitis B patients and HBV carriers were 1145 ± 380 and 1190 ± 278 cells/μL, respectively, both significantly lower than those of the healthy controls ($P < 0.01$). The absolute counts of CD4+ T cells and CD8+ T cells in chronic hepatitis B patients and HBV carriers were also lower than those of the controls. Yet the proportions thereof showed no significant differences between these three groups. The average counts of NK cells in chronic hepatitis B patients showed a moderate decrease compared to those of the carriers and controls. There were no significant differences in B cell counts between the different groups.

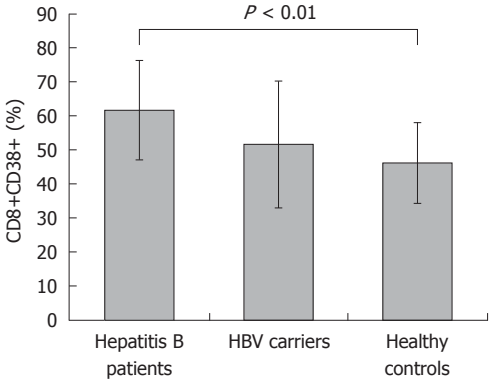

Figure 1 CD8+CD38+ T cell proportions in hepatitis B patients, hepatitis B virus carriers and healthy controls. Levels of CD8+CD38+ T cells in chronic hepatitis B patients were markedly higher than those of the other two groups. HBV: Hepatitis B virus.

Levels of CD8+CD38+ T cells were also examined for each group of patients. We noticed a marked increase of CD8+CD38+ T cell proportions in chronic hepatitis B patients (62.0% ± 14.7%), compared to those of the HBV carriers (51.8% ± 18.5%) and healthy controls (46.3% ± 11.9%) ($P < 0.01$) (Figure 1). No such differences were observed in absolute CD8+CD38+ T counts between these patients. The CD8+CD38+ T cell levels of the HBV carriers and healthy controls showed no differences.

The counts of functional subsets CD4+CD28+ and CD8+CD28+ T cells were both markedly decreased in

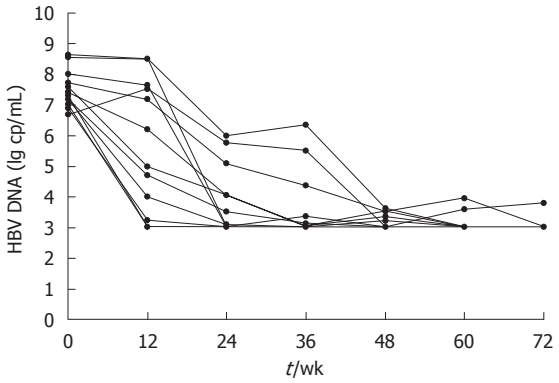

Figure 2 Hepatitis B viral load changes of the 13 patients who responded well to adefovir dipivoxil, by week of treatment. HBV: Hepatitis B virus.

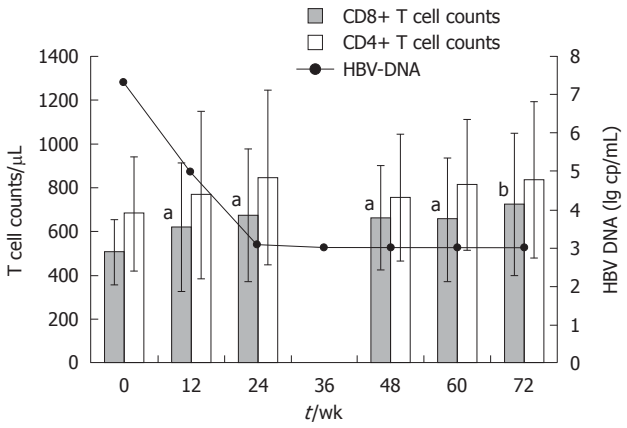

Figure 3 Changes of CD3+CD8+ and CD3+CD4+ T cell counts and median hepatitis B virus DNA in good responders, by week of adefovir dipivoxil treatment. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline T cell counts before adefovir dipivoxil treatment. HBV: Hepatitis B virus.

chronic hepatitis B patients and HBV carriers. However, again, no significant differences were observed as to the proportions thereof compared with the normal controls.

Virological responses

Median change in serum HBV DNA load is presented as $\log_{10}$ copies/mL. Among all the 17 patients receiving adefovir dipivoxil, 13 have achieved significant viral suppression, with their median HBV DNA decreased to undetectable levels ($< 10^3$ copies/mL) since 24 wk of treatment (Figure 2). However, the other 4 failed to respond to the therapy, either with no virological improvement or with a later rebound. As will be discussed later, changes in T lymphocyte subsets were analyzed based on patients' virological responses.

Changes in T lymphocyte subsets

CD3+CD8+ and CD3+CD4+ T lymphocyte subsets: As is shown in Figure 3, in those who responded well to adefovir dipivoxil, we observed a marked increase of 34.5% in absolute CD8+ T cell mean counts, from 505 ± 144 to 723 ± 324 cells/μL ($P < 0.01$) at the end of 72 wk of treatment. This increase began in the first 12 wk of treatment ($P < 0.05$), concomitant with the reduction of HBV DNA loads. Meanwhile, percentage of CD8+ T cells was also elevated at the end of 72 wk, from 30.7% to 33.2% ($P < 0.05$). In contrast, patients with poor response to adefovir dipivoxil showed no increase of CD8+ T cells.

Fluctuations of CD3+CD4+ T cell counts and proportions were also examined throughout the treatment. No significant changes were observed in either population. Absolute counts of CD4+ T cells at each time point are also shown in Figure 3.

As regards NK cells and B lymphocytes, neither the absolute numbers nor the proportions thereof showed any significant changes after the treatment.

CD8+CD38+ T cell subsets: In those who achieved good control of HBV viremia with adefovir dipivoxil, both the proportions and absolute counts of CD8+CD38+ T cells declined dramatically in peripheral blood (Figure 4A). From the first 12 wk after starting therapy, levels of CD8+CD38+ T cells began to decrease sharply. By week 24, the proportions of CD8+CD38+ T cells fell significantly from 53.9% ± 18.4% to 36.8% ± 14.8%, while the absolute counts fell from 271 to 215 cells/μL ($P < 0.01$), and the same trends were sustained afterwards. By week 72, proportion of CD8+CD38+ T cell count had decreased as much as 62.8% from baseline to 20.1%. Absolute counts thereof showed a similar pattern of change. In contrast, the other 4 patients who did not achieve successful responses, though initially having some reduction of CD8+CD38+ T cell levels, showed CD8+CD38+ T cell fluctuations as their HBV load became unstable. CD8+CD38+ T cell changes of those who failed the therapy are depicted in Figure 4B.

As is apparent from Figure 4A, the drop in CD8+CD38+ T cell levels during the first 24 wk of treatment coincided with a marked change in serum HBV DNA load, with a median reduction from 7.30 log copies/mL to undetectable level. Regardless of virological responses to adefovir dipivoxil, in both patient groups the shape of the CD8+CD38+ T cell fluctuation curve followed that of the HBV DNA level. Also, there was a close relationship between changes in viral load and changes in CD8+CD38+ T cell levels. Further analysis from all available data paired for viral loads and T cell subsets indicated that CD8+CD38+ T cell subpopulations in chronic hepatitis B patients are significantly and positively related to serum HBV DNA log values (Figure 5, $r = 0.438$, $P = 0.001$).

Moreover, changes in CD8+CD38+ T cells were examined in 11 individuals who had achieved and maintained a viral load below 10^3 copies/mL, including at least two assessments of CD8+CD38+ T cells during the period of virological suppression. Of these 11 patients, 9 (82.2%) experienced a further fall in CD38+ T cell counts and proportions, whereas changes in HBV DNA were undetectable, all below 10^3 copies/mL. The mean change in CD8+CD38+/CD8+ percentage from

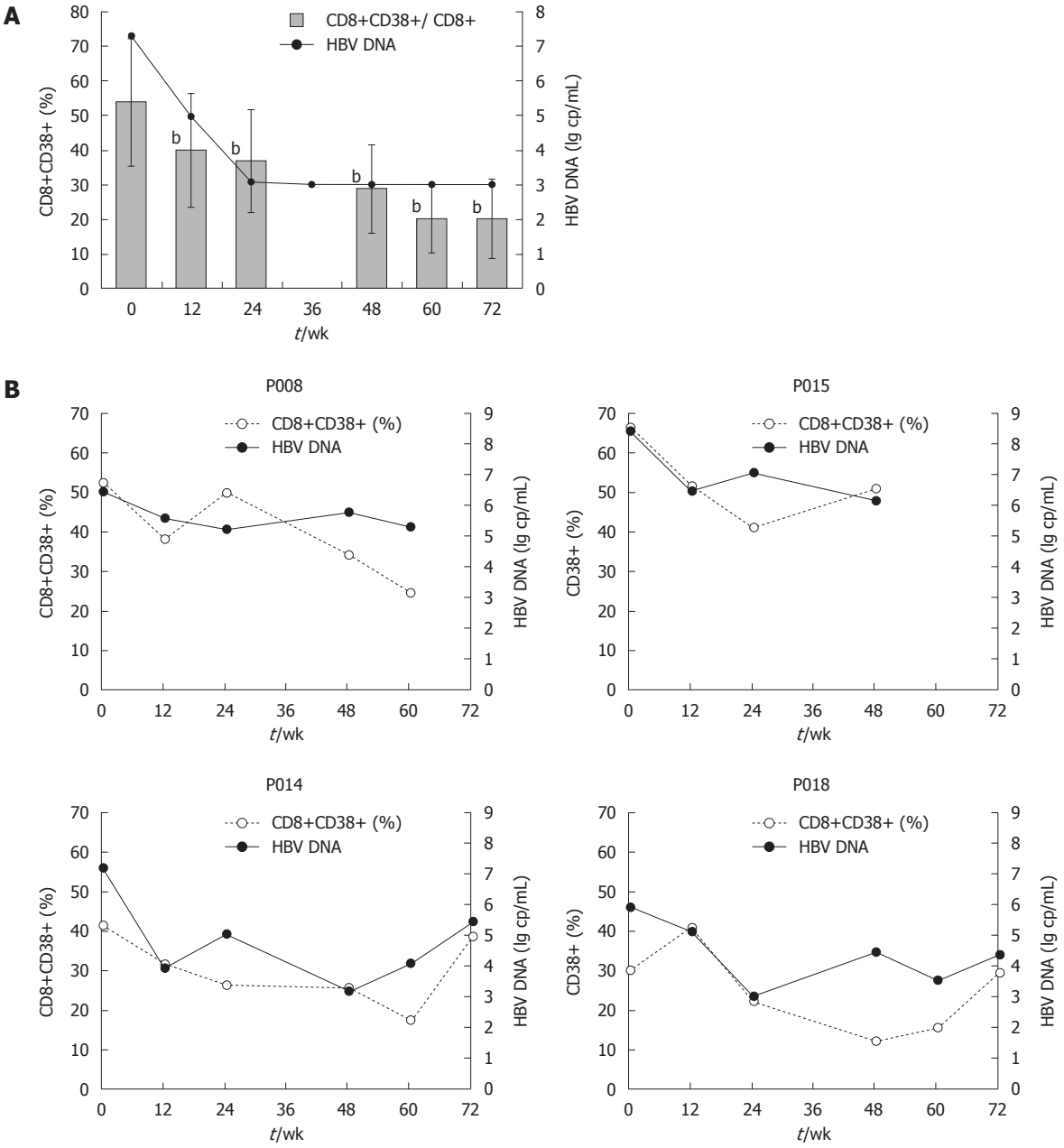

Figure 4 Changes of CD8+CD38+ T cell subsets and median hepatitis B virus DNA, by study week. A: Median CD8+CD38+ T cell proportions in good responders. $^{b}P < 0.01$; B: Changes of CD8+CD38+ T cell subsets in 4 non-responding patients 008, 014, 015, and 018. Among them, Patient 008 and 015 only finished 60 and 48 wk of treatment, respectively. Patient 014 and 018 completed 72 wk of treatment. HBV: Hepatitis B virus.

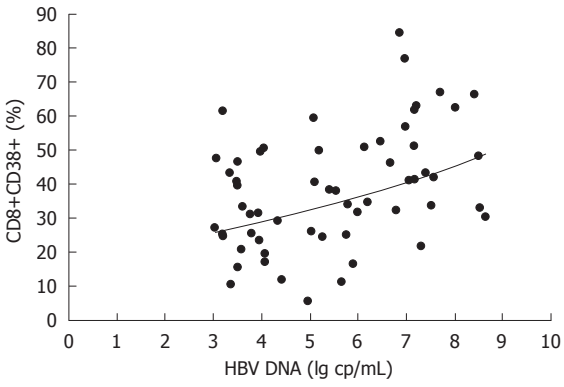

Figure 5 Correlations between CD8+CD38+ T cell levels and median hepatitis B virus DNA. HBV: Hepatitis B virus. $r = 0.438$, $P = 0.001$.

the first available count to the last was -10.6% ($P < 0.01$), indicating that reductions in CD8+CD38+ T cell proportions had continued even after serum HBV DNA become undetectable.

CD4+CD28+ and CD8+CD28+ T cell subsets: We also examined changes of CD28+ subpopulations both in CD8+ and CD4+ T lymphocytes before and after adefovir dipivoxil treatment. No statistically significant changes took place during the treatment (Data not shown).

DISCUSSION

In addition to HBV DNA level and liver function, chronic hepatitis B is further characterized by marked changes

in lymphocyte subpopulations and their activation status, which has only been poorly described in the literature. In the present study, we have identified discoordinate T cell profiles in chronic hepatitis B patients, with decreased counts of CD8+ T cells and robust CD8+ T activation, determined by an increase in the proportions of CD8+CD38+ T cells.

CD8+ and CD4+ T cells are two major components of the cellular immune system. As mentioned above, multiple studies have revealed that CD8+ T cells play an important role in clearance of the virus and progression of the disease. However, reports of CD8+ T cell subset changes in chronic hepatitis B patients have been rather conflicting. In the present study, we examined lymphocyte absolute counts as well as relative proportions in all 55 HBV infected patients and 35 healthy controls. We observed reductions of both CD8+ and CD4+ T cell levels in chronic hepatitis B patients and HBV carriers, which might reflect the T cell disturbance and suppression. Furthermore, in conjunction with the adefovir dipivoxil monotherapy, a marked elevation of CD8+ T cell levels took place, which demonstrated a partial restoration of T cell subsets and T cell immunity after the treatment. Prior studies have suggested that antiviral therapy can also overcome CD8+ T cell hypo-responsiveness in chronic HBV infection[14,15]. We believed that there could be some improvement of T cell functions with adefovir dipivoxil treatment. However, right at this point, more functional studies are needed in order to make a conclusion. On the other hand, neither counts nor proportions of CD4+ T cells demonstrated any significant changes throughout the therapy in our study, despite the initial reduction of CD4+ T cells in chronically infected patients. However, some have suggested that functions of HBV specific CD4+ T cells would be improved in response to antiviral treatment[19]. The peripheral CD4+/CD8+ ratios were much the same level in these three groups, perhaps because of the relatively localized infection and immunity that takes place in subjects with HBV infection. In addition, some prior studies have indicated that CD4+/CD8+ ratio of liver-derived lymphocytes, instead of peripheral lymphocytes, appeared to be more related to the level of HBV replication[20]. In this sense, our analysis of the peripheral T cell composition had its limitations in directly evaluating the local immune functions. However, it is still a much easier way for evaluating the immune status in a more general clinical practice.

The further evaluation of each functional and activated T cell subset in chronic HBV infection highlighted the group of CD8+CD38+ activated T cell subsets. We have found much higher proportions of circulating CD8+CD38+ T cells in chronic hepatitis B patients. CD38 is a surface glycoprotein existing on many immune cells. As a marker of cell activation, it is associated with many infectious diseases such as HIV, EBV or other infections. In certain infections, CD8+CD38+ T cells undergo a rapid up-and-down pattern after the infection once the immune control of acute phase is achieved[21]. However, persistency of immune activation and CD38+ expression throughout the acute and chronic phase is also possible, which may reflect the failure of the host's immune system to fully suppress viral replication. According to our observation of the peripheral CD8+CD38+ T cells in chronic hepatitis B patients, chronic HBV infection showed a mode of persistent T cell activation, just like that of HIV infection. However, elevations of CD8+CD38+ T cells in chronic hepatitis B patients were not so high as those of HIV-, EBV- or cytomegalovirus-infected patients[5,6,13,22], perhaps due to the relatively localized infection of HBV in liver tissue in contrast to other systemic infections. On the other hand, HBV carriers, though also chronically infected, had similar levels of CD8+CD38+ T cells as healthy controls. This difference of CD8+ T activation may help to explain the relative immune quiescence of HBV carriers.

In further assessment of the 17 patients receiving adefovir dipivoxil monotherapy, we found that not only did the treatment effectively inhibit HBV replication, but it also resulted in a concomitant, profound and rapid decline in the CD8+CD38+ T cell levels. This reduction in CD8+CD38+ T cells began to take place after the first 12 weeks of treatment, and in the majority of treated patients, CD8+CD38+ T cell levels finally became fully normalized. This recovery of CD8+CD38+ T cell proportions confirmed the abnormal T cell activation as a result of the virological failure in active HBV infection. A further correlation analysis showed a positive and significant relationship between the CD8+CD38+ T cell proportions and serum HBV DNA, which indicated a T phenotype drift with viral stimulation. However, at this stage, it remains unclear whether the immune activation we observed was only a secondary change of viral insults, or part of HBV pathogenesis. Based on the variance of T cell activation between hepatitis B patients and HBV carriers with similar levels of viremia, CD8+CD38+ activated T cells most probably represent multiple interactions between viral and host factors. Studies of immune activation in HIV have suggested that CD8 T cell activation levels can predict the rate of disease progression independent of viral load, though the causes of this immune activation are likely multifactorial[23,24]. Furthermore, some have tried to target HIV-associated immune activation by using immunomodulatory agents in addition to antiretroviral therapy[25-27]. The role of immune activation in chronic HBV infection has been poorly described. Here, our study of CD8+CD38+ activated T cells raises the possibility that T immune activation helps to shape the fate of chronic HBV infection and, therefore, use of immunomodulatory agents may help with the control of hepatitis B virus. However, a larger sample size and more specific and functional studies are needed to establish this conclusion.

Moreover, a continued decline in CD8+CD38+ T cell levels was observed even after HBV DNA became undetectable and CD8+CD38+ T cells became normalized in successfully treated patients. This finding is interesting,

because we have also observed similar phenomena in other treated viral infections. It could be an indication of presentations of the residual virus below the lower limit of viremia detection. Another possible explanation is that the CD8+CD38+ T cell levels under normal conditions may actually present an overall balance between the human immune system and occasional environmental insults, and that non-specific antiviral medications such as adefovir dipivoxil may also have an effect on these other pathogens, thus reducing the chances of immune activation in these patients. A longer follow-up of these patients during and after treatment may help to better explain the dynamics of the CD8+CD38+ T cell subsets.

The functional subsets of CD8+CD28+ and CD4+CD28+ T cells were also examined in our study. CD28+ acts as a co-stimulating molecule on the surface of T cells. However, according to a few currently available evaluations[12,28], unlike HIV infection which leads to the down-regulation of CD28 on the surface of T cells[29,30], HBV infection seems to have no significant impact on CD28 expression. In our study, the proportions of CD8+CD28+ and CD4+CD28+ T cells in the three groups were similar to each other. The absolute counts, though lower in chronic hepatitis patients and HBV carriers, were actually reflections of the decreased CD8+ and CD4+ T cell levels. Further treatment, which resulted in good control of the virus, did not change CD28 expression on the CD4+ or CD8+ T cells either, which further confirmed our observations before the treatment.

COMMENTS

Background

Chronic Hepatitis B virus (HBV) infection is a global health concern. Studies have identified that T cellular immune responses are essential for disease control. However, profiles of T cell subsets in chronic HBV infection, especially those of activated T cell subsets, have not been fully revealed. The authors studied the peripheral T cell subsets in chronic HBV infection, and their dynamics in response to adefovir dipivoxil monotherapy.

Research frontiers

T cellular immune responses are essential for pathogenesis of chronic HBV infection; impaired balance of peripheral T subpopulations has been reported at various stages of chronic HBV infections, and can be partially restored after antiviral therapy.

Innovations and breakthroughs

The study systemically assessed the peripheral T lymphocyte subsets in different types of chronic HBV infection. For the first time, the authors highlighted the group of CD8+CD38+ activated T cells, and their dynamics before and after treatment. The authors found T cell activation was linked with active HBV infection, and declined with successful antiviral treatment, which suggested the crucial role of immune activation in HBV pathogenesis.

Applications

The authors clarified differences of peripheral T cell subsets between different clinical types of chronic HBV infection, and changes thereof in response to treatment. HBV-associated immune activation may be crucial for the pathogenesis of HBV infection. This adds to current knowledge of T immune functions in chronic HBV infection, and helps with a better understanding of HBV pathogenesis. The abnormal T immune activation status in chronic hepatitis B suggests the possible use of immunomodulatory agents as further treatment.

Peer review

This is an interesting and well-written manuscript that clearly shows the link between CD8+38+ T-cells and HBV DNA levels. The authors described a decrease in total CD4 and CD8 T cell counts in chronic hepatitis B along with a higher proportion of CD8/CD38 cells after treatment. The amount of activated cells decreased in patients responding to an antiviral therapy while the amount of CD8 cells rose. The authors suggest HBV-associated immune activation may be a crucial part of the pathogenesis and a promising target of treatment.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2199

World J Gastroenterol 2011 May 7; 17(17): 2199-2205
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems

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Supported by The Federal Ministry of Research (BMBF - 01 GN0984)
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Received: July 12, 2010 Revised: August 16, 2010
Accepted: August 23, 2010
Published online: May 7, 2011

Abstract

AIM: To investigate effects of hepatotropic growth factors on radical production in rat hepatocytes during sepsis.

METHODS: Rat hepatocytes, isolated by collagenase perfusion, were incubated with a lipopolysaccharide (LPS)-containing cytokine mixture of interleukin-1β, tumor necrosis factor-α and interferon-γ to simulate sepsis and either co-incubated or pre-incubated with hepatotropic growth factors, e.g. hepatocyte growth factor, epidermal growth factor and/or transforming growth factor-α. Cells were analyzed for glutathione levels. Culture supernatants were assayed for production of reactive oxygen intermediates (ROIs) as well as NO_2^-, NO_3^- and S-nitrosothiols. To determine cellular damage, release of aspartate aminotransferase (AST) into the culture medium was analyzed. Activation of nuclear factor (NF)-κB was measured by electrophoretic mobility shift assay.

RESULTS: Rat hepatocytes treated with the LPS-containing cytokine mixture showed a significant increase in ROI and nitrogen oxide intermediate formation. AST leakage was not significantly increased in cells treated with the LPS-containing cytokine mixture, independent of growth-factor co-stimulation. However, pretreatment with growth factors significantly reduced AST leakage and ROI formation while increasing cellular glutathione. Application of growth factors did not result in increased NF-κB activation. Pretreatment with growth factors further increased formation of NO_2^-, NO_3^- and S-nitrosothiols in hepatocytes stimulated with LPS-containing cytokine mixture. Thus, we propose that, together with an increase in glutathione increased NO_2^-, NO_3^- formation might shift their metabolism towards non-toxic products.

CONCLUSION: Our data suggest that hepatotropic growth factors positively influence sepsis-induced hepatocellular injury by reducing cytotoxic ROI formation *via* induction of the cellular protective antioxidative systems.

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Key words: Primary human hepatocytes; Hepatocyte proliferation; Cytokines; Hepatotropic growth factors; Nitric oxide; Glutathione

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Glanemann M, Knobeloch D, Ehnert S, Culmes M, Seeliger C, Seehofer D, Nussler AK. Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems. *World J Gastroenterol* 2011; 17(17): 2199-2205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2199.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2199

INTRODUCTION

After partial hepatectomy, the remaining liver tissue undergoes rapid regeneration of its lost mass. Although it has been studied for many years, the exact mechanisms and interactions of this regenerative process are still the focus of many investigations[1-4]. Despite advances in surgical techniques and perioperative management, liver failure occasionally occurs after extended hepatectomy often being associated with postoperative infections that lead to multiple organ failure and death[5,6].

Although a two-thirds resection of the liver is not fatal, there is increased sensitivity to endotoxin, caused by up-regulation of the toll-like receptor 4, in the period following experimental hepatectomy[7]. Thus, intravenous injection of a sub-lethal dose of lipopolysaccharide (LPS) 48 h after surgery results in a high mortality in rats[8]. LPS directly activates Kupffer cells (the hepatic macrophages) to produce the tumor necrosis factor (TNF)-α and other inflammatory cytokines[9] through activation of the transcription factor, nuclear factor (NF)-κB. During liver regeneration, however, cytokines as well as hepatotropic growth factors have been well demonstrated to be involved in the process of tissue regeneration[10].

Numerous publications suggest a direct link between nitric oxide (NO) production, cellular loss of glutathione (GSH) and reduction of glutathione reductase activity. Thus, depletion of GSH reduces cellular NO levels while increasing superoxide formation, because GSH is an important cofactor for NO synthase[11-16]. Togo *et al*[17] suggest that NF-κB is the major transcription factor regulating the initial steps of liver regeneration. Growth factors, by different mechanisms, play an essential role in cell growth, proliferation, differentiation and DNA synthesis[18-21]. Certain interplays between cytokines and growth factors indeed seem to exist. Inflammatory cytokines increase the intracellular radical formation if not being blocked by intracellular antioxidative systems, e.g. GSH[22]. Therefore, it might be possible that adequate proliferation and regeneration occurs after partial hepatectomy, and the interplay of growth factors and cytokines could be shifted towards protective proliferation rather than hepatocellular injury.

Using an experimental model of sepsis/inflammation, we investigated the effects of hepatotropic growth factors, hepatocyte growth factor (HGF), epidermal growth factor (EGF) and/or transforming growth factor (TGF)-α on radical production and glutathione content in rat hepatocytes that were exposed to an inflammatory cytokine mixture of interferon (IFN)-γ, TNF-α and interleukin (IL)-1β, including LPS.

MATERIALS AND METHODS

Isolation, culture and treatment of primary rat hepatocytes

Rat hepatocytes were isolated from healthy Sprague-Dawley rats with a body weight between 250 and 300 g (Fa. Harlan-Winkelmann, Borchen, Germany) in accordance with the institutional guidelines of the Charité (Berlin, Germany) by collagenase P (Boehringer, Mannheim, Germany) digestion as described previously[23]. Hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 *g*. Cells were further purified by density gradient centrifugation using 30% Percoll (Pharmacia, Piscataway, NJ, USA). Hepatocyte purity, assessed by microscopy, was > 95% and viability, examined by trypan blue exclusion method, was consistently > 90%. Immediately after isolation, hepatocytes were plated onto gelatin-coated culture dishes (5×10^4 cells/cm^2) in Williams medium E (0.5 mmol/L L-arginine, 1 μmol/L insulin, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum). The next day, experiments were performed in serum-free medium. To imitate inflammation, cells were stimulated with a cytokine mixture (CM) consisting of 100 U/mL IFN-γ, 500 U/mL TNF-α, 10 U/mL IL-1β and 10 μg/mL LPS (*Escherichia coli* 111:B4) for 24 h. To investigate the effect of growth factors on inflammation, cells were either co-stimulated or pretreated (12 h) with 20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF-α.

Measurement of NO_2^-, NO_3^- and S-nitrosothiols

Culture supernatants were assayed for the stable end products of NO oxidation (NO_2^- and NO_3^-) and S-nitrosothiols using modified procedures based on the Griess reaction as described previously[24].

Aspartate aminotransferase measurement

In order to evaluate cellular damage, culture supernatants were measured for aspartate aminotransferase (AST) leakage using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany).

Determination of cellular GSH levels

To evaluate total cellular GSH levels [GSH + oxidized glutathione (GSSG)] cells were suspended in 1 mL metaphosphoric acid (3%) and centrifuged at 1000 *g* for 5 min. Supernatants were adjusted to pH 7.5-8.0 with K_2CO_3. Total cellular GSH was assayed, using an enzymatic recycling procedure, as described previously[22]. Reduced GSH was sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic-acid) (DTNB) to GSSG. The rate of DTNB formation was monitored at 412 nm and glutathione content was determined from a standard curve. To determine GSSG, GSH was masked with 2-vinylpyridine. Then, GSSG was reduced by NADPH to GSH in the presence of glutathione reductase to react again with DTNB. Oxidized and

reduced GSH that may be released in the supernatant were measured in the same way. All data were normalized to total protein content determined from cell pellets.

Determination of superoxide (O_2^-)

Release of O_2^- into culture supernatant was measured by monitoring the superoxide dismutase-dependent reduction of 160 μmol/L ferricytochrome c at 550 nm and 37℃, where 1 mol O_2^- reduces 1 mol ferricytochrome c[14].

Measurement of NF-κB activation

The NF-κB binding activity was analyzed by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from cells that were homogenized in sucrose buffer (2.1 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5 mmol/L NaF, 0.5 mmol/L Leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, 1 mmol/L DTT, and 0.1 mmol/L PMSF). Nuclei were separated by centrifugation (35 000 *g*, 3 h, 4℃) and washed twice in sucrose washing buffer. Nuclei were resuspended in high-salt buffer (20 mmol/L HEPES at pH 7.9, 1.5 mmol/L $MgCl_2$, 440 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerin, 5 mmol/L NaF, 0.1 mmol/L PMSF, 0.5 mmol/L leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, and 1 mmol/L DTT). After incubation on ice for 50 min, nuclei were spun down (14 000 *g*, 15 min, 4℃). Following quantification, protein extracts were stored at -70℃. NF-κB binding activity was performed as described previously[25]. The DNA probe used for EMSA corresponded to the high-affinity kB sequence found in the mouse κ light chain enhancer. Two oligonucleotides (sense 5'-AGCTTGGGGACTTTCCACTAGTACG-3', antisense 5'-AATTCGTACTAGTGGAAAGTCCCCA-3') were annealed to generate a double-stranded probe. Labeling was accomplished by the Klenow fragment of DNA polymerase I in the presence of dGTP, dCTP, dTTP and α[^{32}P] dATP. After labeling, the probe was added to 5 μg nuclear protein and 5 μg poly-dI-dC (Pharmacia Biotech Enzyme GmbH, Freiburg, Germany). Binding reactions were carried out in 10 mmol/L Tris-HCl (100 mmol/L NaCl, and 4% glycerol, pH 7.5) for 30 min on ice. DNA-protein complexes were resolved by electrophoresis in a 4% non-denaturing polyacrylamide gel. Monoclonal antibodies raised against various NF-κB subunits (p50, p52, rel A/p65, C rel, and rel B; Santa Cruz Biotechnology, Heidelberg, Germany) were used to confirm the nature of the DNA-protein complex. Competition assay was performed using unlabeled κB probe in 10- 50- and 100-fold concentrations[25].

Statistical analysis

Results are expressed as mean ± SE of at least five independent experiments ($N = 5$) measured in triplicates ($n = 3$). Data sets were compared by Kruskal-Wallis followed by Dunn's multiple comparison test (GraphPad Prism software; El Camino Real, Sunnyvale, CA USA). $P < 0.05$ was taken as minimum level of significance.

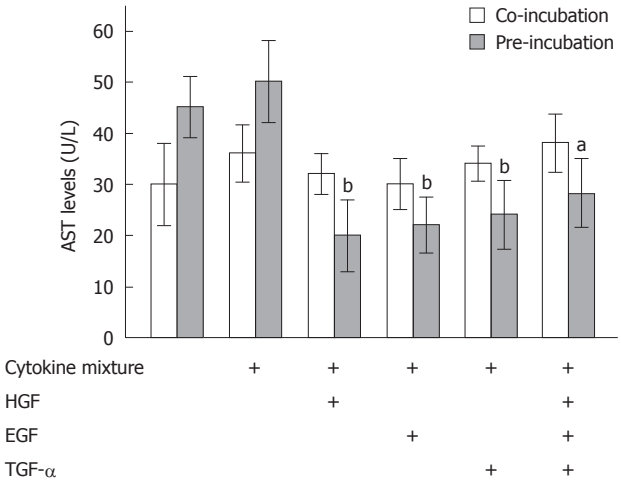

Figure 1 Pre-stimulation with growth factors significantly reduces cellular damage induced by lipopolysaccharide-containing cytokine mixture. Primary rat hepatocytes ($N = 5$, $n = 3$) treated with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h showed a slight increase in aspartate aminotransferase (AST) leakage into the culture supernatant. Co-incubation with the hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-α, individually or in combination, did not reduce AST leakage significantly (empty bars). However, preincubation with these hepatotropic growth factors resulted in a significant reduction in AST leakage (grey bars). $^aP < 0.05$, $^bP < 0.005$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone.

RESULTS

Measurement of AST leakage in rat hepatocytes pre- and co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with LPS-containing CM led to a slight increase in AST levels in the culture supernatant (30.0 ± 8.0 to 36.0 ± 5.6 U/L and 45.0 ± 6.0 to 50.0 ± 8.0 U/L). Co-incubation with growth factors (20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF-α) individually or in combination did not reduce AST leakage (Figure 1, empty bars). However, preincubation with growth factors significantly reduced AST leakage in cells treated with LPS-containing CM (Figure 1, grey bars).

Determination of reactive oxygen intermediate levels in rat hepatocytes co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with the LPS-containing CM slightly increased reactive oxygen intermediate (ROI) production (63.0 ± 6.7 to 80.0 ± 9.0 pmol O_2^-/min every 10^5 cells), while the intracellular glutathione levels were not notably affected as compared to untreated controls. Co-incubation with all growth factors combined slightly reduced ROI levels (Figure 2A and B, empty bars).

Determination of cellular GSH from rat hepatocytes pretreated with growth factors

Rat hepatocytes ($N = 5$, $n = 3$) were pretreated with growth factors 12 h prior to incubation with LPS-containing CM. As observed before, LPS-containing CM

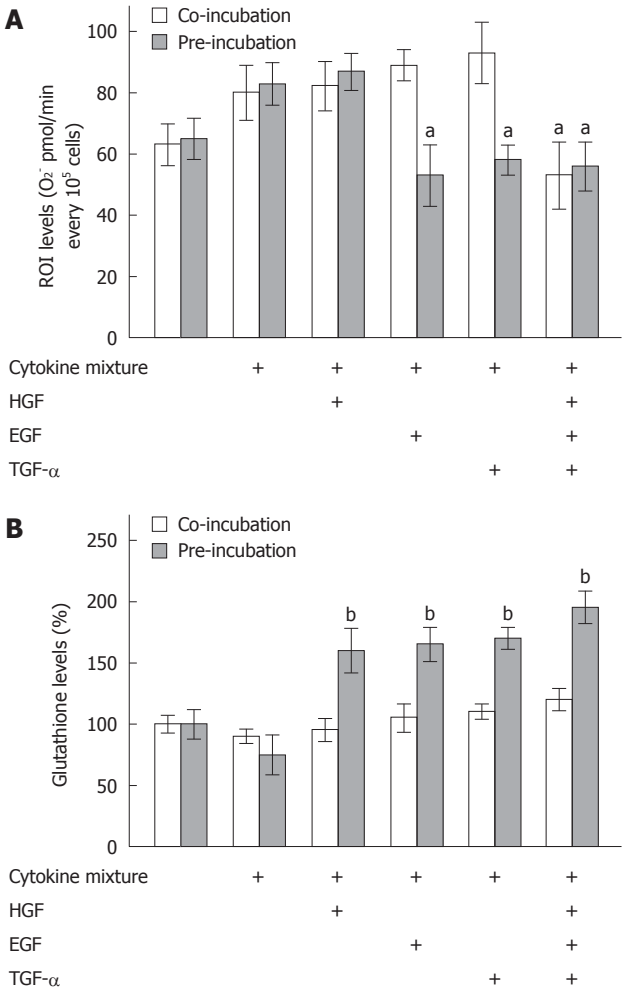

Figure 2 Increased oxidative stress in rat hepatocytes treated with lipopolysaccharide-containing cytokine mixture. A: Treatment of primary rat hepatocytes (N = 5, n = 3) with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h caused a slight increase in O_2^- production. B: Cellular glutathione levels were not significantly affected by this treatment. Co-incubation with single hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor (TGF)-α did not reduce reactive oxygen intermediate (ROI) production significantly. Co-incubation with the hepatotropic growth factor mixture alone was able to reduce ROI production significantly. Furthermore, co-incubation with the growth factors did not alter cellular glutathione levels (empty bars). On the other hand, preincubation with these growth factors, individually or in combination, significantly reduced ROI production (except for pretreatment with HGF alone). Preincubation with the different growth factors increased cellular glutathione significantly (grey bars). $^aP < 0.05$, $^bP < 0.001$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone.

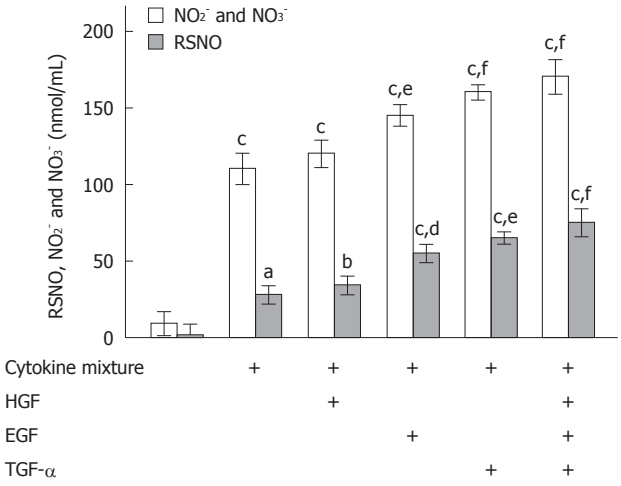

Figure 3 Hepatotropic growth factors increase nitric oxide formation in primary rat hepatocytes. NO_2^- plus NO_3^- (empty bars) and RSNO (grey bars) levels demonstrated a significant increase after hepatocyte (N = 5, n = 3) pretreatment with growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM), if compared to treatment with LPS-containing CM alone [except for pretreatment with hepatocyte growth factor (HGF) alone]. $^aP < 0.05$, $^bP < 0.005$, $^cP < 0.001$ *vs* corresponding untreated rat hepatocytes; $^dP < 0.05$, $^eP < 0.005$, $^fP < 0.001$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone. NO: Nitric oxide; EGF: Epidermal growth factor; TGF: Transforming growth factor.

slightly increased ROI production (65.0 ± 6.7 to 70.0 ± 7.0 pmol O_2^-/min every 10^5 cells) without a notable effect on intracellular glutathione levels (Figure 2A and B, grey bars). However, pretreatment with growth factors, both individually or in combination, significantly reduced ROI production by subsequent stimulation with LPS-containing CM. At the same time, intracellular glutathione levels were significantly increased. This goes along with the reduction in AST leakage observed with growth-factor-pretreated cells (Figure 1, grey bars). Combination of all three growth factors did not further decrease ROI production or increase intracellular glutathione compared to treatment with single growth factors. Pretreatment with HGF alone was not able to reduce ROI production by subsequent stimulation with LPS-containing CM.

Determination of NO_2^-, NO_3^- and S-nitrosothiol formation in rat hepatocytes pretreated with growth factors

Incubation of hepatocytes (N = 5, n = 3) with the LPS-containing CM led to a significant increase in NO production as compared to untreated controls. Formation of stable end products of NO oxidation (NO_2^- and NO_3^-) and S-nitrosothiols was even more increased in hepatocytes pretreated with growth factors when subsequently stimulated with LPS-containing CM. Pretreatment with HGF alone did not further increase NO_2^-, NO_3^- and S-nitrosothiols compared to stimulated cells without pretreatment (Figure 3). This was in accordance with the lack of reduction of ROIs under the same conditions.

Determination of NF-κB activation in rat hepatocytes stimulated with LPS-containing CM pretreated with or without growth factors

Rat hepatocytes, with and without pretreatment with growth factors, were stimulated with LPS-containing CM. NF-κB activation was measured at 0.5, 1, 2, 3 and 6 h after stimulation by EMSA. NF-κB was markedly increased 6 h after stimulation with LPS-containing CM (Figure 4A). Pretreatment with the combined or individual growth factors did not further increase NF-κB activation (Figure 4B). Moreover, growth factors alone (without LPS-containing CM) were not able to cause NF-κB expression (Figure 4B). The competition assay

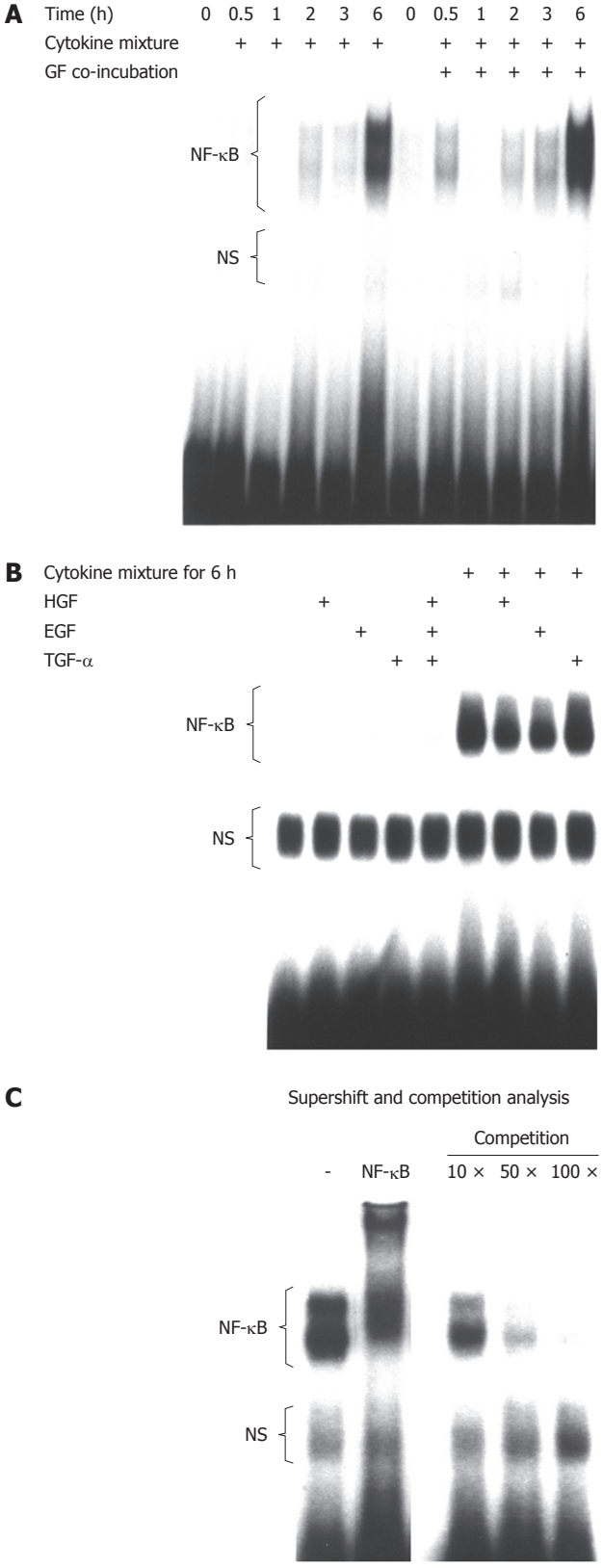

Figure 4 No additional nuclear factor-κB activation by hepatotropic growth factors. Electrophoretic mobility shift assay for nuclear factor (NF)-κB after hepatocyte pretreatment with hepatotropic growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM) demonstrated that stimulation with growth factors combined (A) or individually (B) did not further increase NF-κB expression when compared to stimulation with LPS-containing CM alone. Super shift and competition analysis (C) of NF-κB. HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor.

using an excess of unlabeled κB probes demonstrated the specificity of the signal (Figure 4C).

DISCUSSION

Recovery after partial hepatectomy requires an adequate interplay between hepatotropic growth factors and cytokines, as both factors are markedly involved and obviously well-balanced in the process of residual liver tissue proliferation and regeneration[26,27]. In this context, it has been reported that IL-6 plays a crucial role for regeneration, because it is supposed to prime remnant hepatocytes, in a way that they can fully respond to growth factors and enter a pre-replicative phase (G1)[26-28]. However, in our earlier studies, we have found that addition of IL-6 to hepatocyte cultures does not alter ROI or nitrogen oxide intermediate production in the presence of other inflammatory cytokines. When using the mentioned growth factors, there was also a lack of significant alterations in ROIs, and intracellular glutathione was seen. This suggests that growth factors have no direct impact on radical formation, cellular injury and/or cellular antioxidative protection systems.

Under septic or inflammatory conditions, as in the case of any infectious post-operative complication, when both plasma HGF and inflammatory cytokine levels are increased[29-31], cytokine and growth factor compositions might be different. Indeed, increased cytokine levels and protein--protein interactions may have positive and negative effects on liver regeneration[32,33]. Thus, IL-1β is markedly expressed during inflammation, and acts as a very potent inhibitor of hepatocyte proliferation[34]. Clinically observed, severe infections may seriously affect the post-operative course after liver resection, which results in an increased incidence of liver insufficiency and patient loss[6,35,36].

Obviously, cytokines and growth factors act in a well-balanced process under normal regenerative conditions. To gain a better understanding of the avoidance of the deleterious effects of postoperative infectious complications following liver resection, the interplay of growth factors and cytokines was a focus or our attention.

As cytokine reduction is hard to achieve if inflammation has already occurred, we focused our analysis on the effects of hepatotropic growth factor (pre)treatment in hepatocytes exposed to an inflammatory LPS-containing CM.

In the present study, we could demonstrate that growth factors, namely HGF, EGF and/or TGF-α may positively influence influenced cytokine-induced hepatocellular injury. In pre-treated hepatocytes, we found increased NO levels, while the expression of NF-κB was comparable to untreated controls. Our results confirm the study of Kaido *et al*[37] who have reported on successful prevention of post-operative liver failure in cirrhotic rats by continuous HGF supply. They have shown that rats with HGF-secreting fibroblasts (genetically modified to secret rat HGF and implanted in syngeneic rat spleen 7 d prior to exposition exposure of to hepatotoxins)

showed a dramatic resistance to carbon tetrachloride- and LPS-induced liver injury, which resulted in a significantly improved survival rate (80% *vs* 20%). In the same line of evidence, Kosai *et al*[38] have shown that HGF treatment 6 h and 30 min before and 3 h after intra-peritoneal LPS administration resulted in a significant increase of survival in mice (75% *vs* 0%). Although not focusing on pathophysiological interactions of HGF and cytokines, they clearly described HGF-related hepatic protection in case of severe endotoxemia[37,38].

Although several mechanisms may lead to hepatocyte injury, oxidative stress with increased radical formation as a consequence of inflammation, sepsis or ischemia-reperfusion, plays an important role. Intracellular antioxidative systems, e.g. p38-mitogen activated protein kinase or p21 may protect the cells, but they also decrease the hepatocyte proliferation rate by inhibiting hepatic DNA synthesis during the late G1 phase[39,40]. Other intracellular antioxidative systems include upregulation of enzymes e.g. heme oxygenase-1 by NF-κB[41]. We hypothesize that increased glutathione synthesis reduces the amount of cytotoxic radical formation. As further mechanisms improve oxygen supply, subsequent NO-dependent vasodilatation may contribute to the growth-factor-related protection of rat hepatocytes during sepsis. This could explain the results of Seto *et al*[42] who have observed that HGF pretreatment attenuates LPS-induced sinusoidal endothelial cell injury and intra-sinusoidal fibrin deposition.

However, further studies are required because this kind of cell protection was present only in hepatocyte pretreatment. Indeed, direct stimulation of rat hepatocytes with growth factors had no impact on intracellular ROI levels, glutathione content or AST levels under septic conditions.

Nevertheless, this aspect could provide new therapeutic options in case of partial hepatectomy. Pretreatment with hepatotropic growth factors may potentially decrease the incidence of postoperative liver insufficiency in patients undergoing extended liver resection, and subsequent infectious complications by shifting the postoperative course towards growth-factor-related liver tissue proliferation rather than cytokine-related cellular injury.

COMMENTS

Background

The exact mechanisms and interactions of the regenerative process in the liver after partial hepatectomy remain unclear. The well-balanced interplay of liver growth factors and cytokines is strongly interfered when any infectious postoperative complications occur. This effect leads to higher mortality *via* radical formation.

Research frontiers

The deleterious effects of postoperative infectious complications following liver resection have not been examined adequately. In particular, the interplay of pretreated growth factors and cytokines was studied.

Innovations and breakthroughs

The main reason for increased survival of growth-factor-pre-treated hepatocytes is the intracellular antioxidative system that prevents cell-damaging radical formation. Nitric oxide production during sepsis especially increases cell survival.

Applications

Pretreatment with hepatotropic growth factors can be a new therapeutic option in case of patients undergoing extended liver resection and may potentially decrease the incidence of postoperative liver insufficiency.

Terminology

Partial hepatectomy describes the process by which tumors are surgically removed from the liver. Cytokines are regulatory proteins that are released by cells of the immune system and act as mediators in the generation of an immune response.

Peer review

This paper is interesting and reports a large number of experiments. The methodology is well described and the results are clearly shown.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2206

World J Gastroenterol 2011 May 7; 17(17): 2206-2210
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Value of transient elastography for the prediction of variceal bleeding

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Received: October 15, 2010 Revised: January 11, 2011
Accepted: January 18, 2011
Published online: May 7, 2011

Abstract

AIM: To determine if liver stiffness (LS) measurements by means of transient elastography (TE) correlate with the presence of significant esophageal varices (EV) and if they can predict the occurrence of variceal bleeding.

METHODS: We studied 1000 cases of liver cirrhosis divided into 2 groups: patients without EV or with grade 1 varices (647 cases) and patients with significant varices (grade 2 and 3 EV) (353 cases). We divided the group of 540 cases with EV into another 2 subgroups: without variceal hemorrhage (375 patients) and patients with a history of variceal bleeding (165 cases). We compared the LS values between the groups using the unpaired t-test and we established cut-off LS values for the presence of significant EV and for the risk of bleeding by using the ROC curve.

RESULTS: The mean LS values in the 647 patients without or with grade 1 EV was statistically significantly lower than in the 353 patients with significant EV (26.29 ± 0.60 kPa *vs* 45.21 ± 1.07 kPa, $P < 0.0001$). Using the ROC curve we established a cut-off value of 31 kPa for the presence of EV, with 83% sensitivity (95% CI: 79.73%-85.93%) and 62% specificity (95% CI: 57.15%-66.81%), with 76.2% positive predictive value (PPV) (95% CI: 72.72%-79.43%) and 71.3% negative predictive value (NPV) (95% CI: 66.37%-76.05%) (AUROC 0.7807, $P < 0.0001$). The mean LS values in the group with a history of variceal bleeding (165 patients) was statistically significantly higher than in the group with no bleeding history (375 patients): 51.92 ± 1.56 kPa *vs* 35.20 ± 0.91 kPa, $P < 0.0001$). For a cut-off value of 50.7 kPa, LS had 53.33% sensitivity (95% CI: 45.42%-61.13%) and 82.67% specificity (95% CI: 78.45%-86.36%), with 82.71% PPV (95% CI: 78.5%-86.4%) and 53.66% NPV (95% CI: 45.72%-61.47%) (AUROC 0.7300, $P < 0.0001$) for the prediction of esophageal bleeding.

CONCLUSION: LS measurement by means of TE is a reliable noninvasive method for the detection of EV and for the prediction of variceal bleeding.

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Key words: Liver stiffness; Transient elastography; Esophageal varices; Variceal bleeding

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Sporea I, Raţiu I, Şirli R, Popescu A, Bota S. Value of transient elastography for the prediction of variceal bleeding. *World J Gastroenterol* 2011; 17(17): 2206-2210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2206.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2206

INTRODUCTION

Transient elastography (TE) is a new promising noninva-

sive and rapid method for the diagnosis and quantification of liver fibrosis in patients with chronic liver disease. It was originally developed to detect solid malignancies in soft tissues such as breast cancer and prostate cancer[1]. Liver stiffness (LS) measurement using TE is reproducible and independent of the operator[2]. Some recent extensive studies have demonstrated that LS measurement with TE is a good alternative for liver biopsy. The amount of fibrosis can be quantified very easily and reliably and is feasible in more than 95% of the patients[3-5]. In cirrhotic patients, LS measurements range from 12.5 to 75.5 kPa. However, the clinical relevance of these values is unknown.

The risk of variceal hemorrhage is clearly related to the size of esophageal varices (EV). Therefore primary prevention of variceal bleeding applies in patients with previously diagnosed large EV (grade 2 or 3) detected by periodical upper digestive endoscopy [Baveno V and American Association for the Study of Liver Diseases (AASLD) Consensuses][6,7]. A generalized program of periodical upper tract endoscopy in these patients might result in a heavy economic burden even for developed countries. Furthermore, repeated examinations, when not performed under profound sedation, are often poorly accepted by patients who may refuse further follow up.

TE may be used to predict the presence of portal hypertension. LS measurement may allow prediction of the presence of large EV in patients with cirrhosis and may help to select patients for endoscopic screening[8-11].

The purpose of this study was to determine if TE can be used to predict indirectly the presence of portal hypertension and the risk of variceal bleeding. At present the Baveno V and AASLD Consensuses recommend screening all cirrhotic patients for EV. If LS measurement could predict the presence of large EV in patients with cirrhosis, we could select these patients for endoscopic screening.

MATERIALS AND METHODS

We studied 1000 consecutive patients with liver cirrhosis. Patients with hepatocellular carcinoma were excluded. In all patients, upper digestive endoscopy was performed by 10 experienced endoscopists (at least 300 upper digestive endoscopies performed). The time interval between endoscopy and TE evaluation was less than three months. EV were classified as: small (grade Ⅰ) - small straight varices; medium (grade Ⅱ) - enlarged tortuous varices occupying less than one third of the lumen; large (grade Ⅲ) - large coil-shaped varices occupying more than one third of the lumen.

In accordance with EV diagnosed on endoscopy and the history of variceal bleeding we divided this batch into 2 groups: Group V0,1 - without EV and patients with grade 1 varices (647 cases); Group V2,3 - with significant portal hypertension (grade 2 and 3 EV) (353 cases).

We selected only those cases with EV and we divided them into 2 groups: Group no upper digestive bleeding (UDB) - without variceal hemorrhage (375 patients) and Group UDB - (165 cases) with variceal bleeding.

In all patients we performed LS measurement by TE using a FibroScan device (Echosens, Paris). Measurements were performed in the right lobe of the liver through the intercostal spaces, on patients lying in the dorsal decubitus position with the right arm in maximal abduction. The tip of the transducer probe was covered with coupling gel and placed on the skin, between the rib bones at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion of at least 6 cm thick, free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start an acquisition. Measurement depth was between 25 mm and 65 mm below the skin surface. Measurements which did not had a correct vibration shape or a correct follow up of the vibration propagation were automatically rejected by the software. Ten successful measurements were performed on each patient. The success rate (SR) was calculated as the ratio of the number of successful measurements over the total number of acquisitions. The results are expressed in kilopascal (kPa). The median value of the successful measurements was kept as representative of LS. Only LS measurements obtained with at least 10 successful measurements, with a SR of at least 60% and an IQR < 30% (IQR, the interquartile range interval, is the difference between the 75th and the 25th percentile, essentially the range of middle 50% of the data) were considered reliable.

Both the endoscopist and FibroScan operator were blinded to the result of TE evaluation and endoscopic evaluation of EV, respectively.

For a statistical study of quantitative variables, the mean and standard variation were calculated. T-tests were performed to compare mean values of LS in various subgroups. The diagnostic performance of LS measurements was assessed by using receiver operating characteristics (ROC) curves. ROC curves were thus built for the detection of various grades of EV and for predicting the risk of variceal bleeding. Optimal cut-off values for LS measurements were chosen to maximize the sum of sensitivity and specificity.

The statistical analysis was performed using Microsoft Excel (Microsoft Office 2007) and GraphPad Prism 5 programs.

RESULTS

The mean LS values in the 647 patients without or with grade 1 EV were statistically significantly lower than in the 353 patients with significant EV (26.29 ± 0.60 kPa *vs* 45.21 ± 1.07 kPa, $P < 0.0001$) (Figure 1).

Using the ROC curve we established a cut-off value of 31 kPa for the presence of significant EV, with 83% sensitivity (95% CI: 79.73%-85.93%) and 62% specificity (95% CI: 57.15%-66.81%), with 76.2% positive predictive value (PPV) (95% CI: 72.72%-79.43%) and 71.3% negative predictive value (NPV) (95% CI: 66.37%-76.05%)

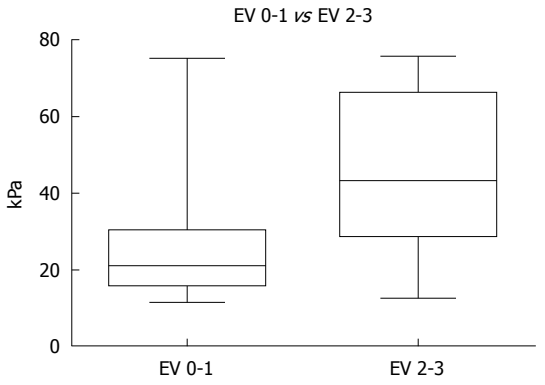

Figure 1 Mean liver stiffness values in patients with no or grade 1 esophageal varices as compared to those with significant esophageal varices (grade 2 and 3). Whiskers = minimum and maximum. EV: Esophageal varices.

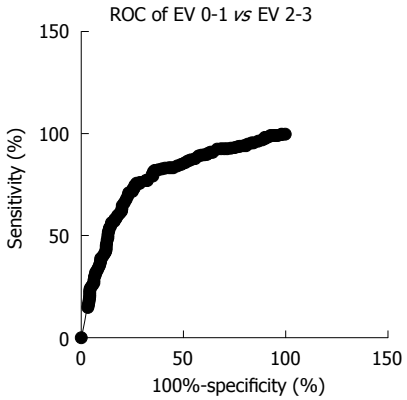

Figure 2 Predictive value of liver stiffness for the presence of at least grade 2 esophageal varices. ROC: Receiver operating characteristic.

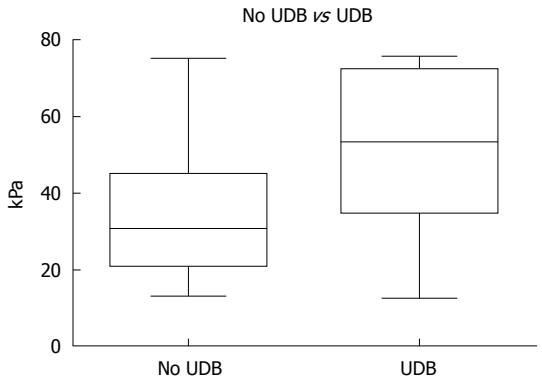

Figure 3 Mean liver stiffness values in patients with upper digestive bleeding due to variceal bleeding as compared to those without upper digestive bleeding. Whiskers = minimum and maximum. UDB: Upper digestive bleeding.

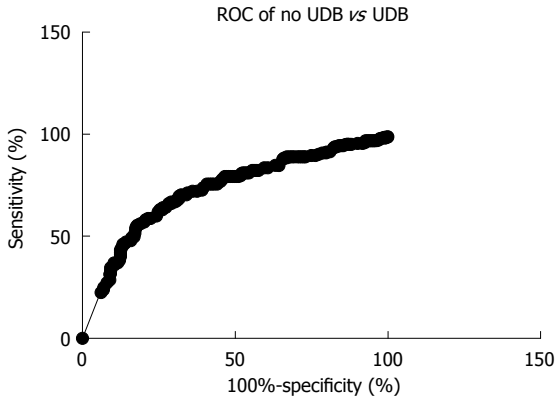

Figure 4 Predictive value of liver stiffness for upper digestive bleeding due to variceal bleeding. UDB: Upper digestive bleeding.

(AUROC 0.7807, $P < 0.0001$) (Figure 2).

The mean LS values in the group with a history of variceal bleeding (165 patients) were statistically significantly higher than in the group with no bleeding history (375 patients): 51.92 ± 1.56 kPa *vs* 35.20 ± 0.91 kPa, $P < 0.0001$) (Figure 3).

For a cut-off value of 50.7 kPa, LS had 53.33% sensitivity (95% CI: 45.42%-61.13%) and 82.67% specificity (95% CI: 78.45%-86.36%), with 82.71% PPV (95% CI: 78.5%-86.4%) and 53.66% NPV (95% CI: 45.72%-61.47%) (AUROC 0.7300, $P < 0.0001$) (Figure 4).

DISCUSSION

In previous studies, LS values < 19 kPa were highly predictive of the absence of significant EV (≥ grade 2), the cut off values for the presence of grade 2 and 3 EV ranging from 27.5 to 35 kPa, and the cut off value for esophageal bleeding being 62.7 kPa[12-14].

In other studies, LS measurement by TE was not accurate for the prediction of EV, with AUROC ranging from 0.76 to 0.84. Although sensitivity was good (71%-96%), specificity and PPV were low (60%-80% and 48%-54%, respectively) and overall accuracy was inferior as compared to simple tests like platelet count/spleen diameter ratio[11]. Another problem arising from these studies is the wide range of proposed cut offs, varying from 13.9 to 21.3 kPa for all varices, from 19 to 30 kPa for grade 2 varices and from 55 to 63 kPa for bleeding varices. The optimal cut offs therefore are still to be defined[15,16].

Foucher *et al*[13] (2006) assessed the accuracy of TE for the detection of large EV and the risk of variceal bleeding in patients with chronic liver disease. For the presence of EV grade 2 and 3, and esophageal bleeding, the cut offs were 27.5 and 62.7 kPa, respectively. The authors concluded that TE use for the follow-up and management of these patients could be of great interest and should be evaluated further.

Rudler *et al*[17] reported their data on consecutive patients admitted to the intensive care unit with variceal hemorrhage who underwent hepatic venous pressure gradient (HVPG) measurement and TE. With an HVPG cut off > 20 mmHg (HVPG > 20 mmHg is an independent predictor of death in patients with cirrhosis and variceal bleeding), 8 patients were enrolled, but 4 could not undergo elastography because of severe ascites. Correlation between LS and HVPG was poor. The authors concluded that TE is unlikely to be of utility in this patient popula-

tion.

Klibansky *et al*[18] was more successful in describing a useful application of TE to predict clinical outcomes in cirrhosis. Clinical endpoints were defined as the development of ascites or encephalopathy, variceal bleeding, development of hepatocellular carcinoma, or liver transplantation. Multivariate analysis indicated that the only independent predictors of outcome were Child-Pugh score and LS.

In 2009, Castéra *et al*[19] showed that TE could be a valuable tool for the diagnosis of cirrhosis but cannot replace endoscopy for variceal screening.

Another study assessed the correlation between LS with FibroScan and HVPG in diagnosing significant portal hypertension in 150 patients who underwent a liver biopsy and hemodynamic measurements. In patients with significant portal hypertension (HVPG > 10 mmHg), AUROC for FibroScan was 0.945. The cut off value of 21 kPa accurately predicted significant portal hypertension in 92% of patients for whom measurements were successful[20].

Due to the controversial results of all the studies described above we wanted to evaluate the value of TE for the prediction of EV and for the risk of bleeding in our patients.

The results of our study showed that TE is a useful technique for evaluating the presence of EV and hemorrhage prediction in cirrhotic patients. For a cut off value of 31 kPa, negative and PPVs were 76.2% and 71.3%, respectively. For > 31 kPa criterion, the cut off value was chosen to maximize the sum of sensitivity and specificity, whereas for > 40 kPa criterion we chose a cut off value to have a PPV of more than 85% with 77.8% sensitivity (95% CI: 74.57%-80.79%), 68.3% specificity (95% CI: 62.55%-73.68%), 86% PPV (95% CI: 83.18%-88.66%) and 55% NPV (95% CI: 49.60%-60.23%). For a cut-off value of 17.1 kPa, chosen to have a NPV close to 90%, we found the NPV to be 89.3%, with 43.2% PPV, 92.6% sensitivity and 33.5% specificity.

In clinical practice, such results could be of major relevance for the follow up of patients with cirrhosis. Cirrhosis places the patient at risk of clinical complications, such as portal hypertension, and variceal rupture is the second cause of death in cirrhosis, justifying early screening for EV. The usual means of diagnosing EV is upper gastrointestinal endoscopy. However, endoscopy can be considered invasive due to the technique and level of discomfort and the last recent consensus did not recommend endoscopic screening for the evaluation of bleeding risk in patients with grade 2 or 3 EV[6,7]. Non-invasive methods for diagnosis need to be developed.

According to our data, in patients with TE values > 40 kPa at least 8/10 cases will have significant portal hypertension. Therefore it is reasonable to recommend prophylactic β-blocker therapy without endoscopy. On the other hand, in patients with TE values < 40 kPa, 5/10 cases will have significant EV (NPV 54.9%), thus we recommend endoscopic evaluation. Below the cut-off value of 17.1 kPa we do not recommend endoscopic evaluation, since the chance of those patients having significant EV is only 1 in 10 (NPV 89.3%).

In our study we established an LS cut off value of 50.7 kPa for the risk of bleeding. A cut off value higher than 64 kPa correlates with a PPV > 90%, which means that those patients have a very high risk of bleeding despite primary prophylaxis with propranolol, with 77.75% sensitivity (95% CI: 73.64%-81.50%), 72.94% specificity (95% CI: 62.21%-82%), 93.88% PPV (95% CI: 90.96%-96.08%), 38.04% NPV (95% CI: 30.56%-45.96%). This data shows that 9 from 10 cases with TE values above 64 kPa will have variceal bleeding and we recommend prophylactic ligation in patients with LS values of more than 64 kPa.

In conclusion, LS measurement by means of TE is accurate for assessing the presence of large EV and the risk of variceal hemorrhage in cirrhotic patients.

COMMENTS

Background

The risk of variceal hemorrhage is related to the size of esophageal varices (EV). Therefore primary prevention of variceal bleeding applies in patients with previously diagnosed large EV (grade 2 or 3) detected by periodical upper digestive endoscopy. A generalized program of periodical upper tract endoscopy in these patients might result in a heavy economic burden even for developed countries. Furthermore, repeated examinations when not performed under profound sedation are often poorly accepted by patients who may refuse further follow up. Transient elastography (TE) may be used to predict the presence of portal hypertension. Liver stiffness (LS) measurement by means of TE may allow prediction of the presence of large EV in patients with cirrhosis and may help select patients for endoscopic screening

Research frontiers

TE may be used to predict the presence of portal hypertension. LS measurement may allow prediction of the presence of large EV in patients with cirrhosis and may help to select patients for endoscopic screening. The purpose of this study was to determine if TE can be used to predict indirectly the presence of portal hypertension and the risk of variceal bleeding. Currently, the Baveno V and AASLD Consensuses recommend screening all cirrhotic patients for EV. If LS measurement could predict the presence of large EV in patients with cirrhosis, we could select these patients for endoscopic screening.

Innovations and breakthroughs

The study showed that, in patients with TE values > 40 kPa at least 8/10 cases will have significant portal hypertension. Therefore it is reasonable to recommend prophylactic β-blocker therapy without endoscopy. In patients with TE values < 40 kPa, 5/10 cases will have significant EV [negative predictive value (NPV) 54.9%], thus endoscopic evaluation is mandatory. Below the cut-off value of 17.1 kPa endoscopic evaluation is not recommended, since the chance of those patients having significant EV is only 1 in 10 (NPV 89.3%). The research team established a LS cut off value of 50.7 kPa for the risk of bleeding. A cut off value higher than 64 kPa correlates with a positive predictive value > 90%, which means that those patients have a very high risk of bleeding despite primary prophylaxis with propranolol. The data shows that 9 from 10 cases with TE values above 64 kPa will have variceal bleeding and they recommend prophylactic ligation in patients with LS values of more than 64 kPa.

Applications

The authors recommend prophylactic β-blocker therapy without endoscopy in all patients with LS measurements > 40 kPa. In patients with LS measurements between 17.1 kPa and 40 kPa, considered to be "the grey zone", we recommend endoscopic evaluation of EV. Below the cut-off value of 17.1 kPa, endoscopic evaluation is not recommended since the chance of those patients to have significant EV is only 1 in 10 (NPV 89.3%). In patients with LS measurements higher than 64 kPa we recommend prophylactic band ligation of EV.

Terminology

TE is a new promising noninvasive and rapid method for the diagnosis and

quantification of liver fibrosis in patients with chronic liver disease. Some recent extensive studies have demonstrated that LS measurement by means of TE is a good alternative for liver biopsy. In cirrhotic patients, LS measurements range from 12.5 to 75.5 kPa.

Peer review

This is a nice study on the important topic of the non invasive diagnosis of large EV. Its strengths are in its size and what appears to be reliable data from TE.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2211

World J Gastroenterol 2011 May 7; 17(17): 2211-2215
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Increased intestinal permeability in inflammatory bowel diseases assessed by iohexol test

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Received: July 29, 2010 Revised: November 10, 2010
Accepted: November 17, 2010
Published online: May 7, 2011

Abstract

AIM: To study intestinal permeability (IP) and its relationship to the disease activity in patients with inflammatory bowel diseases (IBD) - Crohn's disease (CD) and ulcerative colitis (UC).

METHODS: Fifty-eight patients with active IBD (32 with CD and 26 with UC) and 25 healthy controls consented to participate in the study. The clinical activity of CD was estimated using the Crohn's Disease Activity Index (CDAI), and the endoscopic activity of UC using the Mayo scoring system. IP was assessed by the rise in levels of iohexol, which was administered orally (25 mL, 350 mg/mL) 2 h after breakfast. Three and six hours later serum (SIC mg/L) and urine (UIC g/mol) iohexol concentrations were determined by a validated HPLC-UV technique.

RESULTS: In the CD group, SIC values at 3 h (2.95 ± 2.11 mg/L) and at 6 h after ingestion (2.63 ± 2.18 mg/L) were significantly higher compared to those of healthy subjects (1.25 ± 1.40 mg/L and 1.11 ± 1.10 mg/L, respectively, $P < 0.05$). UIC (g/mol) values were also higher in patients, but the differences were significant only for UIC at 6 h. Significant positive correlation ($P < 0.05$) was found between the CDAI and IP, assessed by SIC at 3 h ($r = 0.60$) and 6 h ($r = 0.74$) after the ingestion. In comparison to controls, SIC and UIC of UC patients were higher in the two studied periods, but the differences were significant at 6 h only. Significantly higher values of SIC ($P < 0.05$) were found in patients with severe endoscopic activity of UC compared to those of patients with mild and moderate activity (3.68 ± 3.18 *vs* 0.92 ± 0.69 mg/L).

CONCLUSION: Serum levels of iohexol at 3 h and 6 h after its ingestion reflect increased IP, which is related to the disease activity in patients with IBD.

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Key words: Intestinal permeability; Iohexol test; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Gerova VA, Stoynov SG, Katsarov DS, Svinarov DA. Increased intestinal permeability in inflammatory bowel diseases assessed by iohexol test. *World J Gastroenterol* 2011; 17(17): 2211-2215 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2211.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2211

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two

principal forms of inflammatory bowel disease (IBD), are characterized by a chronic inflammation of the gastrointestinal tract. Over the past decade there has been increasing recognition of the importance of both epithelial barrier function and innate immunity in the genesis of intestinal inflammation[1-5].

Mucosal barrier function in IBD has been investigated in numerous studies and many of them, but not all[6], have demonstrated increased intestinal permeability (IP) in some of the patients[7-11]. Alterations of IP can be evaluated using several different probes such as disaccharides (lactulose, cellobiose), monosaccharides (mannitol), polyethylenglycols of different molecular mass and ^{51}Cr-ethylenediaminetetraacetate (^{51}Cr-EDTA). Studies have utilized different methods for the assessment of permeability, both in regard to administration procedures of the different probes and to outcome measures[12,13].

So far, IP assessment in patients has been performed by measuring urine recovery of ingested permeability substrates. The urine test can be relatively uncomfortable and can lead to potential inaccuracy[14,15] due to incomplete urine collection, renal dysfunction and variable hydration status of the tested subjects. In addition, some analytical difficulties in quantifying carbohydrates in urine have limited the widespread use of those permeability markers. Furthermore, urinary tract infections may compromise the recovery of sugar permeability markers[16]. Measurement of the permeability substrates in plasma or serum could eventually reduce the problems with urine recovery and could potentially have a valuable role, particularly in pediatric patients.

The aim of the present study was to evaluate IP in patients with active IBD (CD and UC) by measuring serum and urine levels of water-soluble contrast medium iohexol, to assess the relationship of IP to the disease activity, and to compare the reliability of serum *vs* urine levels of iohexol as an IP disease marker.

MATERIALS AND METHODS

Patients and controls

The study included 58 patients with active IBD: 32 patients with CD (16 males, 16 females, mean age 38.9 years: range 18-70 years) and 26 patients with UC (11 males, 15 females, mean age 41.5 years: range 21-70 years), hospitalized in the Clinical Centre of Gastroenterology, University Hospital Queen Joanna, Sofia. Diagnosis was based on the commonly accepted clinical, endoscopic and histological criteria. Disease activity of CD patients was estimated using the Crohn's Disease Activity Index (CDAI); values greater than 150 were accepted as a marker for clinical activity[17]. The endoscopic activity (EA) of UC was assessed using the Mayo scoring system (findings on endoscopy): 0: normal or inactive disease; 1: mild disease (erythema, decreased vascular pattern, mild friability, erosions); 2: moderate disease (marked erythema, lack of vascular pattern, friability); 3: severe disease (spontaneous bleeding, ulceration) (Table 1). CD patients were divided in two subgroups with regard to the endoscopic activity score: "EA 3" - severe and "EA 1+2" - mild and moderate endoscopic activity.

Table 1 Characteristics of patients with Crohn's disease and ulcerative colitis

Variables	CD (n = 32)	UC (n = 26)
Males/females	16/16	11/15
Age (yr), mean (range)	38.9 (18-70)	41.5 (21-70)
Duration of the disease (yr), mean (range)	6 (1-31)	6 (1-26)
Crohn's disease		
Area of involvement of the GI tract		
Small intestine only (L1)	4	
Large intestine only (L2)	7	
Ileo-colonic (L3)	21	
UGIT involvement (L4)	0	
Clinical activity		
CDAI, median (range)	211 (156-344)	
CDAI 151-220	17	
CDAI 221-400	15	
CDAI > 400	0	
Ulcerative colitis		
Extent (E)		
Proctitis (E 1)		2
Left-side (distal) colitis (E 2)		12
Total colitis (E 3)		12
Endoscopic activity-Mayo scoring system (EA)		
Endoscopic remission (EA 0)		0
Mild (EA 1)		6
Moderate (EA 2)		5
Severe (EA 3)		15

GI: Gastrointestinal; UGIT: Upper gastrointestinal tract; CDAI: Crohn's Disease Activity Index.

The location and behavior of CD, and extent and severity of UC, were classified using the modified Montreal classification[18]. According to CDAI, all CD patients had active disease (CDAI > 150). All patients with UC had endoscopic features of an active disease. Twenty-five healthy persons (18 males, 7 females, mean age 40.6 years: range 25-68 years) were recruited as a control group. None of them had signs or symptoms of gastrointestinal disorders or renal diseases. None of the investigated subjects took alcohol, non-steroidal anti-inflammatory drugs or any other medications (antidepressants, anticholinergics, metoclopramide, lactulose) that have the potential to affect gastrointestinal motility, for at least 2 wk before the test. Renal function, assessed by serum creatinine levels and calculated Glomerular Filtration Rate, was normal in all investigated subjects (Table 1).

The study was approved by the Ethics Committee of University Hospital Queen Joanna. Written informed consent was obtained from all patients and control subjects.

Assessment of the IP

Iohexol (Omnipaque™, Nycomed-General Electric) was administered orally (25 mL of 350 mg/mL injection solution) in the morning, 2 h after breakfast, immediately after voiding. Drinking was not allowed for the next 3 h. Food was permitted after 5 h. Blood and urine samples

were collected at 3 and 6 h after the iohexol ingestion. Serum was separated within 45 min after the blood withdrawal by centrifugation at 1 500 *g* for 10 min. Serum and urine were kept frozen at -20°C until analysis. Iohexol concentrations were determined by a validated high pressure liquid chromatography technique. Briefly, sample preparation consisted of protein precipitation; separation was performed on a C8 column with a mobile phase of 5% aqueous acetonitrile and detection at 240 nm. Selectivity was confirmed by comparing the signal in blank and spiked samples in 12 individual sources of human serum and urine. Accuracy and precision (within-run and between-runs) were within 12%; extraction recovery was over 90%; linearity range was 0.25-10.00 mg/L for serum samples and 2.50-700.00 mg/L for urine samples; $R^2 > 0.998$. Stability was also validated accordingly. Urine results were presented as iohexol/creatinine ratios (g/mol). Results of the control group (mean + 2SD) were used as a cut-off level for increased IP at 95% confidence interval, and values exceeding that cut-off were considered abnormal.

Statistical analysis

Statistical methods used: descriptive statistics, nonparametric ANOVA (Kruskal-Wallis test), nonparametric Mann-Whitney *U* test for overall testing the difference between subject groups, nonparametric Wilcoxon test for the difference between pairs of groups, Pearson's test (*r*) for correlation. Each hypothesis was tested at a level of significance of 0.05.

RESULTS

All permeability tests were well tolerated and no side effects were reported. Based on the results obtained from healthy subjects, values of SIC over 4.0 and 3.3 mg/L at 3 and 6 h respectively, as well as values of UIC over 35.5 and 31.4 g/mol at 3 and 6 h after ingestion, were accepted as abnormal (cut-offs). One control subject had slightly increased IP at 3 h, and two control subjects at 6 h, post-iohexol ingestion.

The results from applied Kruskal-Wallis test showed that the factor "group" is significant ($P < 0.05$). Mann-Whitney *U* test results indicated that in the group of CD patients the mean values of SIC at 3 h (2.95 ± 2.11 mg/L) and at 6 h (2.63 ± 2.18 mg/L) post-ingestion were significantly higher than those in the control group (1.25 ± 1.40 mg/L and 1.11 ± 1.10 mg/L, respectively). Abnormal IP was found in 10 patients at 3 h (31%) and in 16 patients at 6 h (50%) after iohexol intake. Urine recovery of iohexol was also higher in patients 3 h (18.09 ± 13.13 g/mol) and 6 h (36.92 ± 27.68 g/mol) post-ingestion compared to the control group (14.64 ± 10.44 g/mol and 14.18 ± 7.78 g/mol, respectively), but the difference was significant only for the mean UIC values at 6 h.

In the UC group, the mean serum levels (1.57 ± 1.55 mg/L and 2.49 ± 2.80 mg/L) and urine recovery of iohexol (9.86 ± 9.26 g/mol and 27.76 ± 25.18 g/mol) at 3 and 6 h after its ingestion, respectively, were higher than those for healthy controls, but the differences were significant only at 6 h for both parameters. IP was established as abnormal in 3 (11%) and 8 (31%) patients with UC at 3 and 6 h post-iohexol ingestion, respectively.

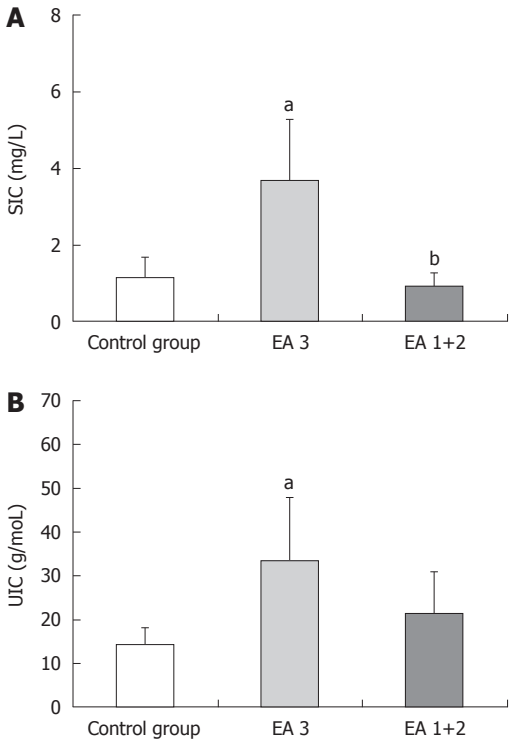

Figure 1 Serum iohexol concentrations (A) and urine iohexol concentrations (B) (mean ± SE) at 6 h post-ingestion according to Endoscopic Activity Mayo score (EA) of patients with ulcerative colitis. EA3: Severe endoscopic activity; EA 1+2 : Mild and moderate endoscopic activity. [a]$P < 0.05$, SIC and UIC *vs* control group; [b]$P < 0.05$, EA 3 *vs* EA 1+2 group. SIC: Serum Iohexol concentrations; UIC: Urine Iohexol concentrations.

There was a significant positive correlation between the parameters SIC and CDAI in the group of CD patients at 3 h ($r = 0.60$) and at 6 h ($r = 0.74$) post-ingestion, while the correlation between CDAI and UIC was insignificant both at 3 h ($r = 0.48$) and at 6 h ($r = 0.32$) post-ingestion of iohexol.

In the subgroup "EA 3" of patients with severe endoscopic activity of UC (n=15), the mean values of SIC at 6 h were significantly higher (3.68 ± 3.18 mg/L), compared to those of the control group (1.11 ± 1.10 mg/L), while the values (0.92 ± 0.69 mg/L) for the patient subgroup "EA 1+2" (n = 11) with mild and moderate endoscopic activity did not differ from the controls (Figure 1A). The mean UIC values for patient subgroup "EA 3" at 6 h were also significantly higher (33.60 ± 28.42 g/mol) compared to those of the control group (14.18 ± 7.78 g/mol), and mean UIC values of the subgroup "EA 1+2" (19.78 ± 18.25 g/mol) were slightly but insignificantly higher from the control values (Figure 1B).

The permeability disturbances were five-fold more frequent in the subgroup of patients with severe activity (47%) in comparison to the subgroup of patients with

mild and moderate activity (9%).

DISCUSSION

Altered barrier function in IBD is documented in experimental and clinical studies, but it is difficult to compare the results of IP due to the existing variability in the chemical nature of the chosen candidate markers in the experimental design and methodology. It is considered that the larger molecular weight markers reflect the changes predominantly in paracellular permeability, while smaller ones register transcellular transfer changes. Paracellular permeability across the epithelial cell monolayers is regulated primarily by the tight junctions (TJs) that encircle the apical poles of the epithelial cells[1,7]. The epithelium in the inflamed intestinal segments of patients with CD is characterized by a reduction of the TJ strands, strand breaks, and alterations of the TJ proteins. In patients with UC the epithelial leaks appear early due to micro-erosions resulting from upregulated epithelial apoptosis and in addition to a prominent increase of claudin-2[3]. Immune regulation of the epithelial functions by cytokines may cause a barrier dysfunction not only by the TJ impairments but also by apoptotic leaks, transcytotic mechanisms and mucosal gross lesions[4].

In our study, IP in patients with IBD was examined by measuring of the serum levels and urine recovery of iohexol, following oral administration. Andersen *et al*[19] demonstrated that water-soluble radiographic contrast media could be of use for evaluation of the altered intestinal barrier function. The contrast agent iohexol is a moderately large molecule (molecular weight 821 Dalton) with a low absorption under normal conditions, and enhanced absorption through the inflamed intestinal mucosa. It does not bind to serum proteins and is filtered through the glomerulus without indications of tubular secretion or reabsorption. Until now, to the best of our knowledge, serum levels of iohexol have not been used for the assessment of IP. The results of this study demonstrate that iohexol permeation through the intestinal mucosa is significantly increased in both CD and UC patients. Using different probes, similar findings have also been reported in numerous studies both in adult and pediatric populations with IBD[7-11,20-24]. The findings of this study support the understanding that patients with active IBD (including both CD and UC) have a mucosal barrier dysfunction, which can be assessed by measurement of IP. The permeability alterations were more frequent in CD than in UC patients: increased iohexol absorption as a marker for the abnormal IP was established in 50% of CD and in 31% of UC patients, figures which were significantly more frequent in comparison to the healthy subjects. Taking into account the fact that 3 h after ingestion iohexol is located in the large intestine[7], the higher SIC and UIC at 6 h after ingestion in patients with UC in our research suggest that this test can be used for evaluation of the colon permeability also.

The relationship between increased IP and disease activity in IBD has been established in some studies[9,11,20,21]. IP in CD patients is increased proportionally to the disease activity; it can be used to predict the clinical relapse of the disease (due to subclinical mucosal inflammation) and to assess prognosis[25,26]. However, the data in the literature are contradictory, most probably due to the usage of different permeability probes as mentioned above. Our study demonstrates that the permeation of iohexol through the intestinal mucosa, evaluated by serum concentrations, correlates positively with the disease activity in CD patients. These results are in agreement with data reported by Halme *et al*[7,20] for increased IP of iohexol (measured by its urinary recovery) and its correlation with the clinical disease activity indices. Furthermore, Halme *et al*[27] concluded that the iohexol test is a superior activity marker compared to the lactulose-mannitol test in patients with IBD. We established a relationship between serum levels of iohexol and the endoscopic activity score for UC patients. Using different permeability markers, Miki *et al*[11], Arslan *et al*[23] and Casellas *et al*[24] found a relationship between IP and disease activity in IBD patients. However, other investigators did not establish such a correlation[8,28,29]. Our data for significant positive correlation between iohexol penetration through the intestinal mucosa and disease activity support the hypothesis of the important role of the impaired intestinal barrier in the pathogenesis of active IBD[2,3,5].

In conclusion, the water-soluble contrast medium iohexol is a suitable marker for assessing the gut barrier function as its penetration through the intestinal mucosa is increased in patients with active IBD (both CD and UC) and is related to the disease activity. In our study, serum iohexol concentration appears to be a superior marker of altered IP, compared to urinary iohexol level. Measurement of a single serum sample of iohexol 6 h following its oral administration makes the proposed permeability test more convenient and provides a possibility for the assessment of altered barrier function in both small and large intestine.

COMMENTS

Background

Increased intestinal permeability (IP) has been implicated in the pathogenesis of the inflammatory bowel diseases (IBD). Mucosal barrier function has been investigated by the measuring of urine recovery of ingested permeability substrates, which is relatively uncomfortable and could lead to potential inaccuracy. To clarify the role of barrier dysfunction and to introduce better diagnostic procedure, the authors decided to evaluate IP in patients with active IBD by measuring serum and urine levels of water-soluble contrast medium iohexol, to assess the relationship of IP to disease activity, and to compare the reliability of serum *vs* urine levels of iohexol as an IP disease marker.

Research frontiers

In the present study the relationship between IP and disease activity in patients with active IBD was investigated by using the iohexol test. The findings support the hypothesis that, by affecting the penetration of macromolecules and pathogens, the permeability disorders are linked to intestinal inflammation and play a role in mucosal damage in IBD.

Innovations and breakthroughs

Mucosal barrier function in IBD has been investigated in numerous studies which differ with respect to methods for the permeability assessment, both in

regard to administration procedures of the different probes and to outcome measures. Measurement of permeability substrates in plasma or serum may reduce the problems with urine recovery and might potentially have a valuable role.

Applications

The assessment of permeability alterations with the suitable, easy to perform, and convenient iohexol test provides a platform for therapeutic modulation of the gut barrier function for better control of IBD patients.

Terminology

IP reflects the integrity of the intestinal mucosa to prevent penetration of macromolecules and bacterial antigens from the gut lumen. Tight junctions are continuous, circumferential, belt-like structures that encircle the apical poles of the epithelial cells. They are a key regulator of paracellular permeability across the intestinal epithelium. Iohexol is a non-ionic, water-soluble contrast agent.

Peer review

The authors found that patients with active ulcerative colitis and Crohn's disease had increased serum and urinary concentration of iohexol, indicating disruptive IP. There was a correlation between severity of intestinal leakiness and disease activity. The authors concluded urinary and serum iohexol are acceptable methods of assessing IP. The authors concluded that IP disrupted in patients with active IBD. It is an interesting study and data appear to support the conclusion.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2216

World J Gastroenterol 2011 May 7; 17(17): 2216-2222
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder

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Received: October 27, 2010 Revised: November 30, 2010
Accepted: December 7, 2010
Published online: May 7, 2011

Abstract

AIM: To investigate the preoperative factors that can predict neoplastic polypoid lesions of the gallbladder (PLGs) as well as malignant PLGs.

METHODS: A retrospective analysis was conducted on the 210 consecutively enrolled patients who underwent cholecystectomy due to a PLG larger than 10 mm, as was determined by preoperative trans-abdominal ultrasonography or endoscopic ultrasonography. We analyzed the medical, laboratory, radiologic data and the pathologic results.

RESULTS: In 210 cases, 146 had non-neoplastic polyps (69.5%) and 64 cases were neoplastic polyps (30.5%). An older age (≥ 65 years), the presence of diabetes mellitus (DM) and the size of polyp (≥ 15 mm) were revealed to be independent predictive variables for neoplastic polyps with odd ratios (OR) of 2.27 (P = 0.044), 2.64 (P = 0.021) and 4.94 (P < 0.01), respectively. Among the neoplastic PLGs, an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) were associated with malignancy with ORs of 4.97 (P = 0.005), 6.13 (P = 0.001) and 20.55 (P < 0.001), respectively.

CONCLUSION: Among patients with PLGs larger than 10 mm in size, higher risk groups such as elderly patients more than 65 years old, those with DM or a large polyp size (≥ 15 mm) should be managed by cholecystectomy.

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Key words: Gallbladder; Polyp; Neoplastic; Cholecystectomy; Diabetes; Pre-operative factors

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Cha BH, Hwang JH, Lee SH, Kim JE, Cho JY, Kim H, Kim SY. Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder. *World J Gastroenterol* 2011; 17(17): 2216-2222 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2216.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2216

INTRODUCTION

A polypoid lesions of the gallbladder (PLGs) is defined

as any elevated lesion of the mucosal surface of the gallbladder wall. Sonographers have described PLGs as an image with similar echogenicity as that of the gallbladder wall; the lesion projects into the lumen and it is fixed, lacks displacement, it may or may not have a pedicle and it shows no acoustic shadow on ultrasonography[1-3]. The prevalence of PLGs varies from 0.3% to 12% in healthy adults who undergo abdominal ultrasonography (US)[4-11]. Although the exact prevalence of PLGs is not clear, the detection of PLGs has been increasing according to the more frequent use of abdominal imaging. Most of the PLGs that are without symptoms are non-neoplastic lesions, but a small portion of them are found to be malignant or premalignant neoplasms. The incidence of malignant polyps has varied from 1% to 20% of the resected PLGs among diverse study populations in previous reports[2,12-17]. The largest PLG series was a review of 172 surgically resected cases, and this showed that the most common type of PLG was the cholesterol polyp (62.8%). They also reported that 7% were inflammatory polyps, 7% were hyperplasia, 5.9%% were adenoma, 9.6% were miscellaneous and 7.7% were malignant polyps in the study population[18]. Due to the considerable incidence of malignant polyps among the PLGs, surgical resection, including laparoscopic cholecystectomy, is widely accepted as the treatment of choice for PLGs that are more than 10 mm in size[18]. This surgical treatment guideline has been supported by many previous published reports[14,15,19]. However, the number of non-neoplastic polyps that are unnecessarily resected exceeds more than 3 times the number of neoplastic polyps when the resected polyps are in accordance with the above mentioned guideline[20]. For this reason, some clinicians hesitate to recommend an operation based on this guideline.

Over the last 10 years, several interesting small trials have attempted to determine the endoscopic or transabdominal ultrasonographic features of neoplastic gallbladder polyps, as compared with those of nonneoplastic polyps[10,11,21-23]. However, these sonographic findings have several limitations such as a mixed component of a benign nature, the lack of standardization and interobserver discrepancy[20].

With this background, this study aimed to reveal the clinical and sonographic predictive findings of neoplastic PLGs, including malignant PLGs, in patients who have PLGs larger than 10 mm. We also tried to demonstrate the guidelines for the decision making for the surgical management of incidentally diagnosed gallbladder polyps.

MATERIALS AND METHODS

Patients

We performed a retrospective analysis of the consecutively enrolled patients who were diagnosed with a PLG larger than 10 mm by preoperative trans-abdominal ultrasonography or endoscopic ultrasonography (EUS) between March 1, 2003 and April 30, 2009 at Seoul National University Bundang Hospital. The study protocol was approved by the Institutional Review Board of our hospital. According to our institution's policy, we recommended cholecystectomy to all patients who had a PLG that was more than 10 mm in size if they were in an operable condition. During the study period, a total of 2281 cases of PLG were diagnosed. Among them, 12 definite adenomyomatosis lesions with a sonographic "comet tail sign" and 20 lesions that were suspected of being gallbladder cancer that had invaded the liver or other adjacent organs were excluded. The 166 cases that did not have sonographic findings available or where polyps were measured by different sonographic equipment were excluded. Among the remaining cases, 1743 patients with small polyps (smaller than 10 mm) and 31 patients who did not undergo an operation were also excluded. Therefore, 210 patients who underwent cholecystectomy were utimately analyzed in this study (Figure 1).

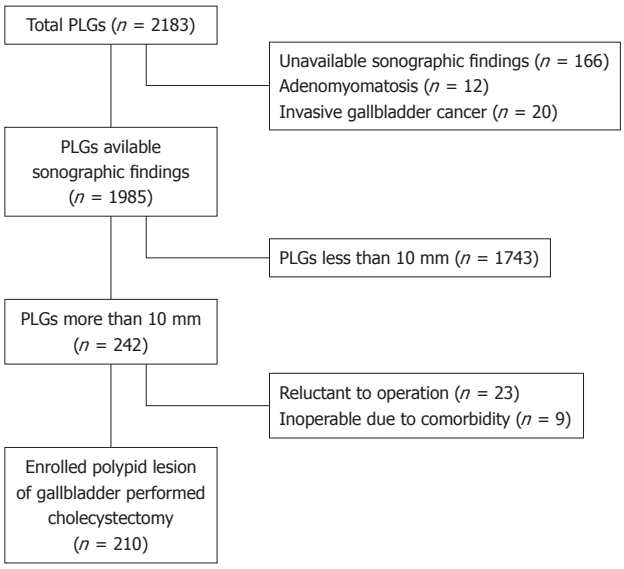

Figure 1 A diagram of the patients' enrollment. PLGs: Polypoid lesions of the gallbladder.

Based on the final diagnosis of the pathologic reports, all the polyps were divided into 2 groups: the non-neoplastic polyps (chronic cholecystitis, inflammatory polyps, adenomyomatosis, cholesterolosis or cholesterol polyps) and the neoplastic polyps (adenomatous polyps with low grade dysplasia, adenomatous polyps with high grade dysplasia, adenocarcinoma)[24].

The following parameters of all patients were recorded and analyzed: the demographic features, including age, gender, a smoking history, a history of drinking alcohol, the presence of diabetes mellitus (DM), the presence of hypertension, clinical symptoms, measurements of obesity, a complete blood count, a routine chemistry panel, the fasting glucose level and the lipid profiles. The body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters. Obesity was defined as a BMI > 25 kg/m^2 according to the Asian-Pacific criteria for obesity[25]. Clinical symptoms were defined as abdominal pain that was compatible with biliary colic, such as right upper quadrant pain with or

without radiation pain that becomes aggravated with eating a fatty meal.

The radiologic reports were retrospectively reviewed by one experienced radiologist to describe and record the polyp size, the echogenicity, the echo pattern, the number of lesions, the location of lesion, lesion combined with gallbladder stones, the size change of the lesion and the duration of the size change. The histologic findings of all the resected specimens were retrospectively reviewed by one experienced pathologist.

Equipment and the definition of the sonographic findings

Abdominal sonography was performed by well trained sonographers who used 6-2 MHz cuvelinear transducers with IU 22 or HDI 5000 units (Phillips). An EUS (endoscopic ultrasonogram) was obtained with 7.5-MHz or 12-MHz radial sector scan transducers (EUS-2000, Olympus Optical Co.), and these procedures were performed by 2 well-trained endosonographers. The EUS probe was advanced to the second portion or bulb of the duodenum and the gallbladder was scanned *via* the water-filled balloon method. All the sonographic findings of the patients were reviewed by two experienced radiologists.

The size of the polypoid lesion was measured by assessing the long diameter of the largest polypoid lesion. The echogenicity was determined on the ultrasonogram by comparing it with the echogenicity of the adjacent liver. For some cases that had a severe fatty liver, the echogenicity of the lesion was compared with the echogenicity of the kidney in same ultrasonographic series of the case. We classified the echogenicity into 3 categories: "hypoechoic", "isoechoic" and "hyperechoic". The surface pattern of the polypoid lesions was divided into 2 groups: "smooth" and "nodular"[26]. The internal echo pattern of the polypoid lesions was divided into 2 categories: "homogeneous" and "inhomogeneous". The number of polyps was diveded into 2 categrories: "multiple" and "solitary". The patients with multiple polyps that consisted of both neoplastic and non-neoplastic polyps in one specimen were classified as having neoplastic polyps. The shape of the polypoid lesions was classified to 2 categories: "pedunculated" and "sessile". Hyperechoic spots were defined "a single 1-5 mm, highly echogenic dot", or "partial aggregates of 1-3 mm sized, multiple, highly echogenic spots"[26].

Statistical analysis

Continuous variables are presented as the mean ± SD, and categorical variables are summarized as frequencies and percents. The variables were compared assuming a 95% probability for rejection of the null hypotheses. Fisher's exact test, Pearson's χ^2 test and student's *t*-test were used, when appropriate, to calculate the statistical significance of the different demographic and clinical variables. Multivariate binary logistic regression analysis was performed to determine the significance of the various predictive variables that were found to be significant by univariate analysis. *P* values of < 0.05 were deemed as significant. All the statistical analyses were carried out using SPSS 15.0 software (SPSS, Chicago, Illinois, USA).

RESULTS

Clinical and sonograhic characteristics of the patients

Of the 210 patients, 145 had non-neoplastic polyps (69.0%) and 65 had neoplastic polyps (31.0%). The histological diagnosis of the resected PLGs revealed that 54 cases (25.7%) were chronic cholecystitis, 3 cases (1.4%) were inflammatory polyps, 78 cases (37.1%) were cholesterol polyps, 10 cases (4.8%) were adenomayomatosis, 29 cases (13.8%) were adenoma with low grade dysplasia, 6 cases (2.9%) were adenoma with high grade dysplasia and 30 cases (14.3%) were adenocarcinoma.

We compared the clinical and laboratory features between the non-neoplastic polyps group and the neoplastic polyps group. The results are described in Table 1. The mean age, the proportion of DM patients and the mean serum alanine transferase (ALT) level were higher in the neoplastic polyp group than that in the non-neoplastic group ($P < 0.001$, $P < 0.001$, $P = 0.041$, respectively). Yet no significant difference was found for gender, medical history and the other laboratory findings between the two groups.

For the sonographic findings, the mean sonographic diameters of the polyps were 13.5 ± 4.5 mm and 22.1 ± 11.1 mm for the non-neoplastic group and the neoplastic group, respectively ($P < 0.001$). In addition, the inhomogeneous echo pattern ($P = 0.019$), a solitary lesion ($P = 0.002$) and a nodular surface pattern of the polyps ($P < 0.001$) revealed significant correlation with neoplastic polyps (Table 1).

For the detailed analysis, maximum diameter was divided to 2 categories by use of reciever-operator characteristic (ROC) curves. At a cutoff value of 15 mm diameter of PLGs' size, the area under the ROC curve (AUC) had the highest sensitivity and specificity. (70.8%, 75.9%, Figure 2).

Predictive variables for neopalastic PLGs

On the univariate analysis, we obtained several important predictive clinical and sonographic values such as an age > 65 years, the presence of DM, the ALT level, a larger sonographic size (≥ 15 mm), solitary lesions and a nodular sonographic surface pattern (Table 1). On multivariate analysis, an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) were found to be the independent predictive variables for neoplastic polyps [odd ratios (OR) = 2.27, $P = 0.044$, OR = 2.64, $P = 0.021$ and OR = 4.94, $P < 0.001$, respectively]. A nodular surface pattern was found to have an association with neoplastic polyps, with borderline significance (OR = 2.31, $P = 0.058$) (Table 2).

Predictive variables for malignant PLGs

In addition, we subdivided the neoplastic group into two

Table 1 Comparative data for the prevalence of the demographic, laboratory and sonographic findings between the non-neoplastic polyp group and the neoplastic polyp group (mean ± SD) *n* (%)

	Total (*n* = 210)	Non-neoplastic (*n* = 146)	Neoplastic (*n* = 64)	*P*
Age (yr)	51.8 ± 13.7	49.1 ± 12.3	57.9 ± 14.7	< 0.001
Age > 65 yr	49 (23.3)	22 (15.1)	27 (42.2)	< 0.001
Gender, male	109 (51.9)	77 (52.7)	32 (50.0)	0.785
BMI (kg/m^2)	24.0 ± 2.97	23.9 ± 3.01	24.1 ± 2.89	0.620
Obesity	79 (38.2)	53 (36.6)	26 (41.9)	0.465
Hypertension	34 (16.3)	20 (13.7)	14 (22.2)	0.126
Diabetes mellitus	46 (21.9)	21 (13.0)	27 (42.1)	< 0.001
Hypercholesterolemia	77 (36.7)	57 (39.0)	20 (31.3)	0.135
RUQ pain	37 (17.6)	24 (16.4)	13 (20.3)	0.498
Total bilirubin (g/dL)	1.22 ± 3.41	0.91 ± 0.41	1.93 ± 6.17	0.189
ALP (g/dL)	69.4 ± 60.9	62.5 ± 20.2	84.7 ± 104.7	0.097
AST (IU/dL)	33.2 ± 61.9	26.2 ± 21.4	49.2 ± 105.9	0.090
ALT (IU/dL)	34.5 ± 42.0	29.1 ± 23.3	47.0 ± 66.2	0.041
Size (mm)	16.1 ± 8.20	13.5 ± 4.5	22.1 ± 11.1	< 0.001
Size > 15 mm	78 (37.1)	33 (22.3)	45 (70.3)	< 0.001
Location				0.977
Fundus	156 (74.3)	109 (74.7)	47 (73.4)	
Body	44 (21.0)	30 (20.5)	14 (21.9)	
Neck	10 (4.8)	7 (4.8)	3 (4.7)	
No. of polyps				0.002
Multiple	76 (36.2)	63 (43.2)	13 (20.3)	
Solitary	134 (63.8)	83 (56.8)	51 (79.7)	
Hyperechoic spots				0.315
No	172 (81.9)	117 (80.1)	55 (85.9)	
Yes	38 (18.1)	29 (19.9)	9 (14.1)	
Echogenecity				0.125
Anechoic or hyperechoic	130 (61.9)	96 (65.8)	34 (53.1)	
Hypoechoic or isoechoic	80 (38.1)	50 (34.2)	30 (46.9)	
Echo pattern				0.093
Homogeneous	115 (52.9)	85 (58.2)	30 (46.9)	
Inhomogeneous	95 (45.2)	60 (41.1)	35 (54.7)	
Sonographic surface pattern				< 0.001
Smooth surface	174 (82.9)	131 (89.7)	43 (67.2)	
Nodular surface	36 (17.1)	15 (10.3)	21 (32.8)	

BMI: Body mass index; RUQ: Right upper quadrant; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. Obesity: BMI higher than 25 kg/m^2.

groups according their histologic results. The polyps that contained adenocarcinoma were classified as the malignant PLGs group and the other neoplastic polyps were classified as the benign PLGs group. We also compared the clinical and sonographic variables to discriminate the malignant PLGs group from the benign group. On univariate analysis, the important predictive clinical and sonographic values for malignant polyps were an older age (≥ 65 years, $P = 0.02$), the presence of DM ($P < 0.001$), the ALT level ($P = 0.033$), a larger sonographic size (≥ 15 mm, $P < 0.001$) and an inhomogeneous echo pattern ($P = 0.016$) (Table 3). But on multivariate analysis, it was observed that an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) had statistical significance

Table 2 Results of the multivariate logistic regression analysis for the factors that were significantly associated with neoplastic polypoid lesions of the gallbladder on univariate analysis

	Hazard ratio	95% CI	*P*-value
Age ≥ 65 yr old	2.27	1.02-5.06	0.044
Gender, male	1.08	0.57-2.51	0.617
DM	2.64	1.15-6.03	0.021
ALT level	1.008	0.99-1.02	0.168
Polyp size > 15 mm	4.94	2.43-10.02	< 0.001
Solitary polyp	0.59	0.26-1.33	0.205
Nodular surface pattern	2.31	0.97-5.50	0.058

DM: Diabetes mellitus; ALT: Alanine transaminase.

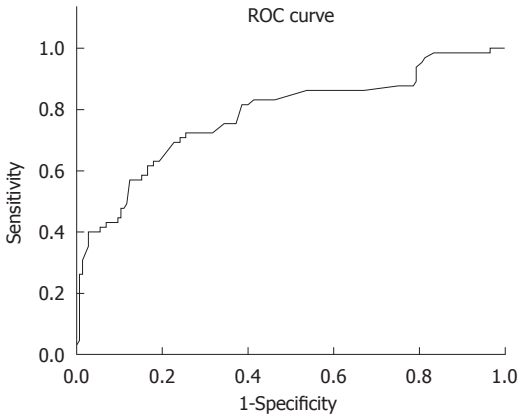

Figure 2 Reciever-operator characteristic curve of the sonographic size of the polypoid lesions of the gallbladder.

with the malignant PLGs group (OR = 4.97, $P = 0.005$, OR = 6.13, $P = 0.001$, OR = 20.55, $P < 0.001$, respectively) (Table 4).

For a more detailed analysis of the chronological change of the neoplastic polyps, we classified all the cases into three subgroups: the adenoma with low grade dysplasia group; the adenoma with high grade dysplasia group; and the adenocarcinoma group. After this subgroup analysis, we found a linear stepwise increase in the mean age of each groups; adenoma low grade dysplasia, high grade dysplasia and adenocarcinoma. The difference of the mean age was 18.9 years between the adenoma with low grade dysplasia group (46.4 ± 13.4 years) and the adenocarcinoma group (65.3 ± 18.0 years) ($P < 0.001$), and the difference of the mean age was 13.2 years between the high grade dysplasia group (52.1 ± 7.4 years) and the adenocarcinoma group ($P = 0.004$) (Figure 3).

DISCUSSION

GB polyps larger than 10 mm in size have generally been recommended for surgical resection despite of the large portion of non-neoplatic polyps among them. Because the current data for making the preoperative differentiation between neoplastic and non-neoplastic polyps is limited, a practical guideline was lacking to decide when to perform cholecystectomy. In this study, we tried to

Table 3 Comparative data for the prevalence of the demographic, laboratory and sonographic findings between the benign polyp group and the malignant polyp group for the 65 neoplastic polypoid lesions of the gallbladder (mean ± SD) *n* (%)

	Total (*n* = 65)	Benign (*n* = 35)	Malignant (*n* = 30)	*P*
Age (yr)	49.8 ± 13.5	47.2 ± 12.4	65.6 ± 7.39	< 0.001
Age > 65 yr	51 (24.3)	31 (17.2)	20 (66.7)	0.002
Gender, male	109 (51.9)	95 (52.6)	14 (46.7)	0.535
BMI (kg/m^2)	23.9 ± 2.97	24.0 ± 3.03	23.8 ± 2.66	0.835
Obesity	79 (38.2)	67 (37.2)	12 (40.0)	0.583
Hypertension	34 (16.3)	25 (13.8)	9 (30.0)	0.244
Diabetes mellitus	46 (21.9)	25 (13.8)	21 (70.0)	< 0.001
Hypercholesterolemia	19 (29.2)	10 (28.6)	7 (10.9)	0.830
RUQ pain	13 (6.2)	6 (4.1)	7 (10.9)	0.534
Total bilirubin (g/dL)	1.22 ± 3.41	0.89 ± 0.41	3.2 ± 6.17	0.166
ALP (g/dL)	69.3 ± 60.9	63.4 ± 20.0	104.9 ± 150.5	0.142
AST (IU/dL)	33.2 ± 61.9	26.6 ± 20.9	72.8 ± 151.3	0.106
ALT (IU/dL)	34.5 ± 42.0	29.2 ± 23.3	67.3 ± 9.09	0.033
Size (mm)	16.1 ± 8.20	14.3 ± 6.3	26.7 ± 10.0	< 0.001
Size >15 mm	45 (69.2)	17 (48.6)	28 (93.3)	< 0.001
Location				0.705
Fundus	40 (61.5)	21 (60.0)	20 (66.7)	
Body	18 (27.7)	10 (28.6)	8 (26.7)	
Neck	3 (6.0)	2 (4.2)	1 (1.5)	
No. of polyps				0.534
Multiple	13 (20.0)	8 (22.9)	5 (16.7)	
Solitary	52 (80.0)	27 (77.1)	25 (83.3)	
Hyperechoic spots				0.912
No	56 (86.2)	30 (85.7)	26 (86.7)	
Yes	9 (13.8)	5 (14.3)	4 (13.3)	
Echogenecity				0.180
Hyperechoic	34 (52.3)	21 (60.0)	13 (43.3)	
Hypoechoic or isoechoic	31 (47.7)	14 (40.0)	17 (56.7)	
Echo pattern				0.016
Homogeneous	30 (46.2)	21 (60.0)	9 (30.0)	
Inhomogeneous	35 (53.8)	14 (40.0)	21 (70.0)	
Sonographic surface pattern				0.135
Smooth surface	45 (69.2)	27 (77.1)	18 (60.0)	
Nodular surface	20 (30.8)	8 (22.9)	12 (40.0)	

BMI: Body mass index; RUQ: Right upper quadrant; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine transaminase; Obesity: BMI higher than 25 kg/m^2.

Table 4 Results of the multivariate logistic regression analysis for the factors that were significantly associated with the malignant gallbladder polyps for the 65 neoplastic polypoid lesions of the gallbladder on univariate analysis

	Hazard ratio	95% CI	*P*-value
Age ⩾ 65 yr old	4.97	1.58-15.61	0.005
Gender, male	1.19	0.38-3.73	0.764
DM	6.13	1.98-18.94	0.001
ALT level	1.01	0.99-1.02	0.075
Polyp size > 15 mm	20.55	4.25-99.39	< 0.001
Inhomogeneous echo pattern	1.60	0.50-5.09	0.418

DM: Diabetes mellitus; ALT: Alanine aminotransferase.

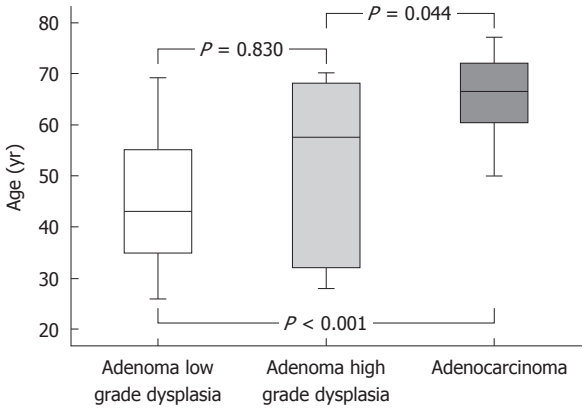

Figure 3 The age distribution according to the pathology subgroups with neoplastic polypoid lesions of the gallbladder.

determine the predictive values for neoplastic PLGs. We evaluated a total of 210 cases of resected GB polyps larger than 10 mm in size and we found that an older age (> 65 years), a history of DM and a large size were the significant predictive values for neoplastic PLGs. We also found that an older age(> 65 years), a history of DM and a large size were significant predictive values for malignant PLGs.

In our study, older patients more than 65 years showed a statistical relation with neoplastic or malignant PLGs, as compared to that of the younger patients (P = 0.021, P = 0.005, respectively). This result corresponds with previous studies about the correlation between age and neoplastic PLGs[12,13,18,27,28]. With this background, we tried to determine a more detailed correlation of age with the subdivided groups among the neoplastic PLGs. According to pathologic results, the neoplastic PLGs were sorted into three subgroups; adenomatous polyp with low grade dysplasia, adenomatous polyp with high grade dysplasia and adenocarcinomas. We then compared the mean ages of each subgroup. After this detailed analysis, we found out a trend for a stepwise increase of mean age in the different neoplastic groups.

A new finding we discovered in this study was the relationship between DM and neoplastic polyps. Our results showed that patients with DM have a strong probability of having neoplastic and malignant polyps, as compared to that of the patients without DM, on univariate (P < 0.001, P < 0.001, respectively) and multivariate analyses, which were adjusted by age and gender (OR = 2.64, P = 0.021, and OR = 6.13, P = 0.001, respectively). There has been one document which reported the relation between diabetes and gallbladder cancer[29]. But the exact mechanism or pathogenesis is not known. There have been a few reports that have found DM or hyperglycemia to be an independent risk factor for gastrointestinal or endocrine malignancies, such as colorectal[30] or pancreatic cancers[31]. Some recent researchers have proposed that the insulin resistance associated with hyperinsulinemia plays an important role as an oncogenic factor[32,33]. According to many etiologic studies, it has become evident that the insulin-like growth fac-

tor (IGF) system plays a permissive role in cancer development and tumor progression[34-38]. But, none of them mentioned any evidence of the IGF-I receptor pathway being involved in the development of gallbladder cancer. So we think that well designed trials are warranted in order to prove that this IGF signal pathway system plays a leading role in developing gallbladder cancer.

We found that the size of polyps (≥ 15 mm) is a powerful predictor for neoplastic polyps (OR = 4.94, $P < 0.001$). There was also a similar trend for malignant polyps (OR = 20.55, $P < 0.001$). Many studies have reported on the size criteria of PLGs as one of the predictive values for neoplastic lesions. The majority of them insisted that a size of gallbladder polyps more than 10 mm may be the most reliable predictor of malignant neoplasm[12,13,18,27,28]. In a retrospective analysis of 354 subjects with resected PLGs, the authors suggested increasing the size criteria for cholecystectomy from 10 to 12 mm[39]. Our study result showed a larger size than the previous noted criteria because small polyps less than 10 mm were not included in the analysis.

For the sonographic findings, solitary polyps ($P = 0.001$), an inhomogeneous echo pattern ($P = 0.019$) and a nodular surface pattern ($P < 0.001$) had a significant correlation with neoplastic PLGs on univariate analysis. However, only one variable, the noduar surface pattern, showed borderline statistical correlation with neoplastic polyps on the multivariate analysis. In addition, a nodular surface pattern did not show statistical significance with malignant polyps. The other sonographic parameters failed to show correlation with neoplastic or malignant PLGs. Many sonographers and endosonographers have recently tried to determine the sonographic characteristics that can reliably predict premalignant polypoid lesions in the gallbladder[20,21,23,40]. They have suggested various sonographic findings as having predictive value for neoplastic lesions; the echo pattern, marginal irregularity, the shape, solitary lesion and preservation or loss of the GB wall layer structure. In spite of vigorous efforts to standardize these ultrasonographic features, inter-observer discrepancy is still the main concern to utilize these values to differentiate malignant polyps from benign polyps.

On the contrary, among 110 cases, which were lower risk groups for neoplastic polyps, such as those younger than 65 years old, those without DM and those with polyps less than 15 mm in sonographic diameter, 15 cases (13.6%) were reported as neoplastic polyps and the remaining 95 cases (86.4%) were non-neoplastic polyps.

The major limitations of this study include the following; first, this is not prospective study, rather, it is a cross-sectional study. There was no additional follow up data about the unresected PLGs more than 10 mm in size. However, because this study included patients who were consecutively enrolled during the study period, we could rule out a common selection bias. To the best of our knowledge, this study is the largest study that has enrolled patients with pathologically comfirmed PLG larger than 10 mm in size. Thus, this data might be valuable when making decisions on how to manage such patients with PLGs.

In conclusion, among patients with PLGs more than 10 mm in size, the higher risk groups, such as elderly patients who are more than 65 years, those with DM and those with a large sized polyp (≥ 15 mm) should be recommended cholecystectomy more seriously than other groups.

COMMENTS

Background

Some neoplastic polypoid lesions of the gallbladder (PLGs) including early cancer show similar appearances to the non-neoplastic PLGs. But there have been no definite guidelines except size criteria (more than 10 mm diameter) for the recommendation of surgical resection.

Research frontiers

Many studies have investigated the relationship between the neoplastic nature of PLGs and their morphological characteristics such as the number of polyps, the polyp shape, the diameter of the largest polyp, the echo level and internal echo pattern, and the polyp margin. But previously published documents showed a lack of case number, pathologic results, and long term follow up data. Also reports about the relationship between other clinical paramenters and neoplastic PLGs were rare.

Innovations and breakthroughs

The authors performed the study using the consecutively enrolled pathologic data of patients with PLGs more than 10 mm in size to eliminate selection bias. This study demonstrated old age and diabetes history are added to the size criteria for predictive values of neoplastic PLGs for the decision about surgical resection.

Applications

Among patients with PLGs more than 10 mm in size considering surgical resection, the higher risk groups such as elderly patients who are more than 65 years, those with diabetes mellitus (DM) and those with a large sized polyp (≥ 15 mm) should be recommended cholecystectomy more seriously than other groups.

Terminology

Neoplastic PLGs: PLGs which have the features of the neoplasm including adenoma and adenocarcinoma. Non-neoplastic PLGs: PLGs which do not have the features of the neoplasm including cholesterol polyps, adenomyomatosis and inflammatory polyps.

Peer review

The authors described that older age, DM and polyp size > 15 mm were independent predictors of neoplasia as well as malignancy. Over all, this paper is well written, concise and information.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2223

World J Gastroenterol 2011 May 7; 17(17): 2223-2228
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Topical application of glycyrrhizin preparation ameliorates experimentally induced colitis in rats

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Received: August 5, 2010 Revised: September 18, 2010
Accepted: September 25, 2010
Published online: May 7, 2011

Abstract

AIM: To examine the efficacy of glycyrrhizin preparation (GL-p) in the treatment of a rat model of ulcerative colitis (UC).

METHODS: Experimental colitis was induced by oral administration of dextran sodium sulfate. Rats with colitis were intrarectally administered GL-p or saline. The extent of colitis was evaluated based on body weight gain, colon wet weight, and macroscopic damage score. The expression levels of pro-inflammatory cytokines and chemokines in the inflamed mucosa were measured by cytokine antibody array analysis. The effect of GL-p on myeloperoxidase (MPO) activity in the inflamed mucosa and purified enzyme was assayed.

RESULTS: GL-p treatment significantly ameliorated the extent of colitis compared to sham treatment with saline. Cytokine antibody array analysis showed that GL-p treatment significantly decreased the expression levels of pro-inflammatory cytokines and chemokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α, cytokine-induced neutrophil chemoattractant-2, and monocyte chemoattractant protein-1 in the inflamed mucosa. Furthermore, GL-p inhibited the oxidative activity of mucosal and purified MPO.

CONCLUSION: GL-p enema has a therapeutic effect on experimental colitis in rats and may be useful in the treatment of UC.

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Key words: Glycyrrhizin; Colitis; Dextran sodium sulfate; Ulcerative colitis; Cytokine; Chemokine; Protein array; Myeloperoxidase; Enema; Carboxymethylcellulose

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Kudo T, Okamura S, Zhang Y, Masuo T, Mori M. Topical application of glycyrrhizin preparation ameliorates experimentally induced colitis in rats. *World J Gastroenterol* 2011; 17(17): 2223-2228 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2223.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2223

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammation of the large intestine, which is characterized by pronounced infiltration of neutrophils into colonic lesions with hyperemia, hemorrhage, and ulcerations. Its etiology is not fully understood but immunological mechanisms seem to be involved[1,2]. Increased pro-inflammatory mediators, including cytokines and chemokines, are observed in patients with colitis[3]. An efficacious treatment strategy comprises controlling the expression and neutralizing the function of these mediators. Agents such as aminosalicylates, corticosteroids, and immunosuppressants inhibit the

production of these factors and are used in the treatment of UC; for example, antibodies that target tumor necrosis factor (TNF)-α are clinically efficacious to neutralize TNF-α actions[1,2]. However, these agents may cause serious adverse effects, such as agranulocytosis, severe infections, osteoporosis, and malignant lymphoid tumors.

Glycyrrhizin (GL), a natural compound extracted from the roots of the Chinese herb *Glycyrrhiza glabra*, has been used for several centuries in traditional Chinese medicine. Its preparation (GL-p) has been clinically used as an anti-allergic agent and also as treatment for chronic hepatitis for more than 30 years[4]. GL-p has useful pharmacological properties, such as anti-inflammatory, immunomodulatory, and cytoprotective effects[5]. Furthermore, treatment with GL-p is rarely accompanied by severe adverse effects, even during long-term use. However, the efficacy of GL-p in the treatment of other inflammatory conditions including UC is not well known.

Several models of experimentally induced colitis have been developed to investigate the mechanisms of inflammation and immunological disturbances[6]. Among them, an animal model with dextran sulfate sodium (DSS)-induced colitis exhibits several symptoms similar to those seen in human UC; therefore, they are considered reliable for studying the pathogenesis of UC[6,7].

To confirm the hypothesis that GL-p ameliorates colitis through its pharmacological effects, we have conducted experiments to investigate the therapeutic efficacy of GL-p for DSS-induced colitis in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (approximately 250 g, 8 wk old) were used for the experiments. All rats were housed in specific pathogen-free conditions in the animal facility of Gunma University and fed standard laboratory chow and tap water *ad libitum*. This study was approved by the Animal Care and Experimentation Committee at Gunma University.

Induction of experimental colitis

To induce colitis, rats were orally administered 3% solution of DSS (molecular weight, 5000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) *via* drinking water for 7 d (from day 0 to day 6).

Treatment design

GL-p is commercially supplied by Minophagen Pharmaceutical (Tokyo, Japan) as a solution (Stronger Neo-Minophagen C®), which contains 2 mg GL monoammonium, 1 mg L-cysteine, and 20 mg glycine per mL in physiological saline solution. Rats in the GL-p and GL groups were respectively administered 1 mL GL-p or 0.2% GL solution transanally under diethyl ether anesthesia, once daily for four consecutive days (day 3 to day 6). Control rats were administered 1 mL saline. Body weight was measured throughout the experiments. All rats were killed on day 7 under excess diethyl ether anesthesia.

Assessment of mucosal damage

Body weight gain was calculated by subtracting the body weight at the beginning of the treatment from that at autopsy. An 8-cm long biopsy specimen of the distal colon was resected at autopsy and opened by longitudinal incision. The wet weight of this biopsy specimen was measured followed by observation of the gross appearance of the mucosa. Mucosal damage was measured and macroscopically scored on a scale of 0 to 10 according to the following criteria[8]: 0, no damage; 1, hyperemia without ulcers; 2, hyperemia and thickening of the bowel wall without ulcers; 3, one ulcer without thickening of the bowel wall; 4, two or more sites of ulceration and inflammation; 5, two or more major sites of ulceration and inflammation or one site of ulceration and inflammation extending > 1 cm along the length of the colon; 6-10, one point being added for each additional centimeter of involvement beyond an initial 2 cm. Tissue specimens were kept at -80℃ until cytokines were evaluated.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the modified method of Bradley *et al*[9]. The mucosal scrapings were homogenized with a Diax 600 homogenizer (Heidolph, Germany) in 1 mL buffer that contained 0.5% hexadecyltrimethylammonium bromide and 50 mmol/L potassium phosphate (pH 6.0). The homogenates were sonicated for 10 s, freeze-thawed three times, and centrifuged at 40 000 × *g* for 15 min at 4℃. From each sample, 100 μL was added to 2.9 mL 50 mmol/L phosphate buffer (pH 6.0) that contained 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide. MPO activity was measured colorimetrically using a spectrometer with a change of absorbance of 460 nm during a 30-min interval at 25℃. One unit of MPO activity was defined as 1 mmol H_2O_2 broken down to H_2O and results were expressed as units per gram mucosal tissue. The MPO activities in the supernatant of mucosal homogenate and in the purified human MPO enzyme (Sigma-Aldrich Japan, Tokyo, Japan) were measured in the presence and absence of various amounts of GL-p.

Measurement of cytokines and chemokines using antibody array

The Cytokine Array (Raybiotech Inc., Norcross, GA, USA)[10] was used to detect 19 different cytokines and chemokines in the supernatant of homogenized colonic mucosal scrapings according to the manufacturer's recommended protocol. After the membranes were exposed to X-ray film (GE Healthcare Bioscience Co. Ltd.), the exposed films were digitized and the relative cytokine levels were compared by densitometrical analysis using ImageJ ver. 1.38x software (National Institute of Health, Rockville Pike, Bethesda, MD, USA). The relative cytokine levels were obtained by subtracting the background staining and normalizing from the positive controls on the same membrane.

Statistical analysis

All data are presented as mean ± SE. Student's *t* test was used for comparison between the data in the two groups. One-way ANOVA followed by Tukey's *post hoc* test was used to analyze the data for multiple groups. For evaluation of the damage score, the non-parametric Kruskal-Wallis test followed by the Steel-Dwass test was used. $P < 0.05$ was considered statistically significant. KyPlot 5.0 (KyensLab. Inc., Tokyo, Japan) was used for the statistical analyses.

RESULTS

Effects of GL-p enema on experimental colitis

After treatment with 3% DSS for 7 d, all the rats developed symptoms of colitis. Diarrhea was first observed on day 4 after the onset of treatment, followed by rectal bleeding and body weight loss. Compared to the control group, the GL-p and GL groups experienced significantly increased body weight gain ($P < 0.05$), decreased colon wet weight ($P < 0.05$), and reduced macroscopic damage score ($P < 0.05$) (Figure 1). Significant differences between GL-p and GL groups were not detected (Figure 1).

Effects of sodium carboxymethylcellulose accompanied with GL-p on experimental colitis

To determine whether the addition of carboxymethylcellulose (CMC) as a viscosity modifier augments the therapeutic effects of GL-p enema, we compared the group treated with GL-p alone to those treated with a combination of GL-p and CMC. The addition of 0.5% CMC seemed to increase body weight gain and significantly decrease the colon wet weight compared to GL-p alone ($P < 0.01$), whereas addition of CMC did not result in any changes in the macroscopic damage score (Figure 2).

Cytokine antibody array analysis

Using cytokine antibody array analysis, the 19 cytokine levels between the GL-p and control groups were compared (Figure 3). The levels of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and TNF-α were significantly decreased in the GL-p group, and those of chemokines, such as cytokine-induced chemoattractant (CINC)-2 and -3, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-3α, were also significantly inhibited in the GL-p group. Among Th1 cytokines, interferon (IFN)-γ was not significantly inhibited. Among Th2 cytokines, IL-4 and IL-6 decreased while IL-10 did not change. The levels of leptin, tissue inhibitor of metalloproteinase (TIMP)-1, fractalkine, and ciliary neurotrophic factor (CNTF) were decreased in the GL-p group.

Effects of GL-p on MPO activity

To evaluate the effect of GL-p on the number of neutrophils infiltrating the colon, levels of mucosal MPO activity were measured. Rats treated with GL-p showed a significantly low level of mucosal MPO activity compared to control rats ($P < 0.05$) (Figure 4A). We conducted *in vitro* experiments to assess the presence of an inhibitory effect of GL-p on MPO activity, and found that GL-p dose-dependently inhibited MPO activity in mucosal tissue (Figure 4B), as well as purified human enzyme (Figure 4C).

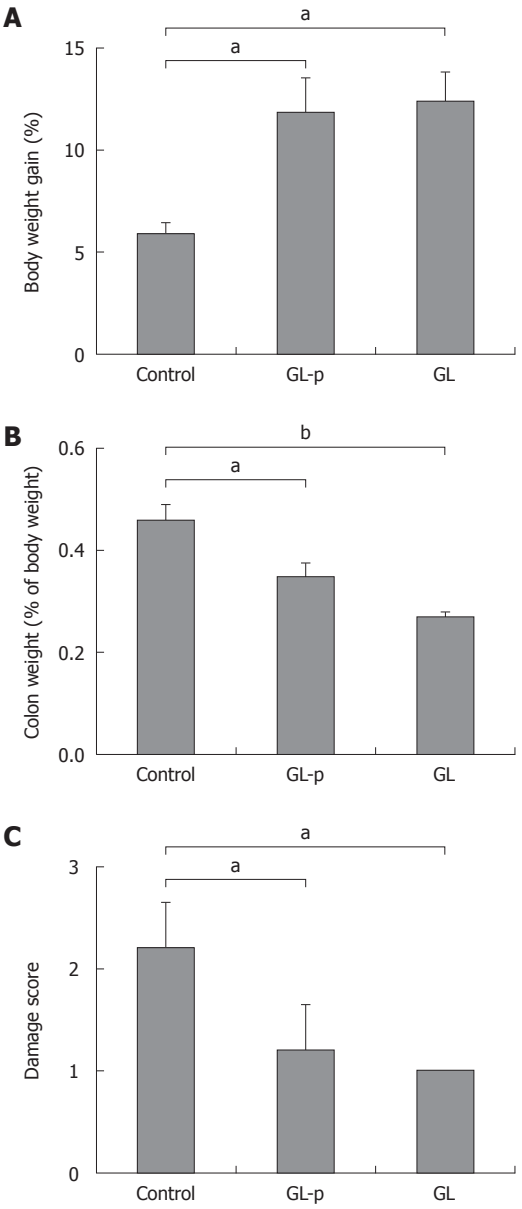

Figure 1 Effects of glycyrrhizin preparation and glycyrrhizin treatments on body weight gain (A), colon wet weight (B), and macroscopic damage score (C) in rats with dextran sulfate sodium-induced colitis. Data are expressed as the mean ± SE of five rats. GL-p: Glycyrrhizin preparation. [a]$P < 0.05$, [b]$P < 0.01$.

DISCUSSION

In the present study, we showed that GL-p ameliorated the extent of DSS-induced colitis in rats. We used cytokine antibody array analyses and revealed that GL-p reduced the level of pro-inflammatory cytokines and chemokines in the colonic mucosa. Furthermore, GL-p was shown to inhibit MPO activity.

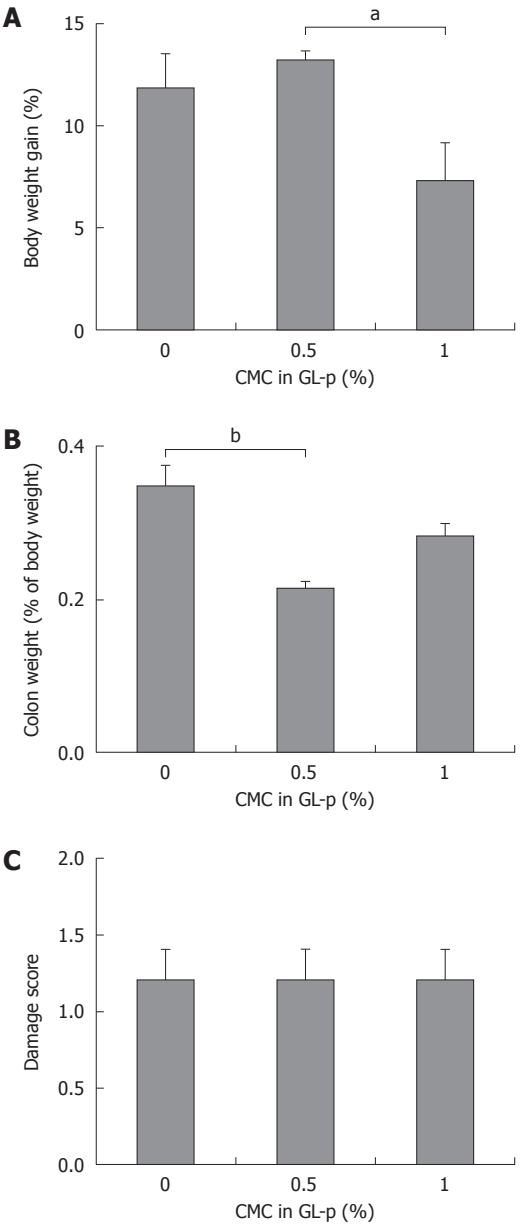

Figure 2 Effects of the addition of sodium CMC to glycyrrhizin preparation on body weight gain (A), colon wet weight (B), and macroscopic damage score (C) in rats with dextran sulfate sodium-induced colitis. Data are expressed as the mean ± SE of five rats. $^{a}P < 0.05$, $^{b}P < 0.01$. CMC: Carboxymethyl cellulose.

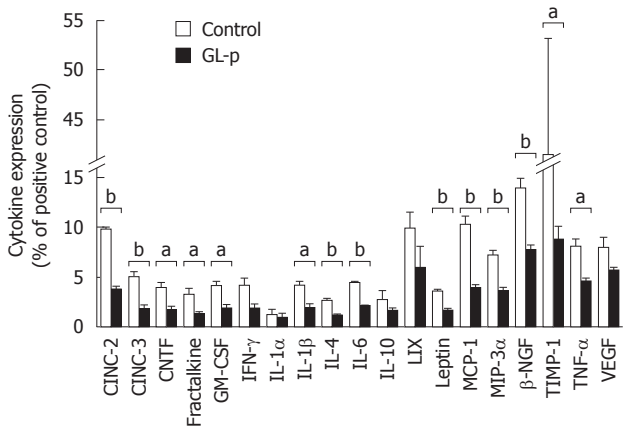

Figure 3 Expressions of cytokines and chemokines in the extract of colonic mucosa by cytokine antibody array analysis. GL-p: Glycyrrhizin preparation; CINC: Cytokine-induced chemoattractant; CNTF: Ciliary neurotrophic factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; IL: Interleukin; LIX: Lipopolysaccharide-induced CXC chemokine; MCP: Monocyte chemoattractant protein; MIP: Macrophage inflammatory protein; NGF: Nerve growth factor; TIMP: Tissue inhibitor of metalloproteinase; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor. $n = 3$ in each group. $^{a}P < 0.05$, $^{b}P < 0.01$.

We demonstrated that GL-p administration ameliorated the production of pro-inflammatory cytokines and chemokines, such as IL-1β, TNF-α, IL-6, CINC-2 and -3, MCP-1, MIP-3α, TIMP-1, fractalkine, CNTF, leptin, and GM-CSF. The relevance of pro-inflammatory cytokines and chemokines to the pathophysiology of chronic inflammation in inflammatory bowel disease (IBD) has recently been elucidated, and their manipulation has successfully reduced disease severity and maintained remission. IL-1β has been well characterized as a potent inflammatory cytokine that is produced by inflammatory and mucosal epithelial cells during colonic inflammation[11]. Its neutralization with antibodies suppresses the development of DSS-induced colitis[12]. IL-6 is a pleiotropic cytokine that exerts its pro-inflammatory effects mainly by means of its soluble IL-6 receptor. The anti-IL-6 receptor monoclonal antibody abrogates murine colitis[13]. TNF-α is a key inflammatory cytokine produced mainly from macrophages and is a target of IBD treatment. Anti-TNF-α antibody and soluble TNF-α receptor are used clinically with significant benefits. Collectively, these findings suggest the efficacy of GL-p in the treatment of human UC.

GL-p used in this study was a combination of GL with 2% of glycine and 0.4% of L-cysteine. A diet that contains 5% glycine has recently been reported to prevent colitis in rats, which is chemically induced by DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS)[14]. Glycine inhibits the induction of pro-inflammatory cytokines and chemokines, such as IL-1β, TNF-α, CINC and MIP-2. On the contrary, dietary L-cysteine supplementation is also reported to have a therapeutic effect on DSS-induced porcine colitis[15]. However, no significant therapeutic differences were observed between GL alone and GL-p in the present study (Figure 1). Although the difference in the experimental settings, such as the route of administration, may be relevant to this contradiction, the precise reasons for this are unknown. One possibility is that the effect of amino acids in GL-p was masked by that of GL in our experimental settings. To evaluate this possibility, further experiments including a control group treated with amino acids alone is required.

Our study demonstrated that GL was beneficial in the treatment of rat colitis if administered intrarectally (Figure 1). However, GL was not proven to be efficacious in a previous study[16]. Although the beneficial effects of oral administration of Hange-shashin-to (HST), a combination of seven herbs[16], on DSS-induced colitis in rats

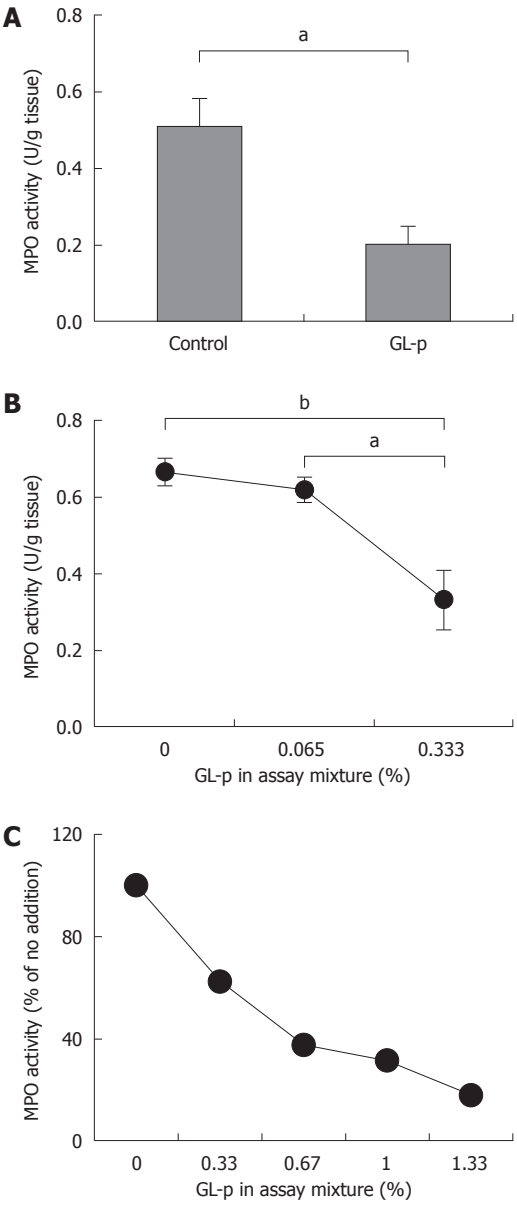

Figure 4 Glycyrrhizin preparation and myeloperoxidase activity. A: Effects of glycyrrhizin preparation (GL-p) treatment on myeloperoxidase (MPO) activity in the mucosa of rats with dextran sulfate sodium-induced colitis (n = 5 in each group); B: Effects of GL-p on MPO activity in the mucosal extract (n = 5 in each group); C: Effects of GL-p on MPO activity of purified human enzyme. [a]$P < 0.05$, [b]$P < 0.01$.

has been demonstrated, the previous study did not detect the effects of oral administration of a single constituent included in HST, such as GL. One possible explanation for the contradiction between their results and ours may be the difference in the type of experimental colitis used. They used a model of colitis induced by intracolonic instillation of TNBS, which is known as a model of Crohn's disease, and has distinct immunological features from those of the DSS-induced colitis that we used[6]. This seems to have caused conflicting responses to GL administration, which suggests that Crohn's disease and UC should be distinguished at the point of clinical application of GL treatment. Another possibility is the differences in dose and route of administration. They administered an oral dose of 2.67 or 5.37 mg/kg GL. We administered a dose of 8 mg/kg (2 mg GL per rat weighing 250 g) intrarectally. We were subsequently able to deliver higher concentrations of GL to the inflamed colon compared to the previous study.

Regarding GL administration for colitis, Yuan *et al*[17] recently have reported the results of experiments using colitis induced by acetic acid installation, which is a model of acute colitis[6]. They found that GL reduced colonic injury with the suppression of nuclear factor-κB, TNF-α, and intercellular adhesion molecule-1 in the affected mucosa. They administered GL at a dose of 40 mg/kg, which was five times greater than that in our study. Furthermore, they chose the intraperitoneal route for administration. Use of this route may raise the systemic concentration of GL sufficiently high to cause adverse effects such as severe hypertension, hypokalemia, and other signs of mineralocorticoid excess, because the incidence of adverse effects is dose-dependent[4,5]. We administered GL and GL-p intrarectally to maintain low systemic concentrations of the agents despite high local concentrations[18]. For clinical application, rectal administration is preferable as it may maximize quality of life by reducing the necessity for frequent visits to outpatient clinics to receive parenteral administration of GL-p.

We demonstrated that GL-p treatment decreased MPO activity in the inflamed mucosa (Figure 4A), which indicates the inhibition of neutrophil accumulation at the site of injury through GL-p administration. MPO is mainly produced by neutrophils that infiltrate the inflammatory sites, therefore, MPO activity in specimens is believed to reflect the number of infiltrating neutrophils[9]. Furthermore, in this study, we demonstrated for the first time that GL-p suppressed MPO activity in a dose-dependent manner in experiments using the inflamed mucosa (Figure 4B), as well as purified human enzyme (Figure 4C). This appears to be a new mechanism in the therapeutic actions of this agent. Although the main function of MPO is to destroy phagocytosed microorganisms by strong oxidative species within the phagosome, the excessive amount and activation of MPO at inflamed sites may cause tissue injury by modification of lipids and proteins through reactive oxidative species[19]. Therefore, modulation of MPO oxidation activity by GL-p has a therapeutic potential for inflammation.

In the treatment of UC, development of colorectal carcinoma is a serious complication. It is noticeable that long-term use of GL-p is effective in preventing development of hepatocellular carcinoma in patients with chronic hepatitis C[20,21]. Amelioration of chronic inflammation is suggested to reduce the possibility of oncogenesis, therefore, GL-p enema may prevent the development of colon cancer.

In conclusion, our study suggests a therapeutic effect of GL-p enemas on experimental colitis in rats. Further studies are necessary to determine an effective administration strategy with higher efficacy and fewer adverse

effects in the treatment of patients with UC.

COMMENTS

Background

Ulcerative colitis (UC) affects many people worldwide. Its pathophysiology involves immunological mechanisms. Glycyrrhizin preparation (GL-p) may be useful in the treatment of UC because it has anti-inflammatory and immunomodulatory effects.

Research frontiers

Although a previous study has demonstrated that oral administration of a combination of seven herbs is beneficial in the treatment of colitis in rats, the effect of GL, one of its constituents, was not detected.

Innovations and breakthroughs

The study demonstrated that GL-p was beneficial in the treatment of rat colitis if administered intrarectally. GL-p decreased the expression of pro-inflammatory cytokines and inhibited myeloperoxidase activity.

Applications

The results of this study suggest a therapeutic effect of GL-p enemas on experimental colitis in rats. Further studies are required to apply this treatment in patients with UC.

Terminology

GL is a natural compound extracted from the roots of the Chinese herb *Glycyrrhiza glabra* and GL-p has been clinically used as an anti-allergic agent and as a treatment for chronic hepatitis in Japan.

Peer review

GL, an active ingredient from licorice root, has long been used for traditional medicinal purposes. Very little work on this subject has been described in the mainstream literature, and this work is a welcome addition. The authors have done a commendable job of comparing their study with the only (to my knowledge) previously published work in the field, primarily that of Yuan *et al*.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2229

World J Gastroenterol 2011 May 7; 17(17): 2229-2235
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Hyperbaric oxygenation promotes regeneration of biliary cells and improves cholestasis in rats

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Received: June 9, 2010 Revised: September 25, 2010
Accepted: October 2, 2010
Published online: May 7, 2011

Abstract

AIM: To investigate the effects of hyperbaric oxygenation (HBO) on regeneration of the biliary ductal system and postoperative cholestasis in hepatectomized rats.

METHODS: HBO was performed in Wistar rats daily starting 12 h after a 70% partial hepatectomy. Regenerated liver weight, serum parameters and the proliferating cell nuclear antigen labeling index of hepatocytes and biliary ductal cells were measured. Hepatocyte growth factor (HGF), c-Met and transforming growth factor (TGF) β-1 mRNA expression levels were analyzed by quantitative reverse transcription polymerase chain reaction.

RESULTS: HBO improved the postoperative serum levels of total bile acid but not transaminase levels. HBO promoted hepatocyte and biliary ductal cell proliferation. The hematoxylin and eosin-stained specimens revealed fewer ballooned hepatocytes and higher cell densities in the HBO group compared to the control group. HBO suppressed c-Met mRNA levels at 15 h but did not modulate HGF or TGF β-1 mRNA expression levels.

CONCLUSION: HBO promoted regeneration of biliary ductal cells and improved postoperative cholestasis after a partial hepatectomy.

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Key words: Hyperbaric oxygenation; Liver regeneration; Partial hepatectomy; Cholestasis; Ballooning

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Idetsu A, Suehiro T, Okada K, Shimura T, Kuwano H. Hyperbaric oxygenation promotes regeneration of biliary cells and improves cholestasis in rats. *World J Gastroenterol* 2011; 17(17): 2229-2235 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2229.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2229

INTRODUCTION

Extended hepatectomy and small graft liver transplantation can result in postoperative cholestasis and liver failure. Hepatectomized liver rapidly regenerates and its volume and function recover, but functional recovery lags behind quantitative recovery. Postoperative liver failure causes cholestasis; hence, it is important to improve cholestasis in order to reduce morbidity and mortality.

Hyperbaric oxygenation (HBO) has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism[1]. Effects of HBO that have been reported include upregulation of growth factors[2], down-regulation of inflammatory cytokines[3] and increased angiogenesis[4].

Studies have reported the effect of HBO on liver re-

generation after partial hepatectomy, but few studies have reported its effect on biliary ductal regeneration and cholestasis. In this study, we investigated the effects of HBO on the biliary system and postoperative cholestasis after a partial hepatectomy.

MATERIALS AND METHODS

Animals and surgery

All experiments were performed on 10-wk-old male Wistar rats (n = 78). Thirty-six rats were used in each group and preoperative data acquired from another 6 rats. Procedures were approved by the Review Committee on Animal Use of Gunma University, Maebashi, Japan. All the rats were housed under temperature- and light-controlled conditions with 12 h light and dark cycles. The rats were fed a standard rat diet and water as desired. Prior to the experiment, the rats were starved for 24 h with free access to water. Under ether anesthesia, a 70% partial hepatectomy was performed using the Higgins and Anderson technique[5]. After the transverse laparotomy, the median and left lobes were ligated and resected. All the rats were allowed free access to the standard diet and water after the surgery. The rats were sacrificed at various time points (12, 15, 24, 48, 72, and 96 h after the partial hepatectomy) by puncture of the inferior vena cava and exsanguination under ether anesthesia. The regenerated livers were removed and either fixed in 20% formalin or frozen and stored at -80℃.

HBO

The rats in the HBO group were placed in a hyperbaric chamber (Model KHO-200; Kawasaki Engineering Co., Ltd., Hyogo, Japan) pressurized to 2 ATA with 100% oxygen for 60 min. HBO was initiated 12 h after the end of surgery and performed once a day until postoperative day 4. HBO was not performed postoperatively in the control group.

Regenerated liver weight/% body weight

The regenerated liver weight/% body weight ratio was calculated using the following equation: 100 × A/B, where A is the weight of the regenerated liver at the time of sacrifice and B is the rat body weight at the time of sacrifice.

Serum parameters

The degree of hepatic injury and biliary function was assessed by determining the serum levels of alanine aminotransferase (ALT) and total bile acid (TBA) using standard laboratory methods.

Hematoxylin and eosin staining

Formalin-fixed liver specimens were embedded in paraffin, and 3-μm sections were prepared. Hematoxylin and eosin (HE) staining was performed according to standard procedures. Hepatocytes were counted in random high power fields (HPFs) without a visible Glisson's sheath or central vein (× 400). The cell density was expressed as the mean number of hepatocytes per HPF.

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) expression was immunohistochemically detected using a monoclonal antibody against PCNA [dilution: 1:400, horseradish peroxidase-labeled, DAKO, PC10, (Code No. M0879)]. PCNA-positive cells were counted in random HPFs (× 400). The PCNA-labeling index was expressed as the percentage of PCNA-positive cells/total number of cells. Hepatocytes and biliary ductal cells were counted.

RNA isolation and cDNA synthesis

Total RNA was extracted from freshly frozen liver using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity of isolated RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). Template cDNA was synthesized from 13.5 μL of total RNA using an Omniscript reverse transcriptase kit (Qiagen), a random primer (hexadeoxyribonucleotide mixture) (TaKaRa, Shiga, Japan), and a ribonuclease inhibitor (porcine liver) (TaKaRa). Total RNA was reverse transcribed using 4 U of Omniscript reverse transcriptase in a reaction volume of 20 μL (60 min at 37℃, 5 min at 93℃, then placed on ice). The resultant cDNA samples were stored at -20℃ until analysis.

Quantitative reverse transcription polymerase chain reaction analysis of hepatocyte growth factor, c-Met, and transforming growth factor β-1 mRNA expression levels

Quantitative reverse transcription-polymerase chain reaction (PCR) analyses were performed using the ABI Prism 7000 Sequence detection system (Applied Biosystems, CA, USA). The standard reaction volume was 20 μL and contained 1 × SYBR Green PCR Master Mix (Applied Biosystems), 2.0 μL of cDNA template, and 0.5 μmol/L each of the forward and reverse primers. The initial PCR denaturation step was performed for 10 min at 95℃ followed by 45 cycles of 15 s at 95℃ (denaturation), 10 s at 60℃ (annealing), and 30 s at 72℃ (extension). All reactions were performed in duplicate. The data were adjusted using the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and expressed as a ratio of the GAPDH levels. The primers for hepatocyte growth factor (HGF) and transforming growth factor (TGF) β-1 were used as described in previously published assays[6], and the primers for c-Met and GAPDH were designed using Primer 3 (primer3_www.results.cgi) with sequences from Ensembl Genome Browser (http://www.ensembl.org/index.html) (Table 1).

Statistical analysis

All values are expressed as mean ± SD. Statistical significance was determined using the post-hoc test of Bonferroni-Dunn. $P < 0.05$ was considered statistically significant.

Table 1 Primer sequence for reverse transcription polymerase chain reaction

Gene		Primer sequence	Annealing temperature (℃)
HGF	Sense	5'-TTATGGGGAATGAGAAATGC	60
	Antisense	5'-TCGAACAAAAATACCAGGAC	
c-Met (ENSRNOT00000009662)	Sense	5'-CAGCGGCAATTCTAGACACA	60
	Antisense	5'-CTGAAGCTGCTTGTCACTCG	
TGF β-1	Sense	5'-ATGACATGAACCGACCCTTC	60
	Antisense	5'-TGTGTTGGTTGTAGAGGGCA	
GAPDH (ENSRNOT00000002652)	Sense	5'-AGACTATGGATGGCCCCTCT	60
	Antisense	5'-GGTAGGAACACGGAAGACCA	

HGF: Hepatocyte growth factor; TGF: Transforming growth factor.

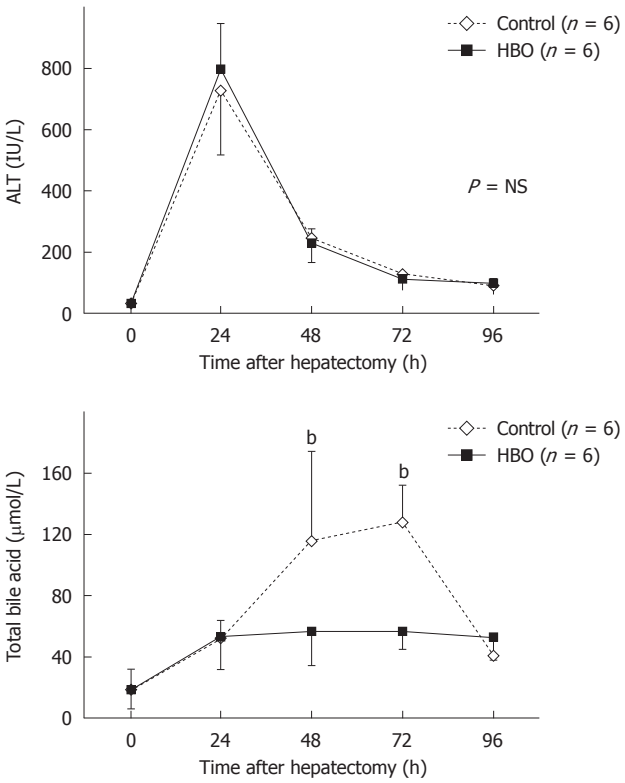

Figure 1 Serum parameters after hepatectomy. No significant difference was observed in the postoperative serum alanine aminotransferase (ALT) levels between the 2 groups. Total bile acid levels at 48 and 72 h after hepatectomy were significantly lower in the hyperbaric oxygenation (HBO) group than in the control group. Results are expressed as mean ± SD of $n = 6$ for each period in each group. NS: No significant. $^{b}P < 0.01$.

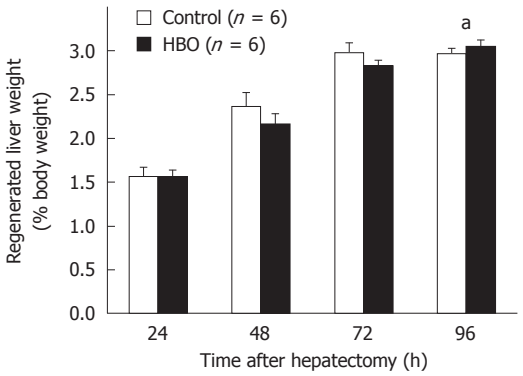

Figure 2 Regenerated liver weight /(% body weight). In both groups, the percentage weight of the regenerated liver increased daily after hepatectomy. At 96 h after hepatectomy, the percentage weight was higher in the hyperbaric oxygenation (HBO) group than in the control group. Results are expressed as mean ± SD of $n = 6$ for each period in each group. $^{a}P < 0.05$.

RESULTS

Liver injury and biliary function after the partial hepatectomy

Blood parameters were measured at various time points. ALT levels peaked at 24 h after the partial hepatectomy in the control and HBO groups and then decreased rapidly (Figure 1). No significant differences were observed between the groups at any time point as the serum ALT level decreased. Serum TBA levels increased and peaked at 72 h (Figure 1); these were decreased by HBO. Significant differences were observed in TBA levels at 48 h and 72 h after the partial hepatectomy. These data indicate that HBO improved the postoperative biliary function.

Liver regeneration after the partial hepatectomy

Data in Figure 2 reveal that the regenerated liver weight/% body weight increased daily after the partial hepatectomy. At 96 h, the regenerated liver weight ratio was higher in the HBO group than in the control group. The PCNA labeling index of the hepatocytes was higher at 48 and 72 h and lower at 24 h in the HBO group than in the control group (Figure 3A). The PCNA labeling index of the biliary ductal cells peaked at 48 h in the HBO group and was significantly higher in the HBO group than in the control group (Figure 3B). Combined, these data indicate that biliary ductal cell proliferation was greater in the HBO group than in the control group.

HE staining after the partial hepatectomy

An edematous cytoplasm and ballooning hepatocytes were observed in the regenerated livers of the control group at 96 h after surgery; however, these findings were not observed in the HBO group (Figure 4). The cell density of the hepatocytes was higher in the HBO group at

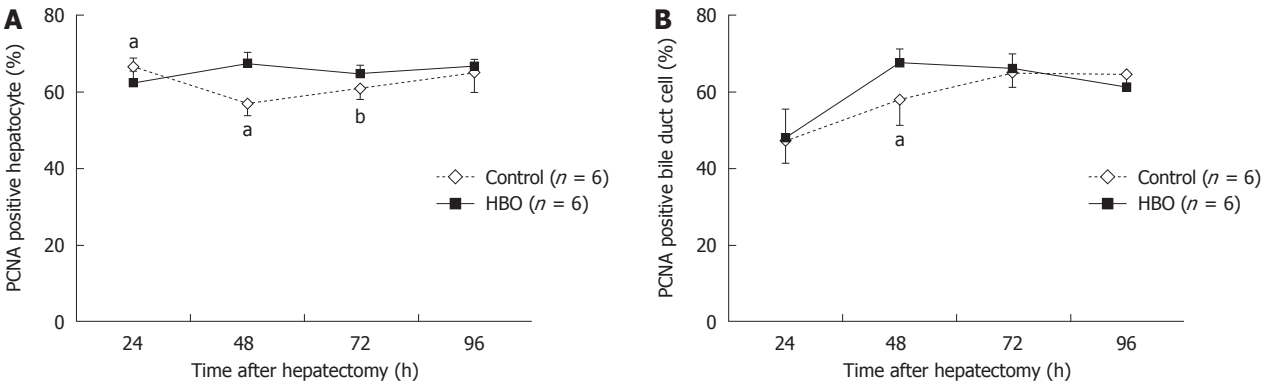

Figure 3 Percentage of proliferating cell nuclear antigen-positive cells in both groups at 24, 48, 72 and 96 h after hepatectomy. A: The percentage of proliferating cell nuclear antigen (PCNA)-positive hepatocytes was higher at 48 and 72 h and lower at 24 h in the hyperbaric oxygenation (HBO) group than in the control group. The percentage of PCNA-positive biliary ductal cells in the HBO group was higher at 48 h compared with the control group; B: The proliferation of biliary ductal cells was increased in the HBO group, with an early peak at 48 h. Results are expressed as mean ± SD of $n = 6$ for each period in each group. $^{a}P < 0.05$, $^{b}P < 0.01$.

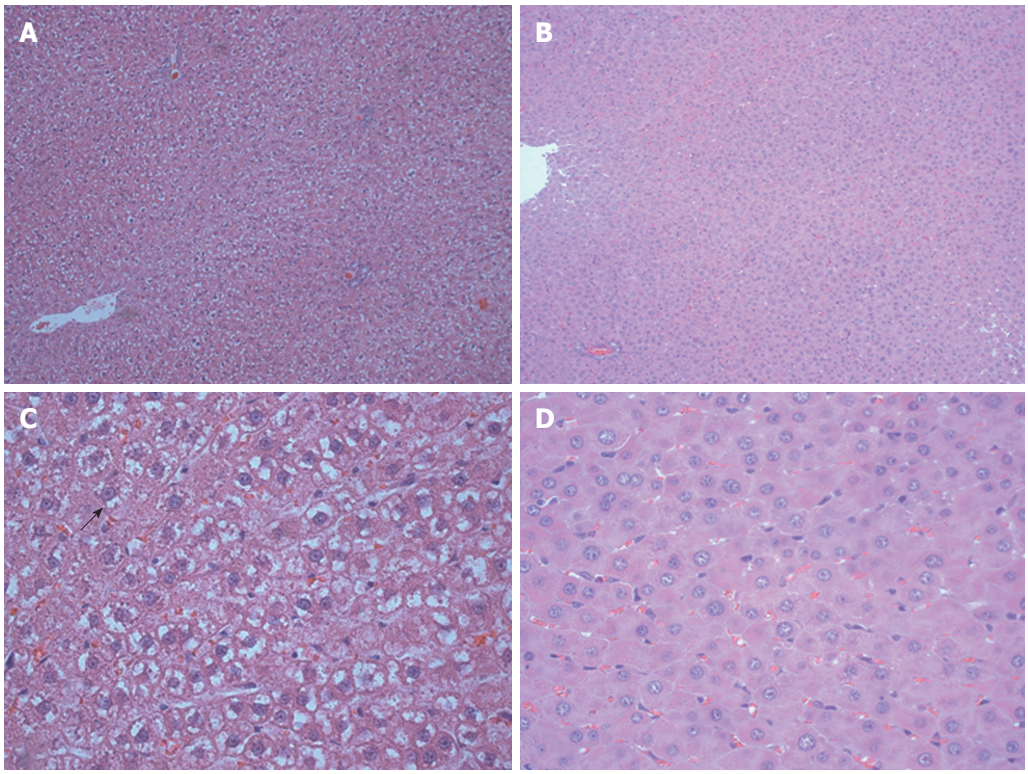

Figure 4 Hematoxylin and eosin staining of the liver slices at 96 h after hepatectomy. Control group (A: 100 × magnification, C: 400 ×). Hyperbaric oxygenation (HBO) group (B: 100 ×, D: 400 ×). In the control group, the regenerating livers were edematous and showed ballooned hepatocytes (C, arrow). In addition, the number of hepatocytes per high power field was lower in the control group than in the HBO group.

72 and 96 h (Figure 5). These findings suggest that HBO inhibited cell edema and protected the hepatocytes during liver regeneration.

Expression of HGF, c-Met and TGF β-1 mRNA

The expression of HGF and TGF β-1 mRNA peaked at 15 h in both groups and no significant differences in the mRNA levels of the HBO and control groups were observed. While the mRNA expression of c-Met increased and peaked at 15 h in both the groups, expression was significantly suppressed in the HBO group (Figure 6).

DISCUSSION

The goal of this study was to determine the effect of HBO on liver regeneration, particularly in biliary system restoration and improvement of postoperative cholestasis.

HBO involves the inspiration of a high concentration of oxygen in a pressure chamber at pressures higher than 1 ATA. During HBO, the partial pressure of oxygen increases, thereby increasing oxygen delivery throughout the body. The beneficial effects of HBO have been reported in ischemia-reperfusion injuries[6-8], acute liver fail-

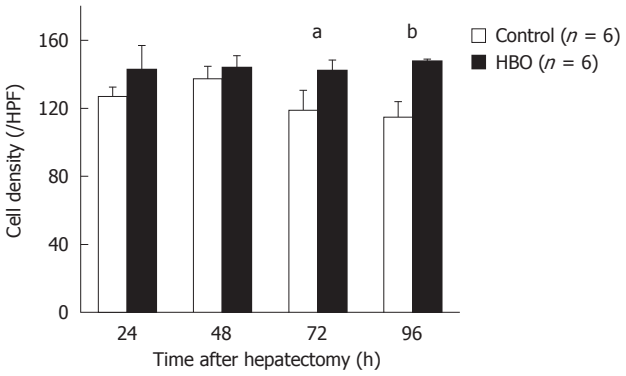

Figure 5 Cell density calculated as the number of hepatocytes per high power field. The cell density was higher in the hyperbaric oxygenation (HBO) group than in the control group at 72 and 96 h after hepatectomy. Results are expressed as mean ± SD of $n = 6$ for each period in each group. $^{a}P < 0.05$, $^{b}P < 0.01$.

ure after major hepatectomy[9], hepatic artery thrombosis after liver transplantation[10] and liver regeneration after portal vein embolization[11].

The hepatectomized liver enlarges to compensate for the mass of the resected lobes. In the early phase of liver regeneration, avascular clusters of hepatocytes are formed. Following hepatocyte proliferation, the vascular architecture and biliary ductal system are reconstructed in the regenerating liver. HBO promotes the proliferation of hepatocytes[11-13] and sinusoidal cells[14]. In the present study, data from the PCNA labeling index revealed that HBO promotes not only proliferation of hepatocytes but also biliary ductal cells. HBO probably improved bile drainage function during early phases of liver regeneration and decreased postoperative cholestasis.

During liver regeneration, hepatocytes exhibit morphologic changes such as cytoplasmic edema and ballooning. Ballooned hepatocytes are commonly observed in viral[15], drug-induced[16-18] and alcoholic[19,20] hepatic injuries and non-alcoholic steatohepatitis[21,22]. Hepatocytes are swollen in biopsies of orthotopic liver grafts after transplantation. Ballooning is associated with, but not directly caused by, bile retention. One of the pathogenetic factors of ballooned hepatocytes is ischemic damage. In a rat steatotic liver model, swollen hepatocytes caused abnormal microcirculation, manifested as reduced sinusoidal density[23]. The edematous state of hepatocytes can cause obstruction of the sinusoids and biliary ducts, secondary cholestasis and circulatory failure in the liver. HBO increases oxygen delivery and improves the ischemic state of the regenerated liver, breaking the cycle of hepatocyte ballooning and abnormal microcirculation. Our results suggest that HBO suppresses the edematous state of hepatocytes, accelerates bile drainage and improves postoperative cholestasis.

The present study revealed that HBO did not lower postoperative ALT levels. One study has suggested that HBO reduces serum ALT levels after a partial hepatectomy[14], while others have revealed no significant differences in ALT levels during liver regeneration[12,13,24]. The

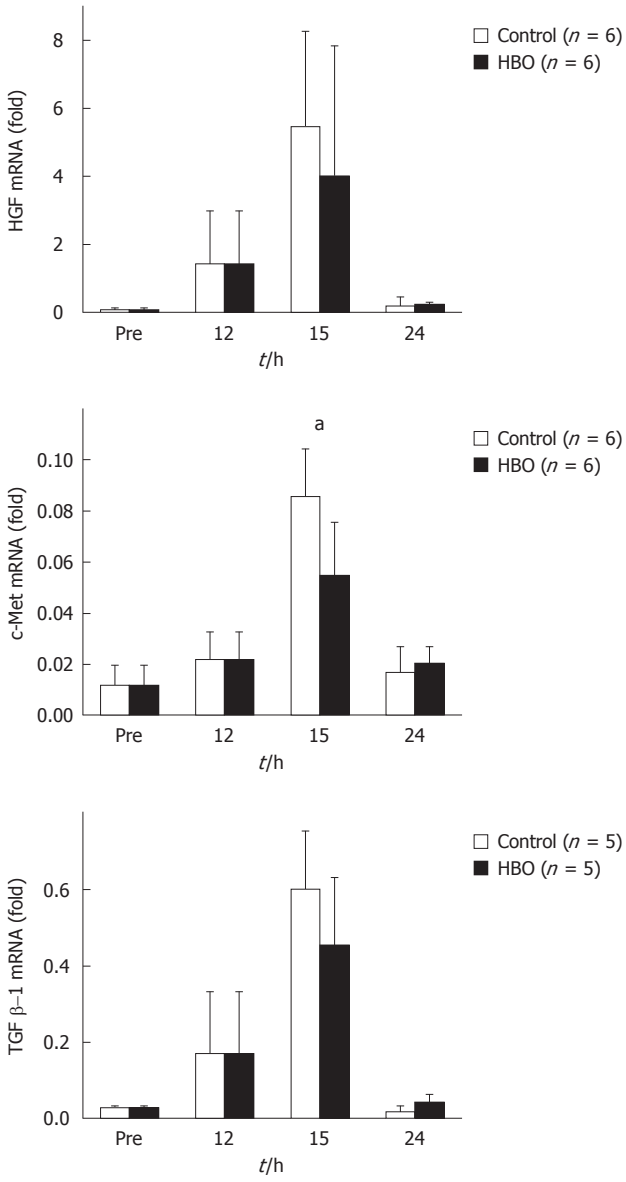

Figure 6 Expressions of hepatocyte growth factor, c-Met and transforming growth factor β-1 mRNA in the liver tissues determined at various time points. Results are expressed as mean ± SD. Hepatocyte growth factor (HGF) and c-Met: $n = 6$ for each period in each group, transforming growth factor (TGF) β-1: $n = 5$ for each period in each group. $^{a}P < 0.05$.

mechanisms of reduction of liver damage are mediated by various effects of HBO, which directly increases the oxygen concentration in the remnant liver and augments the expressions of HGF[11] and vascular endothelial growth factor (VEGF)[14].

HGF, known as the scatter factor, has mitogenic, motogenic, morpho-organogenic, angiogenic, and anti-apoptotic effects. HGF and its receptor c-Met are key factors for liver growth and function. TGF β-1 is an inhibitor of proliferation in hepatocyte cultures[25]. Data from the present study did not reveal significant differences in mRNA levels of HGF and TGF β-1 with HBO treatment after the partial hepatectomy. However, data did reveal that HBO suppressed c-Met mRNA expression at 15 h after the partial hepatectomy. HGF and c-Met promote liver

regeneration, while TGF β-1 inhibits it[25]. A balance of these promoter and inhibitor levels is important for liver regeneration[26]. HBO modulated the expression of c-Met mRNA and might cause an improvement in cholestasis and liver edema.

In conclusion, data from this study suggests that HBO has beneficial effects on the regeneration of biliary ductal cells and postoperative cholestasis after a partial hepatectomy. HBO also improved cell edema of hepatocytes in the regenerating liver. HBO might be useful for improving resectability and for decreasing the frequency of postoperative complications, such as liver failure, after a major hepatectomy.

ACKNOWLEDGMENTS

The authors thank Professor Saito S (Department of Anesthesiology, Gunma University, Graduate School of Medicine) for his excellent technical advice.

COMMENTS

Background

Extended hepatectomy and small graft liver transplantation can result in postoperative cholestasis and liver failure. Postoperative liver failure causes cholestasis; hence, it is important to improve cholestasis in order to reduce morbidity and mortality.

Research frontiers

Hyperbaric oxygenation (HBO) has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism. Effects of HBO which have been reported include upregulation of growth factors, down-regulation of inflammatory cytokines and increased angiogenesis.

Innovations and breakthroughs

The beneficial effects of HBO have been reported in ischemia-reperfusion injuries, acute liver failure after major hepatectomy, hepatic artery thrombosis after liver transplantation and liver regeneration after portal vein embolization. Further to this, the study would suggest that HBO promotes regeneration of biliary cells and improves cholestasis in rats after a partial hepatectomy.

Applications

The study would suggest that HBO has beneficial effects on the regeneration of biliary cells and postoperative cholestasis after a partial hepatectomy. HBO also improved cell edema of hepatocytes in the regenerating liver. HBO might be useful for improving resectability and for decreasing the frequency of postoperative complications, such as liver failure after a major hepatectomy.

Terminology

HBO involves the inspiration of a high concentration of oxygen in a pressure chamber at pressures higher than 1 ATA. During HBO, the partial pressure of oxygen increases, thereby increasing oxygen delivery throughout the body. In general, HBO has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism. Other beneficial effects of HBO in hepatic surgery and liver regeneration have been reported.

Peer review

The authors investigated the effect of HBO administration on bile duct cellular regeneration and cholestasis in a 70% model of hepatectomy. This is an area of high significance as findings may have a potential impact on therapeutic strategies in liver failure and regeneration following major hepatectomy. In limitation, the conclusions are based on results up to 96 h. The regeneration cycle is usually complete by day 8 and it may be appropriate to extend the time interval for the results to be obtained to this stage, before an appropriate conclusion may be reached. It may very well be the optimum results evident are a transient phenomena during the regenerative cycle.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2236

World J Gastroenterol 2011 May 7; 17(17): 2236-2240
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Limited water infusion decreases pain during minimally sedated colonoscopy

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Supported by Research funds from Buddhist Dalin Tzu Chi General Hospital
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Received: September 25, 2010 Revised: January 10, 2011
Accepted: January 17, 2011
Published online: May 7, 2011

Abstract

AIM: To investigate a limited water infusion method in colonoscopy.

METHODS: Consecutive patients undergoing minimally sedated colonoscopy were randomized to receive air insufflation (n = 89) or water infusion limited to the rectum, sigmoid colon and descending colon (n = 90). Completion rates, cecal intubation times, procedure times, need for abdominal compression, turning of patients and levels of discomfort were evaluated.

RESULTS: Completion rates, total procedure times, need for abdominal compression, and turning of patients were similar between groups. Less pain was experienced in the water group than in the air group (2.5 ± 2.5 *vs* 3.4 ± 2.8, mean ± SD, P = 0.021). The cecal intubation time was significantly longer in the water group than in the air group (6.4 ± 3.1 min *vs* 4.5 ± 2.4 min, P < 0.001). More water was infused in the water group (322 ± 80.9 mL *vs* 26.2 ± 39.4 mL, P < 0.001).

CONCLUSION: Limited airless water infusion in the distal colon reduces patients' pain during colonoscopy.

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Key words: Water; Pain; Colonoscopy; Looping; Intubation time

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Hsieh YH, Lin HJ, Tseng KC. Limited water infusion decreases pain during minimally sedated colonoscopy. *World J Gastroenterol* 2011; 17(17): 2236-2240 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2236.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2236

INTRODUCTION

Colonoscopy is currently the gold standard for the evaluation and treatment of colon disease[1,2]. The procedure can sometimes be quite painful and the success rate of intubation varies with the skills of the endoscopists[3]. Various methods have been used to reduce the pain of colonoscopy, including variable stiffness colonoscopy, magnetic endoscope imaging and oil lubrication[4-6]. Some of these measures require new instruments and their effects are controversial.

Pain during colonoscopy may originate from colon distension caused by air insufflation during the procedure. Adequate air insufflation opens the lumen of the

colon and improves the colonoscopic view. Overinflation, however, elongates the colon, accentuates the angles and results in difficult intubation. In addition, overinflation reduces mucosal blood flow in the laboratory setting and could possibly cause clinically significant ischemia[7].

Water instillation into the colon can facilitate intubation by straightening the sigmoid colon and decreasing the friction between the scope and the colonic mucosa during colonoscopy[6]. Recently, an airless water intubation method was proposed in which water, in lieu of air, was used to open the lumen of the colon[8]. In previous studies, this method was found to decrease the use of sedative medication during sedated colonoscopy and to increase patient willingness to receive unsedated colonoscopy[8-10]. However, a large amount of water was needed to inflate the colon adequately, and if water was infused manually with a syringe, the procedure time was prolonged[8]. Otherwise, an automatic infusion pump was needed[9-11].

In this randomized, controlled study, we simplified the water intubation method by limiting the infusion of water to the rectum and sigmoid colon. We aimed to investigate whether this limited water infusion method could reduce patient discomfort during minimally sedated colonoscopy.

MATERIALS AND METHODS

This prospective study was conducted between May and November 2009 at Buddhist Dalin Tzu Chi General Hospital. Patients undergoing minimally sedated colonoscopy performed by two endoscopists (Hsieh YH and Tseng KC) at our endoscopic suite were included. Patients with the following conditions were excluded: obstructive lesions of the colon, inadequate bowel preparation, allergy to meperidine, massive ascites, past history of partial colectomy, or refusal to provide written informed consent. The study was approved by the Institutional Review Board of the Buddhist Dalin Tzu Chi General Hospital.

Bowel cleansing was accomplished by asking the patients to ingest either 90 mL of sodium phosphate, or 4 bisacodyl tablets (4 × 5 mg) plus 250 mL of magnesium citrate (Purzer Pharmaceutical Co., Taipei, Taiwan) oral solution in two divided doses the night before the procedure.

Buscopan (hyoscine N-butylbromide, 20 mg), if not contraindicated, and 25 mg of meperidine were given intramuscularly immediately before the procedure to produce good colonic relaxation and reduce patient discomfort.

The colonoscopic examinations were performed by using a video colonoscope (CF 240AL, Olympus Optical Co. Ltd., Tokyo, Japan). All procedures were performed with the standard method, including starting with the patient in the left lateral decubitus position, avoiding and reducing loops as much as possible. The variable stiffness function was activated at the discretion of the endoscopist, usually after the sigmoid colon was passed and before manual abdominal pressure was applied. Intubation of the cecum was defined as successful only if the base of the cecum could be touched with the tip of the colonoscope. Detailed examinations were undertaken during the withdrawal phase.

The patients were allocated into two groups randomly by means of a computer-generated list. In the air group, air insufflation was used throughout the procedure. In the water group, the air pump was turned off initially during the procedure. Instead, tap water in 50 mL syringes was instilled into the rectum and the sigmoid colon through the accessory channel to open the colon lumen. Water was not usually instilled into the descending colon because the infused water already accumulated in this dependent segment. If the tip of the scope came out of the water or could not find its way at the transverse colon, the air pump was then turned on. In addition, if the lumen could not be opened with water in the sigmoid colon after a 5-min attempt, the air pump was turned on.

Demographic data (age, gender, height, weight, and education level), indications for colonoscopy, history of abdominal surgery or hysterectomy, constipation and chronic use of laxatives were obtained before colonoscopy. At the end of the procedure, the following parameters were evaluated and recorded on the patient data sheet: quality of bowel preparation, cecal intubation time, total procedure time, activation of the variable stiffness function, use of abdominal pressure, use of changing position, presence of polyps and reasons for incomplete colonoscopy. Body mass index was calculated as body weight divided by body height squared (kg/m^2). The quality of bowel cleansing was classified by the endoscopist as: (1) good: dry colon or only a small amount of clear liquid; (2) fair: large amount of clear liquid fluid or minimal solid stools; (3) poor: significant amount of solid residue; and (4) inadequate: when stool residue precludes complete insertion.

A trained research assistant who was unaware of the randomization status administered a questionnaire to the subjects after the procedure. In the questionnaire, abdominal pain was graded according to 10 levels (VAS scale). The degree of satisfaction was also assessed by a similar scale.

Statistical analysis

A sample size of about 75 patients was chosen to detect a difference of 1 in the patient abdominal pain score during colonoscopy given a standard deviation of 2.2 with a two-tailed α of 0.05 and a β of 0.20. Statistical analysis was performed using SPSS version 12.0 software (SPSS Inc., Chicago, IL). All continuous variables were expressed as mean ± SD. The Student's *t*-test and an analysis of variance to compare the means of continuous variables were used where appropriate. The χ^2 test, with Yates' correction for continuity, was used for comparison of categorical data, while the Fisher exact test was used when numbers were small. Multivariate logistic regression was performed using the application of assistant-administered abdominal pressure as the end point. A *P*

Table 1 Baseline characteristics of the patients undergoing colonoscopy (mean ± SD) *n* (%)

	Air group (*n* = 89)	Water group (*n* = 90)	*P* value
Male	51 (57.3)	49 (54.4)	0.764[2]
Age (yr)	58.3 ± 13.3	57.2 ± 13.3	0.612[1]
BMI (kg/m^2)	24.0 ± 3.5	24.2 ± 3.5	0.678[1]
Previous abdomino-pelvic surgery	27 (36.0)	23 (30.7)	0.604[2]
Constipation	34 (38.2)	22 (24.4)	0.054[2]
Inpatients	5 (5.6)	3 (3.3)	0.497[2]
Indications			0.482[2]
Follow-up of polyps	34 (38.2)	29 (32.2)	
Abdominal pain	5 (5.6)	9 (10.0)	
Rectal bleeding	13 (14.6)	11 (12.2)	
Stool occult blood	12 (13.5)	18 (20.0)	
Change of bowel habit	16 (18.0)	18 (20.0)	
Anemia	3 (3.4)	1 (1.1)	
Loss of body weight	3 (3.4)	3 (3.3)	
Other	3 (3.4)	1 (1.1)	
Education level			0.969[2]
Primary school and less	21 (24.1)	21 (24.4)	
High school	38 (42.7)	40 (44.4)	
College and higher	30 (34.5)	29 (33.7)	
Bowel cleansing regimens			0.179[2]
Sodium phosphate	41 (46.1)	51 (56.7)	
Magnesium citrate-bisacodyl	48 (53.9)	39 (43.3)	
Anxiety	3.0 ± 2.9	3.9 ± 2.9	0.110[1]
Antispasmodic agent use	72 (80.9)	75 (83.3)	0.700[2]
Colon preparation			0.276[2]
Good	49 (55.1)	60 (66.7)	
Fair	25 (28.1)	18 (20.2)	
Poor	15 (16.9)	12 (13.3)	

[1]Student's *t*-test; [2]χ^2 test. BMI: Body mass index.

value of less than 0.05 was considered to be statistically significant.

RESULTS

Between May 2009 and February 2010, 255 patients underwent colonoscopies performed by the two endoscopists at our hospital. Sixty-one patients wished to receive sedation, so they were not invited to participate in the study. Twelve patients were excluded because of prior hemicolectomy (n = 6), unwillingness to give written informed consent (n = 5), or massive ascites due to carcinomatosis (n = 1). The remaining 182 subjects were randomized to the air group (n = 90) or the water group (n = 92). Three patients were subsequently excluded because of inadequate preparation (one in each group) and severe colitis (one in the water group). The baseline characteristics of the remaining patients in both groups were similar (Table 1).

Incomplete colonoscopy occurred in 1 patient in the air group (1.1%) due to looping. Incomplete colonoscopy also occurred in 1 patient in the water group (1.1%) due to intolerance. However, air insufflation had to be used in 8 patients in the water group before reaching the transverse colon due to poor visibility in 2 patients, and an inability to open the lumen in 6 patients (4 at the sigmoid and 2 at the descending colon). These 8 patients were all subsequently intubated to the cecum.

Table 2 Outcomes of colonoscopy (mean ± SD) *n* (%)

	Air group (*n* = 89)	Water group (*n* = 90)	*P* value
No. of failed cecal intubations	1 (1.1)	1 (1.1)	1.000[4]
Cecal intubation time (min)	4.5 ± 2.4	6.4 ± 3.1	< 0.001[3]
Procedure time (min)	13.8 ± 5.6	14.5 ± 4.7	0.333[3]
Volume of water used (mL)	26.2 ± 39.4	322 ± 80.9	< 0.001[3]
Time of air insufflation during intubation (min)	4.5 ± 2.4	2.3 ± 2.6	< 0.001[3]
No. of cases requiring assistant-administered abdominal pressure	51 (57.3)	49 (54.4)	0.764[4]
No. of cases requiring change of position	24 (27.0)	17 (18.9)	0.217[4]
Patient pain score[1]	3.4 ± 2.8	2.5 ± 2.5	0.021[3]
No. of cases without pain	18 (20.2)	32 (35.6)	0.030[4]
Patient satisfaction score[2]	9.6 ± 0.8	9.6 ± 0.7	0.980[3]
No. of cases with polyps	52 (58.4)	44 (48.9)	0.232[4]
No. of cases with adenomas	31 (34.8)	32 (35.6)	1.000[4]

[1]0 = no pain, 10 = worst pain imaginable; [2]0 = not satisfied at all, 10 = completely satisfied; [3]Student's *t*-test; [4]χ^2 test.

The need for abdominal compression, need for changing position and total procedure time were similar between groups (Table 2). The cecal intubation time was shorter in the air group than in the water group (4.5 ± 2.4 min *vs* 6.4 ± 3.1 min, $P < 0.001$).

Significantly less water was infused in the air group than in the water group during the procedure (26.2 ± 39.4 mL *vs* 322 ± 80.9 mL, $P < 0.001$). The time of air insufflation during the insertion phase was less in the water group than in the air group (4.5 ± 2.4 min *vs* 2.3 ± 2.6 min, $P < 0.001$). The mean pain scores, as rated by the patients, were higher in the air group than in the water group (3.4 ± 2.8 *vs* 2.5 ± 2.5, $P = 0.021$). Also, more patients in the water group had no pain at all compared to patients in the air group (35.6% *vs* 20.2%, $P = 0.030$). Overall satisfaction with the procedure was similar between groups (9.6 ± 0.8 *vs* 9.6 ± 0.7, $P > 0.05$) (Table 2).

Polyps were detected in 52 (58.4%) patients in the air group. Thirty-one (34.8%) of these polyps were adenoma, 5 (5.6%) were tubulovillous adenoma and 3 (3.4%) were carcinoma. Polyps were detected in 44 (48.9%) patients in the water group. Thirty-two (35.6%) polyps were adenoma, 4 (4.4%) were tubulovillous adenoma and 1 (1.1%) was carcinoma (Table 2).

DISCUSSION

The results of this study show that water infusion rather than air insufflation at the rectum and sigmoid is associated with less pain during minimally sedated colonoscopy. In experienced hands, about 35.6% of the patients felt no pain at all. The water was infused manually with a syringe without additional instruments, making this method readily available. Although the limited water infusion group had a longer intubation time than the air insufflation group, the difference was small (less than 2 min). To the best of our knowledge, this is the first study to compare this new water infusion method with traditional air insufflation.

Water infusion to facilitate colonoscopy has been reported by several authors in the past; however it has not been applied frequently. Most authors used water infusion in conjunction with air insufflation. Falchuk *et al*[12] found it helpful to infuse up to 300 mL of water into the sigmoid colon while intubating patients with severe diverticulosis. Baumann compared water infusion (200 mL) with traditional air insufflation during colonoscopic examination[13]. He found that passing through the left colon was faster with the water method than with the air method. Brocchi *et al*[6] compared warm water (300 mL) infusion with seed oil and traditional air insufflation during colonoscopic examination. They found that water infusion was associated with a higher cecal intubation rate, shorter intubation time and less pain than in the control group.

Recently, Leung *et al*[9] employed a novel method with infusion of a large amount of water during the insertion phase. In their pilot study, airless water intubation permitted 52% of patients to complete the procedure without sedation. A mean volume of more than 1 L of water was infused in aliquots of 30 to 60 mL. The major drawback of this technique was the long intubation time of up to 22.6 min[9]. In a subsequent randomized, controlled study comparing water intubation with air insufflation, the water was infused intermittently with a peristaltic pump with a blunt needle adaptor through the biopsy channel[10]. The cecal intubation times were comparable between the two groups. The endoscopists found that the increments of medications and the maximum pain scores were significantly lower with the water method[10].

Our study showed that a lower mean pain score was experienced in the water group (2.5 ± 2.5) than in the air group (3.4 ± 2.8, $P = 0.021$). A previous study has shown that patient discomfort occurs when the colonoscope tip reaches the sigmoid colon[14]. Loop formation of the colonoscope occurring mostly in the sigmoid colon is the major cause of pain[15]. We infused water instead of air in the rectum and sigmoid colon in our study. By eliminating air in the sigmoid colon, we reduced the loop formation and caused less pain in these patients. The weight of the water might also be helpful in reducing looping over the sigmoid colon.

In the present study, we did not use air insufflation until the scope reached the transverse colon. The infused water accumulated at the descending colon due to gravity when patients were in the left lateral decubitus position. When the scope reached the transverse colon the air was switched on, otherwise much more water would have been needed to open the more proximal colon, which was at non-dependent areas. By limiting the use of water infusion to the distal colon, we could achieve intubation with little water (around 300 mL). This amount of water could easily be infused with a syringe instead of a peristaltic pump.

The intubation time was longer in the water group than in the air group (6.4 ± 3.1 min *vs* 4.5 ± 2.4 min). However, air had to be turned on prematurely in 8 (8.9%) patients in the water group. There are several reasons for the longer time in the water group. Firstly, it took time to infuse water into the biopsy channel repeatedly with a syringe. Secondly, it was difficult to open a collapsed segment or acute angle at a non-dependent portion without enough water. Thirdly, the view was less clear and the lumen was more difficult to find under water than with air insufflation, especially when the preparation was less than optimal. With a limited amount of water infused, our method had little cleansing effect on colon contents[8].

The polyp detection rates were similar in our study groups. After opening the lumen adequately with air insufflation and aspirating the residual stools and fluid, we inspected the mucosa closely when we withdrew the scope in both groups, so the infused water did not impair our ability to detect polyps. When the bowel preparation was adequate, we had no difficulty finding a polyp underwater during insertion.

In this study, we infused water at room temperature. Some previous studies used warm water[6,10,16], but others have used water at room temperature[13,17]. Church *et al*[16] showed that warm water minimized colon spasms and decreased patient discomfort. Most of our patients had received intravenous buscopan before colonoscopy, so we did not encounter any colon spasms in this study.

This study has several limitations. The procedures were performed by two experienced endoscopists, since the procedure conditions cannot appropriately be handled by less experienced doctors. The endoscopists were not blinded to the methods, but the patients were blinded. The use of the water method only reduced a small proportion (about 26%) of the pain scores; however the procedure was simple and cost-effective. We did not compare the syringe infusion method with a peristaltic pump method, although this does warrant further studies.

In conclusion, compared with traditional air insufflation, limited infusion of water at the distal colon resulted in less pain in patients undergoing minimally sedated colonoscopy, although a longer intubation time was required.

COMMENTS

Background

Colonoscopy can sometimes be quite painful. Pain during colonoscopy may originate from colon distension caused by air insufflation. Water instillation into the colon can facilitate intubation by straightening the sigmoid colon and decreasing the friction between the scope and the colonic mucosa. The weight of water also helps prevent loop formation.

Research frontiers

Recently, an airless water intubation method was proposed in which water, in lieu of air, was used to open the lumen of the colon. In previous studies, this method was found to decrease the use of sedative medication during sedated colonoscopy. However, a large amount of water was needed to inflate the colon adequately, which might increase procedure time or require an additional peristaltic pump. In this randomized, controlled study, the authors simplified the water intubation method by limiting the infusion of water to the rectum and sigmoid colon.

Innovations and breakthroughs

In the limited water group in the present study, the air pump was turned off initially during the procedure. Tap water in 50 mL syringes was instilled into the rectum and the sigmoid colon through the accessory channel to open the colon lumen. Water was not usually instilled in the descending colon because the

infused water already accumulated in this dependent segment. The air pump was opened when the colonoscope reached the transverse colon. No additional instrument was needed with this method and only about 300 mL of water was used to complete the colonoscopy.

Applications

This article shows that limited airless water infusion in the distal colon reduces patients' pain during colonoscopy. Further research should be done by less experienced endoscopists. In addition, head-to-head comparison of the limited water infusion method with total water infusion is needed.

Peer review

Hsieh *et al* present the results of the largest randomized trial yet performed to assess the value of water instillation to facilitate colonoscopy, here in minimally sedated patients. In general the paper is well written, the methodology is good and the results will generate widespread interest amongst endoscopists.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2241

World J Gastroenterol 2011 May 7; 17(17): 2241-2247
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats

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Supported by Two grants from National Natural Science Foundation of China, No. 30300465 and No. 30772883
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Received: October 17, 2010 Revised: March 24, 2011
Accepted: March 31, 2011
Published online: May 7, 2011

Abstract

AIM: To determine the optimal dosage and mechanism of Ginkgolide B (BN52021) on severe acute pancreatitis (SAP) of rats.

METHODS: Seventy male Wistar rats were randomly divided into seven groups (10 for each group). Sham-operation group (SO), SAP model group (SAP), dimethyl sulfoxide (DMSO) contrast group (DMSO), and groups treated with 2.5 mg/kg BN52021 (BN1), 5 mg/kg BN52021 (BN2), 10 mg/kg BN52021 (BN3), and 20 μg/kg Sandostatin (SS). The SAP model was established in Wistar rats by injecting 5% sodium taurocholate retrogradely into the common bilio-pancreatic duct. The rats of SO, DMSO and BN52021 were injected with 0.9% NaCl, 0.5% DMSO and BN52021 through femoral vein 15 min after the operation. The SS group was injected with Sandostatin subcutaneously. All rats were anaesthetized at 6 h after operation, and venous blood was collected to determine the levels of serum amylase and phospholipase A2 (PLA_2), and pancreas tissue was harvested and stained.

RESULTS: There was no significant difference between the SAP and DMSO groups in serum amylase level, PLA_2, ascites and pathologic score, but significant difference was found in SAP/DMSO groups compared with those in SO group ($P < 0.05$) and the levels of serum amylase, PLA_2, ascites, and pathologic score were lower in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups ($P < 0.05$). However, among BN1, BN2, BN3 and SS groups, BN2 had the best effect in decreasing the levels of serum amylase and PLA_2 ($P < 0.05$). Expression of platelet activating factor (PAF) receptor (PAFR) mRNA and protein showed no significant difference between the SAP and DMSO groups, or among BN1, BN2, BN3 and SS groups, but there was remarkable difference between SAP/DMSO group and SO group ($P < 0.05$), and expression of PAFR mRNA and protein was higher in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups ($P < 0.05$). PAFR expression was observed in the nucleus and cytoplasm of pancreatic islet cells in Wistar rats by immunohistochemistry.

CONCLUSION: By iv injection, 5 mg/kg of BN52021 is the optimal dosage for SAP rats. BN52021 may inhibit the interaction/binding of PAF with PAFR.

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Key words: Pancreatitis; Ginkgolide B; Dose-effect; Phospholipase A2; Platelet activating factor receptor

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Ji RL, Xia SH, Di Y, Xu W. Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats. *World J Gastroenterol* 2011; 17(17): 2241-2247 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2241.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2241

INTRODUCTION

Platelet activating factor (PAF) is a biologically active phospholipid mediator, playing its role by binding to PAF receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways[1]. Flickinger *et al*[2] revealed specific localization of PAFR in the pancreatic vascular endothelium, but not in other pancreatic cell types. Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP)[3].

Ginkgolide B (code: BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of Ginkgo biloba leavies. It has been found that BN52021 possesses activity as antagonists of the action of PAF. It can inhibit PAF-induced cascade effect in inflammatory reactions. For example, it can reduce the portal vein pressure of liver cirrhosis, exhibit an anti-shock effect, and exert protective functions on experimental asthma[4,5].

SAP is dangerous to life. It can cause multiple organ system failure (MOSF) and if the patients do not receive effective treatment in time, they may die. But up till now, there has been no effective drug for this disease. Recent studies have shown that BN52021 has best effect on experimental SAP[6,7], but there is no report on its optimal dosage, and its mechanism has not been fully understood. Especially as an extract of Ginkgo biloba, it is hypotoxic, and can be used in clinical treatment of SAP. We investigated the optimal dosage of BN52021 and its mechanism on SAP.

MATERIALS AND METHODS

Main reagents and apparatus

BN52021 and sodium taurocholate were purchased from Sigma (St. Louis, MO, USA), amylase kit from Beijing Kemei Reagent Co. (Beijing, China), trizol and diethyl pyrocarbonate (DEPC) from Invitrogen (Carlsbad, CA, USA), RT kit from MBI Fermentas (Ontario, Canada), DNTPs and RNA enzyme inhibitor from TaKaRa Dalian Co., Ltd. (Dalian, China), DNA Tag enzyme from Promega (Madison, WI, USA), primary antibody of PAF-R rabbit-anti-rat serum and the enhanced chemiluminescence system from Santa Cruz Biotechnology (Santa Cruz, CA, USA), secondary antibody of sheep-anti-rabbit from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China), β-actin from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China), DYY-12 electrophoresis system and electric transblot SD from Beijing Liuyi Instrument Factory (Beijing, China), Type-2720 polymerase chain reaction (PCR) apparatus from ABI (Foster, CA, USA), and gel scanning and imaging system and vertical electrophoresis system from Bio-Rad (Hercules, CA, USA).

Primer sequences

PAF-R and β-actin primer series are provided by Invitrogen (Carlsbad, CA, USA): PAF-R: forward primer (F): 5'-CCGCTGTGGATTGTCTATTA-3', reverse primer (R): 5'-AGGAGG TGATGAAGATGTGG-3' (377 bp)[8]; β-actin: F: 5'-TCC TAGCACCATGAAGATC-3', R: 5'-AAACGCAGCTCAGTAACAG-3' (190 bp)[9].

Experimental animal grouping and model preparation

Seventy Wistar male rats (Laboratory Animal Center of PLA, Academy of Military Medical Sciences, Beijing, China), aged 6-8 wk, and weighing 200-220 g (Grade Ⅱ, Certificate SCXK 2002-001) are used for this study. All rats were maintained in an environment of controlled temperature (22-25℃), humidity (55%-58%), and lighting (12 h light/12 h dark), with free access to tap water and regular chow diet. Rats were divided randomly into 7 groups: Sham-operation group (SO group, n = 10), SAP model group (SAP group, n = 10), DMSO contrast group (DMSO group, n = 10), and treatment groups of 2.5 mg/kg BN52021 (BN1 group, n = 10), 5.0 mg/kg BN52021 (BN2 group, n = 10), 10.0 mg/kg BN52021 (BN3 group, n = 10), and 20.0 μg/kg Sandostatin (SS group, n = 10)[10]. SAP model was established according to Aho *et al*[10]. All rats were weighed, marked and fasted for 24 h before operation. The rats were anesthetized by abdominal injection of 0.4% pentobarbital sodium (40 mg/kg), and fixed in dorsal decubitus. A 2-cm cut was made at the center of the upper belly, entering the abdominal cavity to look for rat's duodenum and pancreaticobiliary duct. The hepatic end of the pancreaticobiliary duct was clipped with a non-invasive vascular clip, and pancreaticobiliary duct retrograde centesis was performed with an obtuse needle through duodenum seromuscular layer. Then 5% sodium taurocholate (0.1 mL/100 g) was injected in the retrograde direction of pancreaticobiliary duct with a micro-syringe at an injection rate of 0.20 mL/min. After injection, the port of pancreaticobiliary duct entering duodenum was clipped with a non-invasive vascular clip and observed for 10 min. When no active bleeding was confirmed in the abdominal cavity, the abdomen was closed in two layers and the incision was covered with sterile gauze. Same treatment was given to the SO group but without injections into the duct. Fifteen min after the model was completed, the rats in SO and SAP groups were injected with physiological saline (0.9% NaCl) through femoral vein at a dose of 5ml/kg; the rats of DMSO group were injected with DMSO (0.5% DMSO) at a dose of 5ml/kg; the BN1, BN2, and BN3 groups were rapidly injected with BN52021 at doses of 2.5, 5.0 and 10.0 mg/kg, dissolved with 0.5% DMSO; and SS group was injected with Sandostatin subcutaneously at a dose of 20.0 μg/kg[11].

Collection and storage of samples

Rats in each group were anaesthetized at 6 h after first injection of Sodium taurocholate, because the $T_{1/2}$ of BN52021 is 5.23 h[12], venous blood was collected from right atrium and was centrifuged for 10 min at 3000 *g*/min after a 10-min water bath at 37℃. The supernatant was placed into a sterilized EP tube, and stored in refrigerator at -20℃ for serum amylase determination. Portions of pancreatic tissues were placed in liquid nitrogen overnight and frozen in refrigerator at -80℃, meanwhile, another portion of pancreatic tissues was fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, cut into slices and stained with HE for pathological observation and scoring. Pneumonic tissues were fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, and cut into slices for immunohistochemical studies.

Determination of serum amylase, PLA2 and pathological observation, scoring of pancreatic tissues

Serum amylase and PLA_2 were detected with fully automatic biochemical apparatus, amylase and PLA_2 kits. Pancreatic tissue samples were observed pathologically and scored according to the reference[13].

Determination of PAFRmRNA expression by reverse transcription PCR

After total RNA from pancreatic tissue in each group was extracted, its integrality was checked with agarose electrophoresis, and its concentration and purity were determined with an UV spectrophotometer, and the concentration was calculated. RNA 5 μg and Oligo DT_{15} 1.25 μg were placed into a water bath at 70℃ for 5 min, rapidly put into ice for 5 min, and centrifuged just for 15 s. 5 × M-MLV reverse transcription buffer 5 mL, DNTPS 0.05 μmol, RNA enzyme inhibitor 40 U, M-MLV and reverse transcriptase 1 μL were added, and diluted to 25 μL with deionized water treated by DEPC. The whole mixture was maintained at 42℃ for 60 min. Reverse transcription was conducted and reverse transcriptase was deactivated at 95℃ for 5 min. The mixture was maintained at 4℃ for 5 min and preserved at -20℃. Fifty microliters PCR reaction system consists of 3 μL reverse transcription product, 0.01 μmol DNTPS, 5 μL 10 × PCR buffer, 0.075 mol $MgCl_2$ (Magnesium Chloride), 50 pmol of each PAF-R specific sense strand and anti-sense strand, and 50 pmol of each β-actin sense strand and anti-sense strand. Reaction conditions were as follows: pre-denaturizing at 94℃ for 3 min, denaturizing at 94℃ for 45 s, annealing at 58℃ for 45 s, and extending at 72℃ for 45 s, 38 cycles in total; extending at 72℃ for 7 min, and preserving at 4℃. The PCR product was included in the PAF-R gene sequence of rat spleen (gi:470384), which was performed by Beijing Boya Biotechnology Co., Ltd. The PCR product was analyzed by 2% agarose gel electrophoresis, ethidium bromide staining, and a gel imaging system scanning. Gray level of PCR product was treated by PAF-R and β-actin was analyzed using the Quantity-One Software, and the change of PAF-R mRNA expression was evaluated semi-quantitatively.

Determination of PAFR protein expression by immunohistochemistry

Paraffin slices of pancreas (5 μm) were made, mounted on slides and treated with paraformaldehyde-lysine-periodate solution. The slides were immersed in 100% dimethylbenzene for 15 min, 100% ethanol for 10 min, 95% ethanol for 5 min, 80% ethanol for 5 min, 70% ethanol for 5min, and washed with phosphate-buffered saline (PBS). The slides were immersed in citrate solution for 15 min in microwave at high temperature, cooled at room temperature, washed for 3 times with PBS, incubated with the rabbit polyclonal antibody against a recombinant protein corresponding to amino acids at the amino terminus of PAFR (1:100) for 18 h at 4℃, then incubated with goat anti-rabbit IgG-HRP polymer (1:500) for 0.5 h at 4℃, washed with PBS after incubation, and then incubated with $DABH_2O_2$ solution for color development, and washed with lotic water. The slices were counterstained with hematoxylin and mounted with coverslips. The slices were observed and analyzed, and the quantity of PAFR protein expression was determined according to the density of positive cells. Some frozen slices of pancreatic tissues and paraffin slices of lung tissues were made to observe the location of PAFR in pancreatic and lung tissues by immunohistochemistry.

Statistical analysis

The experiments were repeated for 3 times and their average values served as the final values; all values were expressed as mean ± SD. Data were processed with SPSS 11.5 Statistical Software, and LSD and Dunnett's post-hoc method were used for comparison of ANOVA. Statistical significance was established at $P < 0.05$.

RESULTS

Serum level of amylase and PLA_2, quantity of ascites and pathological scores

There were no significant differences in the serum levels of amylase and PLA_2, quantity of ascites and pathological scores between SAP and DMSO groups. It was higher in SAP/DMSO group than in SO group ($P < 0.05$), and lower in BN1, BN2, BN3 and SS group than in SAP/DMSO group ($P < 0.05$). However, BN2 had the best effect in decreasing the serum level of amylase and PLA_2 among BN1, BN2, BN3 and SS groups ($P < 0.05$) (Table 1, Figure 1).

Expression of PAFR mRNA and its proteins in pancreatic tissues

There was no significant difference in expressions of PAFR mRNA and PAFR protein between SAP and DMSO groups, neither among BN1, BN2, BN3 and SS groups. Expression of PAFR mRNA and its proteins in pancreatic tissues was lower in SAP/DMSO group than in SO group ($P < 0.05$), and higher in BN1, BN2, BN3 and SS groups than in SAP/DMSO group ($P < 0.05$) (Table 2, Figures 2 and 3).

Location of PAFR in pancreatic and lung tissues

PAFR was located in pancreatic microvascular endothelial

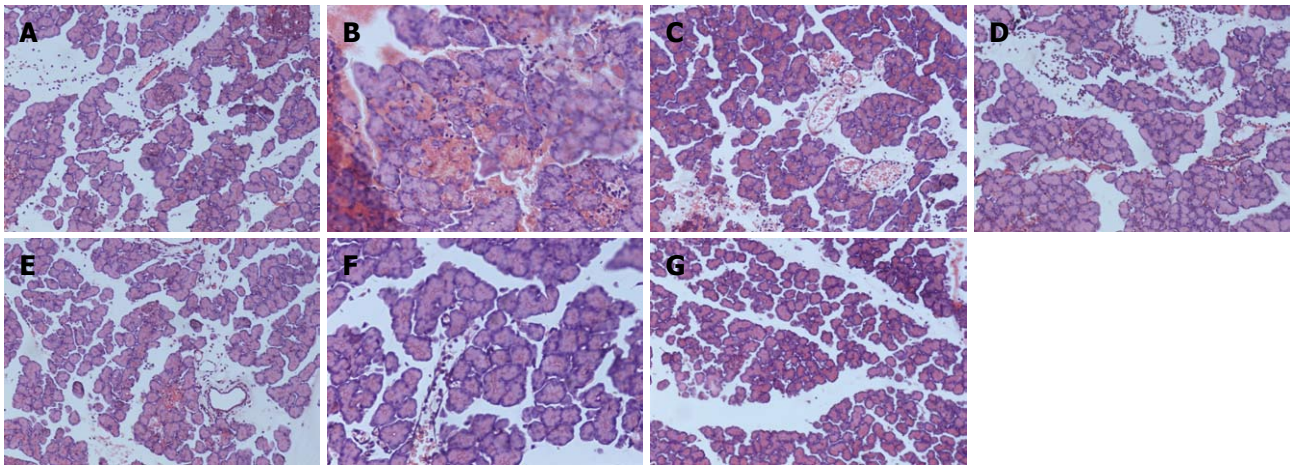

Figure 1 Pathological change of pancreatic tissues (HE staining, × 200). A: Sham-operation group; B: Severe acute pancreatitis group; C: DMSO group; D: 2.5 mg/kg BN52021 group; E: 5.0 mg/kg BN52021 group; F: 10 mg/kg BN52021 group; G: 20 μg/kg Sandostatin group.

Table 1 Serum levels of amylase and PLA_2, quantity of ascites and pathological scores (n = 10) (mean ± SD)

Group	Amylase (U/L)	PLA_2 (μmol/min per mililiter)	Ascites (mL)	Pathological scores
SO	1923 ± 527[a,c]	0.0434 ± 0.019[a,c]	0.0[a,c]	0[a,c]
SAP	6448 ± 240[c]	0.2990 ± 0.0225[c]	5.4 ± 1.3[c]	9.15 ± 0.55[c]
DMSO	6070 ± 464[c]	0.2728 ± 0.4461[c]	5.0 ± 2.0[c]	9.10 ± 0.65[c]
BN1	4744 ± 1031[a,c]	0.2320 ± 0.0890[a,c]	2.4 ± 0.9[a]	6.65 ± 0.42[a]
BN2	3452 ± 548[a]	0.1049 ± 0.0528[a]	1.6 ± 0.5[a]	5.55 ± 0.36[a]
BN3	4777 ± 168[a,c]	0.1069 ± 0.0440[a]	2.6 ± 0.9[a]	5.90 ± 0.19[a]
SS	4646 ± 675[a,c]	0.1632 ± 0.0523[a,c]	2.4 ± 0.5[a]	5.70 ± 0.30[a]

PLA_2: Phospholipase A2; SO: Sham-operation; SAP: Severe acute pancreatitis; BN1: 2.5 mg/kg BN52021; BN2: 5.0 mg/kg BN52021; BN3: 10 mg/kg BN52021; SS: 20 μg/kg Sandostatin. [a]$P < 0.05$ *vs* SAP group; [c]$P < 0.05$ *vs* BN2 group.

Table 2 Expression of platelet activating factor receptor in pancreatic tissues (n = 10) (mean ± SD)

Group	Ratio of PAFR/β-actin mRNA	IOD of PAFR
SO	0.46 ± 0.10[a]	5394.6 ± 1739.4[a]
SAP	0.38 ± 0.08[c]	1881.2 ± 376.7[c]
DMSO	0.39 ± 0.09[c]	2105.4 ± 345.2[c]
BN1	0.54 ± 0.14[a]	19966.7 ± 6488.7[a]
BN2	0.60 ± 0.09[a]	20935.6 ± 6551.9[a]
BN3	0.60 ± 0.09[a]	2169.7 ± 6872.5[a]
SS	0.59 ± 0.08[a]	20897.5 ± 666.2[a]

PAFR: Platelet activating factor receptor; IOD: Integrated optical density; SO: Sham-operation; SAP: Severe acute pancreatitis; BN1: 2.5 mg/kg BN52021; BN2: 5.0 mg/kg BN52021; BN3: 10 mg/kg BN52021; SS: 20 μg/kg Sandostatin. [a]$P < 0.05$ *vs* SAP group, [c]$P < 0.05$ *vs* BN2 group.

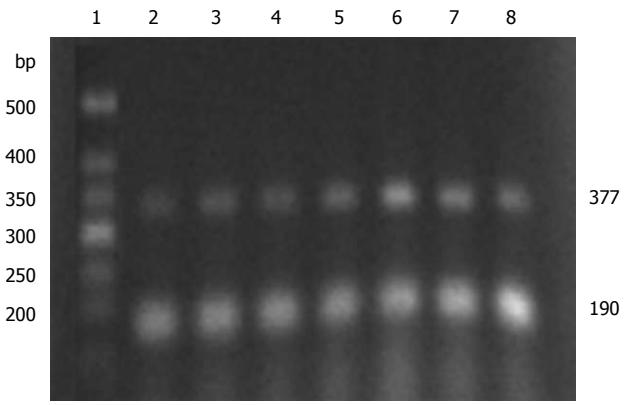

Figure 2 Expression of platelet activating factor receptor mRNA in pancreatic tissues by reverse transcription polymerase chain reaction. 1: Marker; 2: Sham-operation group; 3: Severe acute pancreatitis group; 4: DMSO group; 5: 2.5 mg/kg BN52021 group; 6: 5.0 mg/kg BN52021 group; 7: 10 mg/kg BN52021 group; 8: 20 μg/kg Sandostatin group.

cells and bronchia epithelial cells. It was first found in the nuclei of pancreatic islet cells and cytoplasm of Wistar rats (Figure 4).

DISCUSSION

Amylase, PLA_2, score of pathologic changes of pancreas and quantity of ascites were reduced after administration of BN52021 at 2.510 mg/kg, and the pancreas PAFR mRNA or protein was protected. PAFR expression was found in the pancreas' islet, which has not been reported by others up till now. The best effect was shown at 5 mg/kg among BN groups. BN52021 at 5 and 10 mg/kg showed equivalent effects on SAP, and 10 mg/kg was less effective such as in serum amylase. This may be caused by the dose range between 10 and 5 mg/kg.

PLA_2 is pivotal in the pathogenesis of SAP. There are two types of PLA_2: cytosolic phospholipase A_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$).There is a cross-talk between

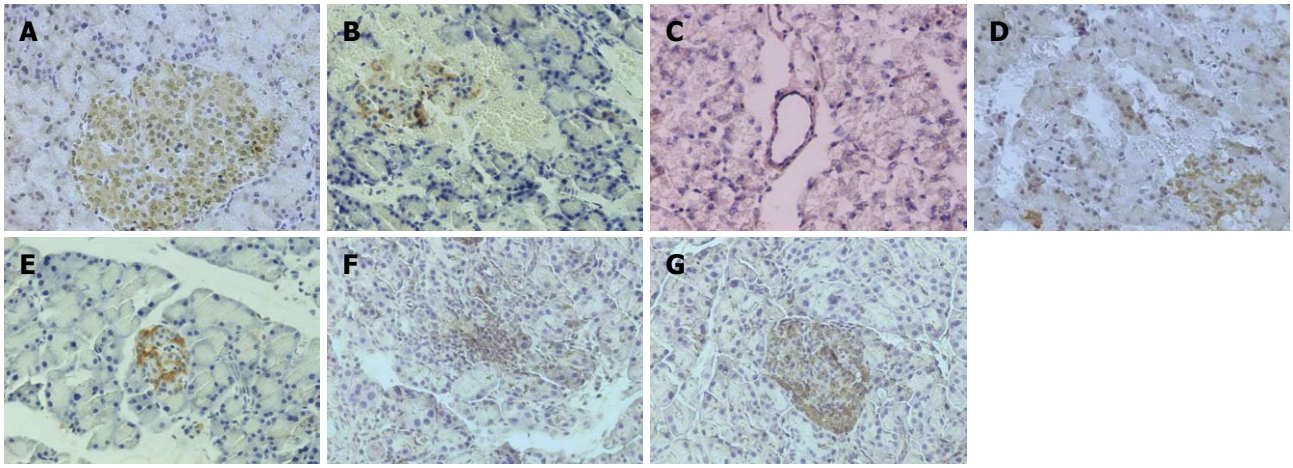

Figure 3 Expression of platelet activating factor receptor in pancreatic tissues by immunohistochemistry (HE staining, × 200). A: Sham-operation group; B: Severe acute pancreatitis group; C: DMSO group; D: 2.5 mg/kg BN52021 group; E: 5.0 mg/kg BN52021 group; F: 10 mg/kg BN52021 group; G: 20 μg/kg Sandostatin group.

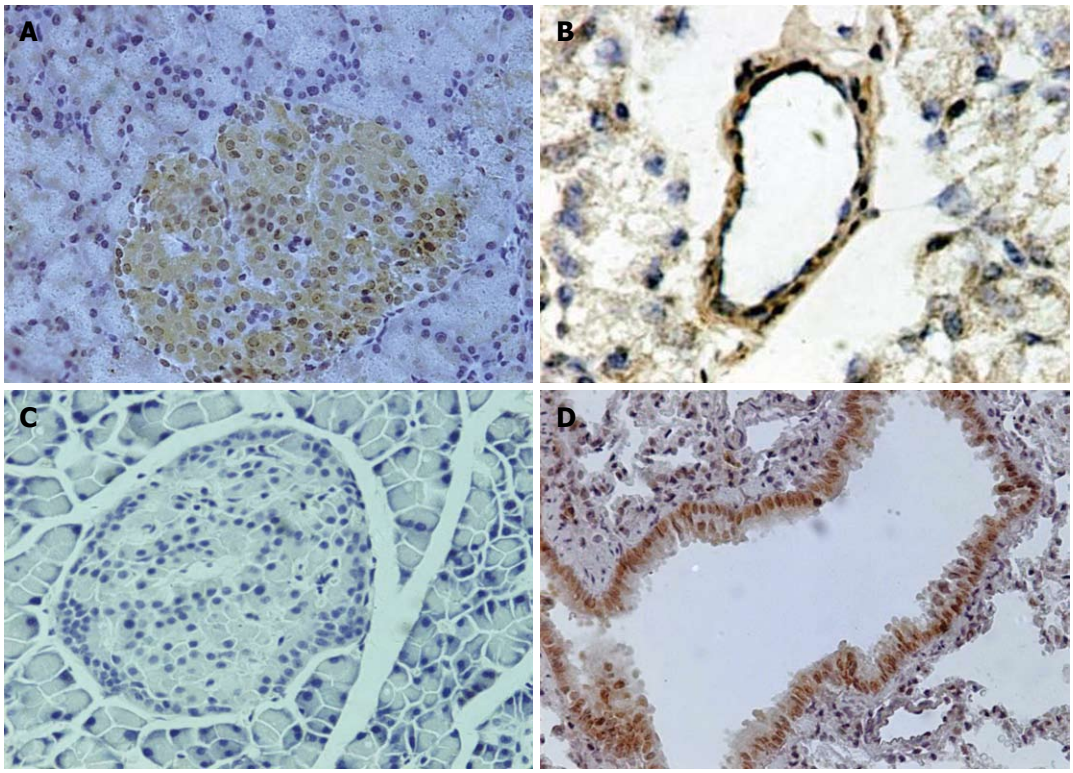

Figure 4 Location of platelet activating factor receptor in pancreatic and lung tissues in sham-operation group shown by immunohistochemistry (HE staining, × 200). A: Paraffin slice of pancreatic tissues; B: Frozen slice of pancreatic tissues; C: Negative control of pancreatic tissues; D: Positive control of lung tissues.

sPLA2 and cPLA2. sPLA2 activates a signaling cascade, including activation of cPLA2, arachidonic acid release, PAF production, and induction of COX-2[14]. PLA2 can decompose the membrane to release PAF. It is known that PAF is an initiative factor in the pathogenesis of SAP, and the cascade exacerbates the course of pancreatitis by activating the PLA2 in membrane. BN52021 antagonizes PAF by restraining the activation of PLA2 and this is the first report about the relation between BN52021 and PLA2 in SAP.

Quantity of ascites showed the severity of pathological changes of pancreas. When pancreas is involved in inflammation, hemorrhage and putrescence, it would irritate peritoneum and peritoneal permeability was increased, resulting in the release of ascites. PLA2 was closely correlated with the release of ascites[15]. The results indicated that BN52021 can reduce the PLA2 in plasma and decrease the quantity of ascites, and that BN52021 may interact defi-

nitely on other inflammatory factors to reduce peritoneal permeability.

The level or regulation of PAFR mRNA and PAFR protein has not been clearly studied. In our experiments, expression of PAFR in pancreas was up-regulated in the initial 3 h in SAP, but down-regulated after 6 h[16]. The reason for this may be in the first 3 h, pancreas could release more inflammatory factors to induce the up-regulation of PAFR, such as tumor necrosis factor α[17] and interferon-β, but cellular membrane was destroyed completely along with severe damages occurring in the pancreas after 6 h. In addition, protein kinase C excess activation was reported to decrease expression of PAFR[18], followed by a down-regulation of mRNA. BN52021 protected the membrane so that it maintained the quantity of PAFR. There was significant difference between SAP and BN52021 groups. The ratio of IOD of PAFR to β-actin was gradually improved in 2.5 and 10 mg/kg BN52021 groups, although there was no significant difference among BN52021 groups, and remarkable difference was found among SO group, 5 mg/kg SAP group and 5 mg/kg BN group.

It was firstly reported that PAFR was located in pancreas' islet, and it is important to study the mechanism of pancreas inflammation. BN52021 exerted its protective effects in patients with diabetes and pancreatitis, but the active mechanism is not clear. When inflammation in pancreas happened, PAFR was up-regulated, and the cells released more PAF, which damaged the pancreas. As BN52021 competitively inhibited the combination of PAF and PAFR and released less PAF, the pancreas was protected. It meant that BN52021 achieved its effects on SAP not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas.

In conclusion, 5 mg/kg BN52021 is the optimal dose in treating SAP rats, which can reduce the amylase and PLA_2 in serum and the quantity of ascites. It prevents the pancreas from severe damage induced by sodium taurocholate. Compared with Sandostatin, a classical drug for SAP, BN52021 exerts the best effects in treating SAP rats. This paper also showed that BN52021 achieves therapeutic effects in SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. Because of its hypotoxicity as an extract of *Ginkgo bibola*, it will be of significance in clinical application. More experiments are needed for the extensive application of BN52021 in clinic.

COMMENTS

Background

Platelet activating factor (PAF) is a family of phosphorylcholine esters with diverse potent physiological effects. It is closely associated with the cell membrane and is found in a variety of cells. Numerous cell types and tissues have been shown to synthesize and release PAF upon stimulation and at the same time to exhibit biological responses to it. Flickinger *et al* found the specific localization of PAF receptor (PAFR) in the pancreatic vascular endothelium, but not in other pancreatic cell types.

Research frontiers

Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP). It has been found that BN52021 possesses activity as antagonists of the action of PAF. However, there is no report on its optimal dosage. Although BN52021 has shown best effects on treating SAP, its mechanism has not been elucidated in details. This paper investigated the optimal dosage of BN52021 and its mechanism on SAP.

Innovations and breakthroughs

Recent reports have highlighted the importance of PAF in SAP and have shown that BN52021 has best effect on experimental SAP. This is the first study to report the optimal dosage of BN52021. The authors discovered that there is PAFR expression in the pancreas' islet, which has not been reported by others up to now. The *in vitro* studies suggest that this protein may be the cause of the repression in E-cadherin observed in this cancer.

Applications

By understanding how PAF is induced and by blocking its expression, this study may represent a future strategy for SAP treatment with BN52021.

Terminology

PAF is a biologically active phospholipid mediator, playing its role by binding to platelet activating factor receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways, including the nucleal PAFR. Ginkgolide B (code:BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of *Ginkgo biloba leavies*.

Peer review

The authors examined the level of serum amylase and PLA_2, quantity of ascites, and pathological scores, the expression of PAFR mRNA and its proteins in pancreatic tissues and location of PAFR in pancreatic and lung tissues. It revealed that PAFR expressed in the pancreas' islet and the best effect on SAP was shown in the 5 mg/kg group. This paper also showed that BN52021 achieves the therapeutic action on SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. The results are interesting and may represent a molecular mechanism of BN52021 on SAP.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2248

World J Gastroenterol 2011 May 7; 17(17): 2248-2254
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Specific HLA-DQB1 alleles associated with risk for development of hepatocellular carcinoma: A meta-analysis

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Supported by Shandong Provincial Natural Science Foundation, China, No. ZR2009CQ031
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Received: September 10, 2010 Revised: December 9, 2010
Accepted: December 16, 2010
Published online: May 7, 2011

Abstract

AIM: To evaluate the association of human leukocyte antigen (HLA)-DQB1 alleles with hepatocellular carcinoma (HCC) through meta-analysis of published data.

METHODS: Case-control studies on HLA-DQB1 allele association with HCC published up to January 2010 were included in the analyses. The odds ratios (ORs) of HLA-DQB1 allele distributions in HCC patients were analyzed and compared with healthy controls. The meta-analysis software REVMAN 5.0 was applied for investigating heterogeneity among individual studies and for summarizing all the studies. A meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Seven case-control studies containing 398 cases and 594 controls were included in the final analysis.

RESULTS: Among the five family alleles, two (DQB1*02 and DQB1*03) were found to be significantly associated with the risk of HCC. The combined OR for the association of DQB1*02 and DQB1*03 allele with the risk for HCC was 1.78 (95% CI: 1.05-3.03, P = 0.03) and 0.65 (95% CI: 0.48-0.89, P = 0.007), respectively. Among the 13 specific alleles, two (DQB1*0502 and DQB1*0602) were significantly associated with risk of HCC. The combined OR for the association of DQB1*0502 and DQB1*0602 allele with the risk for HCC was 1.82 (95% CI: 1.14-2.92, P = 0.01) and 0.58 (95% CI: 0.36-0.95, P = 0.03), respectively. No significant association was established for other HLA-DQB1 family alleles and specific alleles.

CONCLUSION: Our results support the hypothesis that specific HLA-DQB1 allele families and alleles might influence the susceptibility or resistance to HCC, although it needs further investigations.

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Key words: Hepatocellular carcinoma; Human leukocyte antigen-DQB1 alleles; Meta-analysis

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Xin YN, Lin ZH, Jiang XJ, Zhan SH, Dong QJ, Wang Q, Xuan SY. Specific HLA-DQB1 alleles associated with risk for development of hepatocellular carcinoma: A meta-analysis. *World J Gastroenterol* 2011; 17(17): 2248-2254 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2248.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2248

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most com-

mon cause of cancer-related deaths worldwide and about 600 000 patients died from the disease annually[1]. Nearly 78% of the 600 000 cases are from Asian countries[2]. China alone accounts for more than 50% of the world's cases[3]. The development of HCC is linked to the interaction of the genetic, immunologic, environmental, dietary, and lifestyle factors. Its incidence and distribution vary widely among ethnic groups, sex, and geographic regions. HBV and HCV infection, liver cirrhosis, male gender, and old age are important risk factors of HCC. The clustering of HCC within families raises the possibility that genetic factors are also involved in the susceptibility to HCC.

The major histocompatibility complex (MHC) plays a key role in anti-virus and tumor defense. Human leukocyte antigens (HLA) function in the regulation of immune response to foreign antigens and discrimination of self from non-self antigens. They are encoded by a series of closely linked genetic loci found on chromosome 6[4,5]. HLA polymorphism is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers. Given the pivotal role of HLA molecules in the immune system, several studies have investigated the association between specific HLA alleles and HCC. However, the association between HLA-DQB1 alleles and HCC in different ethnic populations is controversial. As many conflicting reports have been published to date, we conducted a systematic review of all the relevant studies published in the literature to evaluate the association between HLA-DQB1 alleles and HCC. Our principal objectives were to clarify the specific HLA-DQB1 allele families or alleles that are associated with the risk of HCC development.

MATERIALS AND METHODS

Literature search

Electronic databases (PubMed, EMBASE, Cochrane Library and China National Knowledge Infrastructure) were used to search for all genetic association studies evaluating the HLA-DQB1 polymorphism and HCC in humans in all languages up to January 2010. The search strategy was based on combinations of the key words, "HLA" or "human leukocyte antigen" and "hepatocellular carcinoma" or "HCC", and was not restricted by period. We also did a full manual search from the bibliographies of selected papers. In addition, we contacted the authors of studies containing relevant information, who did not report the results necessary for this analysis. Unpublished data were also accepted if an abstract was available and further information was obtained from the authors.

Inclusion and exclusion criteria

In the meta-analysis, the following inclusion criteria were set and reviewed by two independent investigators: (1) an independent case-control study; (2) studies with similar purpose and statistical methods; (3) studies providing enough information to calculate the odds ratio (OR); (4) HLA-DQB1 alleles were molecularly typed (high or low resolution level); and (5) the diagnosis of HCC was based on at least one of the following criteria: typical histological characteristics or serum α-fetoprotein (AFP) levels higher than 400 ng/mL together with radiological findings (ultrasound and CT) consistent with HCC.

The following exclusion criteria were set: (1) incomplete raw data; (2) repetitive reports (if more than one version of the same study was retrieved, only the most recent was used); and (3) materials and methods were not well-described and reliable.

Although assessment of study quality is considered important for systematic reviews and meta-analyses, scoring methods have been considered problematic[6] and may not accurately assess the quality measures of interest[7]. Therefore, we used reliability of patient selection, molecular typing method, and statistical analysis method as quality variables.

The frequency of HLA-DQB1 alleles varies according to ethnic and racial background, with some alleles being extremely rare. Therefore, articles were not required to identify all alleles for inclusion.

Data extraction

The studies were independently evaluated by two researchers (Xin YN and Lin ZH). Discrepancies in the evaluations of studies were resolved by discussion between the reviewers.

The following data were collected from each study: authors, publication year, journal, publication type and language, HLA genotyping method, allele genotype, number of cases and controls, definitions used for HCC, HCC sample description, control sample description (if there was more than one control group, we choose the healthy group as the control group in order to minimize the confounder). The main features of the trials included in the meta-analysis are shown in Table 1.

Statistical analysis

Homogeneity was calculated by Cochran's Q test ($\alpha = 0.05$). If the results of the Q test had no significant heterogeneity, the Mantel-Haenszel fixed-effect model (Peto method) was used for the combined data. If the results of the Q test had significant heterogeneity, the Dersimonian-Laird random-effects model (DL method) was used for the combination of data[8]. A pooled OR was presented as a standard plot with 95% CIs. In the absence of heterogeneity, the two methods provided identical results. As a measure of association between HCC and HLA-DQB1 alleles, we combined ORs with 95% CIs stratified by gene subtype of patients and controls in a study. Funnel plots and the Egger's regression asymmetry test were used to evaluate publication bias[9]. We performed a sensitivity analysis to assess the stability of the results by sequential omission of individual studies. All P values presented are two-tailed. The analyses were performed using Revman 5.0 provided by the Cochrane Collaboration Internet.

Table 1 Characteristics of studies included in the meta-analysis

Study	Country/ region	No. of HCC (M/F), age (yr)	No. of controls (M/F), age (yr)	No. of DQB1 alleles studied	Type of control	HLA genotyping method
Li *et al*[10], 1995	Hong Kong	49 (-/-), ND	78 (-/-), ND	11	Healthy	PCR-SSP
Donaldson *et al*[11], 2001	Hong Kong	84 (79/5), 55	124 (-/-), ND	10	Healthy	PCR-SSOP
De Re *et al*[12], 2004	Italy	29 (-/-), ND	144 (-/-), ND	5	Healthy	PCR-SSP
López-Vázquez *et al*[13], 2004	Spain	46 (27/19), 62 ± 8	48 (19/29), 56 ± 12	10	HCV carriers	PCR-SSOP
Liu *et al*[14], 2007	China	78 (63/15), 58.8 ± 11.5	100 (-/-), ND	19	Healthy	PCR-SSP
El-Chennawi *et al*[15], 2008	Egypt	50 (45/5), 51.16 ± 6.16	50 (44/6), 48.88 ± 9.22	5	Healthy	PCR-SSP
Pan[16], 2009	China	62 (52/10), 53.58	50 (29/21), 30.12	8	Healthy	PCR-SSP

HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; ND: Not described; PCR-SSOP: Polymerase chain reaction-sequence-specific oligonucleotides probes; PCR-SSP: Polymerase chain reaction-sequence specific primer; (-/-): No data was mentioned in the included studies.

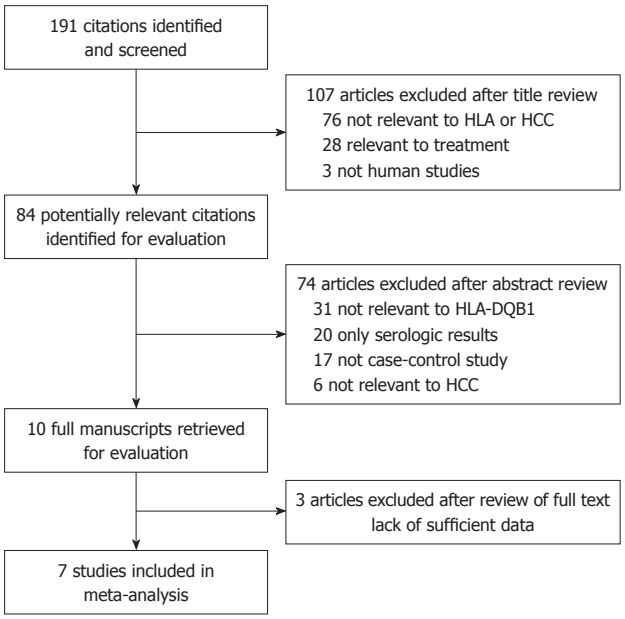

Figure 1 Flow diagram of study identification. HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen.

RESULTS

Literature assessment

Figure 1 shows the flow diagram of publications identified by the literature search. The search strategy allowed us to identify 60 studies for potential inclusion in the meta-analysis. Only 7 case-control studies relating to HLA-DQB1 alleles polymorphism and susceptibility to HCC qualified on the basis of our selection criteria[10-16]. A total of 991 subjects were studied (398 patients and 593 controls) (Table 1). Two of these studies were conducted in mainland China, two in Hong Kong, and one each in Italy, Egypt, and Spain. The diagnosis of HCC was based on at least one of the following criteria: typical histological characteristics or serum AFP levels higher than 400 ng/mL together with radiological findings (ultrasound and/or CT) consistent with HCC. The control group of six studies consisted of healthy individuals, while only one study used HCV carriers as the control group[13]. Records of mean or median age and sex were incomplete in 4/7 reports. HIV status was determined in two reports[12,15]. HLA-DQB1 alleles were considered only when they were classified in at least two independent studies.

HLA-DQB1 alleles were molecularly typed (high or low resolution level). Six studies used high resolution molecular typing for HLA, while the Italian study used both high and low resolution molecular typing for HLA. Low resolution molecular typing methods for HLA could not identify the specific alleles. Accurate methods for HLA class Ⅱ typing should involve the combination of polymerase chain reaction (PCR)-sequence-specific oligonucleotides probes, PCR-sequence specific primer and PCR-single strand conformation polymorphism[17].

Association between HLA-DQB1 alleles and HCC

A total of five HLA-DQB1 allele families and 13 specific alleles were extracted from the studies to investigate their association with HCC. Six specific alleles were excluded because each was identified in only one study.

Among the allele families, two (DQB1*02 and DQB 1*03) were significantly associated with risk of HCC. In the meta-analysis, the overall frequency of DQB1*02 was 32.9% (131/398) in HCC, and 24.5% (145/593) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was statistically significant (χ^2 = 15.28, P = 0.02 and < 0.05); the random-effect method was used to combine the results. The combined OR was 1.78 (95% CI: 1.05-3.03) and was statistically significant (P = 0.03 and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the studies by Li *et al*[10], López-Vázquez *et al*[13], Liu *et al*[14] and El-Chennawi *et al*[15], which produced a non-significant association. Overall, the frequency of DQB1*03 was 50.0% (199/398) in HCC and 62.7% (372/593) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant (χ^2 = 11.53, P = 0.07 and > 0.05). The fixed-effect method was used to combine the results. The combined OR was 0.65 (95% CI: 0.48-0.89) and was statistically significant (P = 0.007 and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by De Re *et al*[12], which produced a non-significant association. Statistics calculated for each study about DQB1*03 are shown in the forest plot (Figures 2 and 3). Meta-analysis for another 3 HLA-DQB1 allele

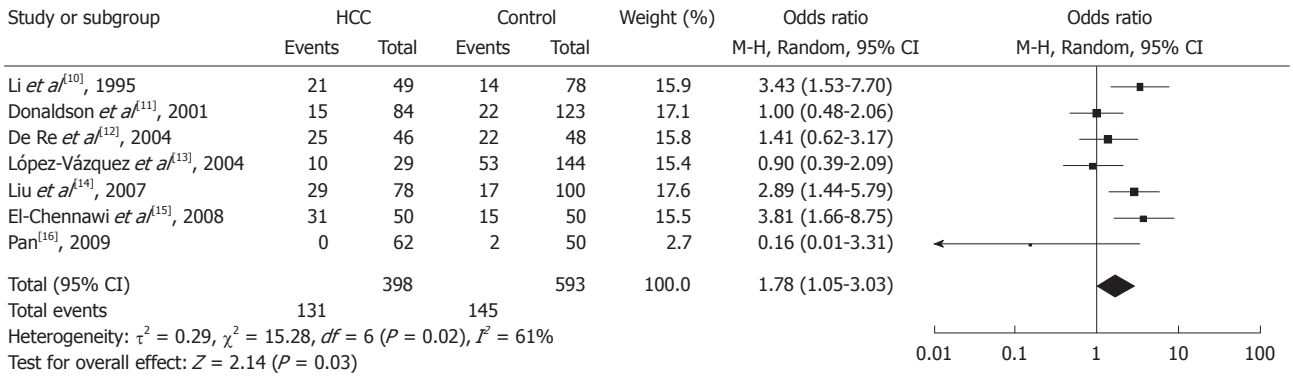

Figure 2 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*02 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

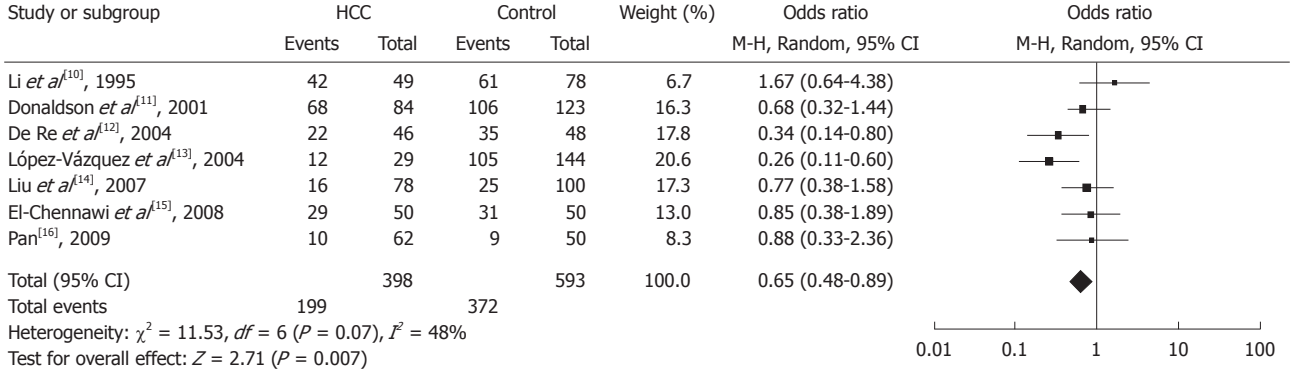

Figure 3 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*03 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

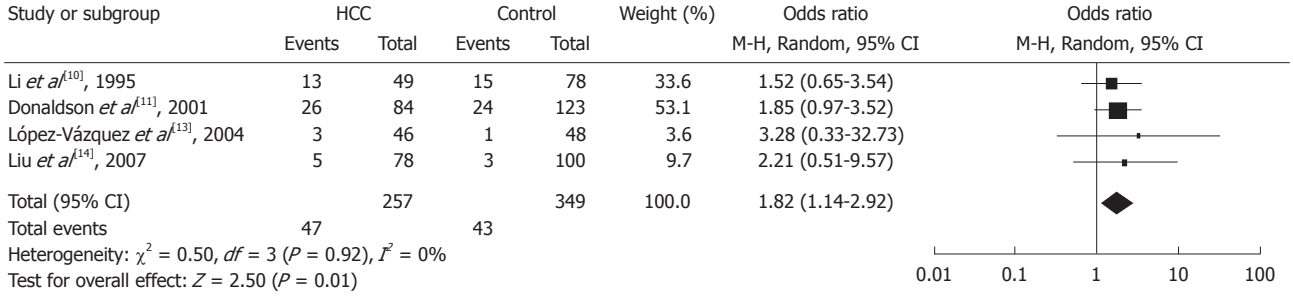

Figure 4 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*0502 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

families (DQB1*04, DQB1*05 and DQB1*06) was carried out, but did not show any statistical effect.

Among the specific alleles, two (DQB1*0502 and DQB1*0602) were significantly associated with risk of HCC. In the meta-analysis, the overall frequency of HLA-DQB1*0502 allele was 18.3% (47/257) in HCC, and 12.3% (43/349) in the controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant (χ^2 = 0.50, P = 0.92 and > 0.05); the fixed-effect method was used to combine the results. The combined OR was 1.82 (95% CI: 1.14-2.92), and was statistically significant (P = 0.01 and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by Donaldson *et al*[11], which produced a non-significant association. Overall, the frequency of HLA- DQB1*0602 allele was 23.7% (41/173) in HCC and 31.4% (71/226) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant (χ^2 = 1.26, P = 0.53 and > 0.05); the fixed-effect method was used to combine the results. The combined OR was 0.58 (95% CI: 0.36-0.95) and was statistically significant (P = 0.03 and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by Liu *et al*[14], which produced a non-significant association. Statistics calculated for each study of DQB1*0502 and DQB1*0602 are shown in the forest plot (Figures 4 and 5). Meta-analysis for another 11 HLA-DQB1 specific alleles

Study or subgroup	HCC		Control		Weight (%)	Odds ratio	Odds ratio
	Events	Total	Events	Total		M-H, Random, 95% CI	M-H, Random, 95% CI
Li *et al*[10], 1995	1	49	6	78	10.6	0.25 (0.03-2.14)	
López-Vázquez *et al*[13], 2004	11	46	13	48	22.6	0.85 (0.33-2.14)	
Liu *et al*[14], 2007	29	78	52	100	66.8	0.55 (0.30-1.00)	
Total (95% CI)		173		226	100.0	0.58 (0.36-0.95)	
Total events	41		71				

Heterogeneity: $\chi^2 = 1.26$, $df = 2$ ($P = 0.53$), $I^2 = 0\%$

Test for overall effect: $Z = 2.17$ ($P = 0.03$)

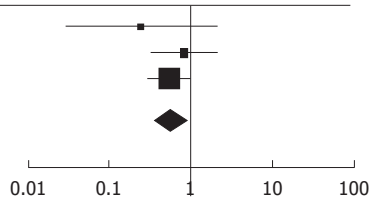

Figure 5 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*0602 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

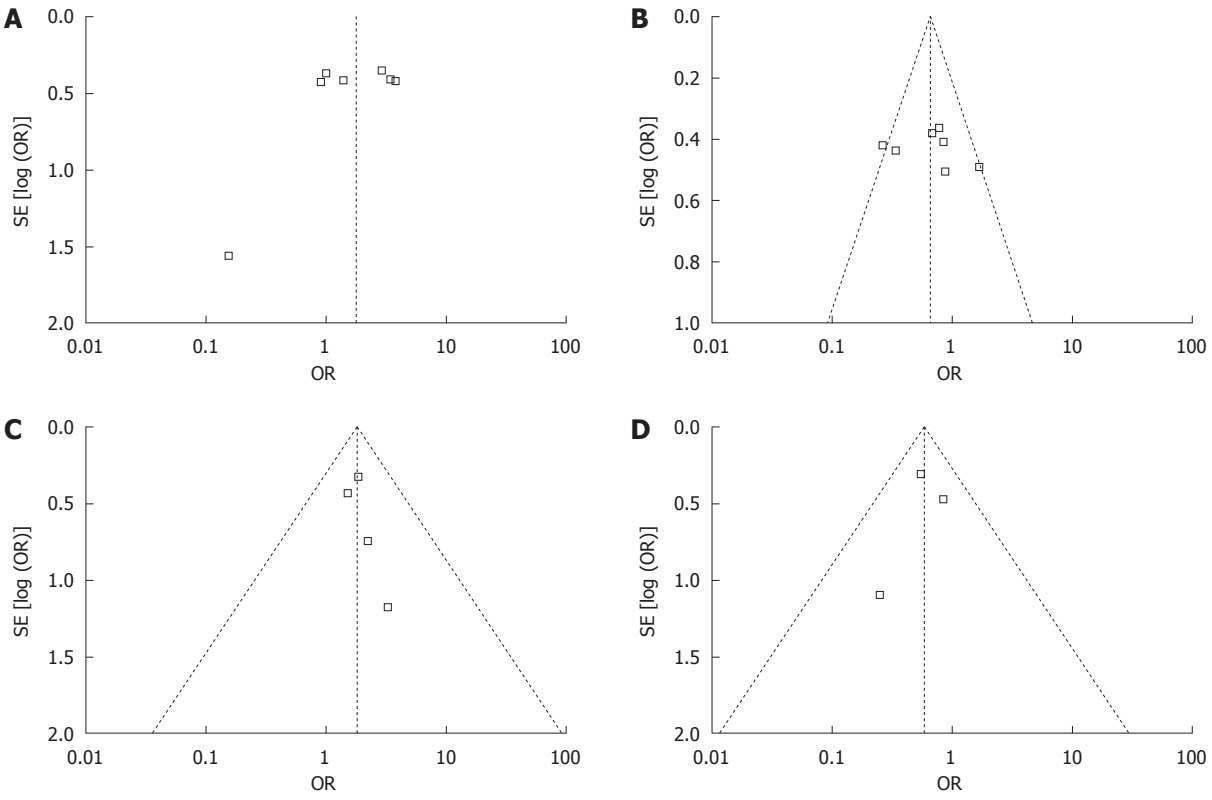

Figure 6 The funnel plot to detect publication bias of each study for DQB1*02, DQB1*03, DQB1*0502 and DQB1*0602. Each point represents a separate study for the indicated association. A: Funnel plot analysis of human leukocyte antigen (HLA)-DQB1*02 allele and hepatocellular carcinoma (HCC) to detect publication bias ; B: Funnel plot analysis of HLA-DQB1*03 allele and HCC to detect publication bias; C: Funnel plot analysis of HLA-DQB1*0502 allele and HCC to detect publication bias; D: Funnel plot analysis of HLA-DQB1*0602 allele and HCC to detect publication bias.

(DQB1*0201, DQB1*0301, DQB1*0302, DQB1*0303, DQB1*0401, DQB1*0402, DQB1*0501, DQB1*0503, DQB1*0601, DQB1*0603 and DQB1*0604) was carried out, but did not show any statistical relationships.

Our meta-analysis of seven studies revealed that DQB1*02 and DQB1*0502 were risk factors for HCC, while DQB1*03 and DQB1*0602 were protective factors. These results suggest that patients with DQB1*02 and DQB1*0502 alleles were at a higher risk of developing HCC than those with DQB1*03 and DQB1*0602 alleles. These analyses were based on the data from seven studies irrespective of the ethnicity of the study populations. The shape of the funnel plot to detect publication bias of each study for DQB1*02, DQB1*03, DQB1*0502 and DQB1*0602 seemed to be asymmetrical (Figure 6), suggesting that publication bias might affect the findings of our meta-analysis.

DISCUSSION

To our knowledge, this is the first published meta-analysis to comprehensively investigate the association between HLA-DQB1 alleles and HCC. Numerous studies have reported associations of HLA with malignant tumors, such as thyroid cancer, renal cell cancer, Burkett's lymphoma, Kaposi's sarcoma, Hodgkin's disease, nasopharyngeal carcinoma, cervical carcinoma, esophageal carcinoma, gastric cancer, and HCC. However, most of these studies focused on the association between HLA antigen and cancer based on HLA serotyping which has limited reso-

lution, and may be inaccurate for the assignment of many HLA antigens. With the development of DNA-based methods for HLA genotyping, HLA genotyping has become more accurate in the determination of the HLA-DQB1 alleles, and more precise than serotyping in the determination of the peptide-binding domain of MHC class Ⅱ molecules. Hence, HLA genotyping is being used more frequently in the field of immunogenetics.

Recent studies on the association between HLA-DQB1 allele polymorphisms and HCC have been inconclusive. Pan[16] reported that some HLA-DQB1 alleles are significantly associated with HCC (DRB1*04 and DQB1*02), while others (DQB1*06) are not. Therefore, it is possible that the DRB1*04 and DQB1*02 alleles might be risk factors for the occurrence of HCC (OR = 4.373 and 3.807, respectively), and DQB1*06 may be a protective allele (OR = 0.259). Lopez-Vazquez *et al*[13] confirmed that HLA-DQB1*0301 carriers rarely develop end-stage liver disease (ESLD). This allele may, therefore, be considered to be a good prognostic factor. De Re *et al*[12] have shown that the DQB1*0301 allele played a major protective role in patients with HCV-associated HCC. Donaldson *et al*[11] observed that the alleles DRB1*1501, DQA1*0302 and to a lesser extent DQB1*0302 appeared to confer resistance to HCC.

The current meta-analysis indicates that specific HLA-DQB1 alleles are associated with HCC. Among the 5 family alleles, DQB1*02 and DQB1*03 were significantly associated with risk of HCC. The combined OR was 1.78 (95% CI: 1.05-3.03) and 0.65 (95% CI: 0.48-0.89), respectively. Among the 13 specific alleles, DQB1*0502 and DQB1*0602 were significantly associated with risk of HCC. The combined OR was 1.82 (95% CI: 1.14-2.92) and 0.58 (95% CI: 0.36-0.95), respectively. These results suggest that DQB1*02 and DQB1*0502 alleles may have a higher risk for HCC, while DQB1*03 and DQB1*0602 alleles may have a protective effect for HCC.

The DQB1*02 allele family is negatively associated with the incidence of breast cancer in the Tunisian population[18], while the DQB1*03 allele family is associated with susceptibility to cervical carcinoma in Europe (OR = 3.03)[19], cutaneous T-cell lymphoma in USA (OR = 2.7)[20], and HCV-related non-Hodgkin's lymphoma in Italy (OR = 0.23)[12]. The DQB1*0502 allele is also associated with the susceptibility to Sezary syndrome of cutaneous T-cell lymphoma in USA (OR = 7.75)[20], while the DQB1*0602 allele is associated with an increased gastric cancer risk in Taiwan (OR = 2.79)[21] and increased distal gastric cancer risk in southern European population (OR = 4.82)[22]. DQB1*0602 also appears to be a candidate protective allele for testicular germ cell carcinoma in the Japanese population (RR = 0.26, $P = 0.02$)[23]. The mechanisms for these effects are unknown, but larger-scale studies may confirm this observation.

Human tumor cells express diverse types of antigens, depending on the etiology and pathogenesis of the disease[24]. Because tumor development is preceded by chronic inflammation, immune responses, whether towards the infectious agent itself or against tumor antigens, may be critical for development of tumor. Different types of HLA molecules have different capability of binding and presenting tumor antigens. The association of specific HLA alleles with susceptibility or resistance to malignant tumors is probably attributable to a direct involvement of the HLA molecules as an antigen presenter or possibly due to a neighboring linked gene[14].

Several other points should be considered when interpreting the results of our study. Most studies did not control for the matching variables in the analysis, and the risk for HCC was not controlled for possible confounders such as HBV or HCV. These observational studies are more prone to bias than randomized clinical trial (RCT) studies. The shape of the funnel plot seemed to be asymmetrical, suggesting that publication bias might affect the findings of our meta-analysis.

In conclusion, this meta-analysis reveals that DQB 1*02 and DQB1*0502 are risk factors for HCC, while DQB1*03 and DQB1*0602 are protective factors. These findings are consistent with the previous studies about the association of HLA alleles and other cancers. In the present study, no attempt has been made to account for the potential confounding influence of cirrhosis in the development of HCC, although future studies of HLA should take this into consideration.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide and about 600 000 patients died from the disease annually. Human leukocyte antigen (HLA) polymorphism is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers.

Research frontiers

The clustering of HCC within families raises the possibility that genetic factors are involved in susceptibility to HCC. Much attention has been paid to the potential role of HLA in the pathogenesis of HCC in an attempt to uncover the underlying mechanisms. However, the relationship between HLA-DQB1 and HCC remains controversial and no meta-analysis has been conducted.

Innovations and breakthroughs

This is the first meta-analysis which systemically studied the relationship between HLA-DQB1 and HCC susceptibility, and suggested that specific HLA-DQB1 allele families and alleles might influence the susceptibility or resistance to HCC, which needs further investigations.

Applications

The study concludes that DQB1*02 and DQB1*0502 are risk factors for HCC, while DQB1*03 and DQB1*0602 are protective factors, which provides an insight into the role of HLA-DQB1 in the pathogenesis of HCC. Furthermore, these findings are meaningful to early diagnosis and provide a new strategic approach to the prevention of HCC.

Terminology

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis.

Peer review

This is a very interesting meta-analytic study dealing with an important topic in HCC. The described analysis has been performed with precision and accuracy.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i17.2255

World J Gastroenterol 2011 May 7; 17(17): 2255-2258
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Neoadjuvant sorafenib combined with gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma

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Received: November 2, 2010 Revised: January 18, 2011
Accepted: January 25, 2011
Published online: May 7, 2011

Abstract

This paper reports the first case of a patient with hepatocellular carcinoma with lymph node metastasis treated by sorafenib combined with gemcitabine plus oxaliplatin, with a partial response and normalization of α fetoprotein, which allowed curative surgery. The potential synergy between these three drugs needs to be confirmed, and is currently being investigated in a randomized phase Ⅱ trial.

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Key words: Hepatocellular carcinoma; Gemcitabine; Oxaliplatin; Sorafenib; Neoadjuvant therapy

Peer reviewer: Fernando J Corrales, Associate Professor of Biochemistry, Division of Hepatology and Gene Therapy, Proteomics Laboratory, CIMA, University of Navarra, Avd. Pío XII, 55, Pamplona, 31008, Spain

Williet N, Dubreuil O, Boussaha T, Trouilloud I, Landi B, Housset M, Botti M, Rougier P, Belghiti J, Taieb J. Neoadjuvant sorafenib combined with gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(17): 2255-2258 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i17/2255.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i17.2255

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality[1]. Underlying cirrhosis is present in 90% of cases. Its prognosis remains poor with lots of advanced forms, and there is no curative therapeutic approach.

Since 2007, sorafenib has been the standard treatment for first-line therapy of advanced or metastatic disease that is not eligible for intra-arterial chemo-embolization. It has been shown to significantly improve progression-free and overall survival[2]. However, sorafenib is not able to induce any objective response and tumor shrinkage that allows secondary surgery. Its role in neoadjuvant therapy has not been established either.

Although HCC has been documented as a chemoresistant tumor, some chemotherapeutic regimens, such as gemcitabine plus oxaliplatin, have shown some interesting efficacy results, with objective response rates around 20% and disease control rates > 50% in phase Ⅱ trials[3-5]. Here, we report what is believed to be the first case of a patient with advanced HCC who received sorafenib together with gemcitabine plus oxaliplatin, which allowed secondary curative surgery.

CASE REPORT

A 61-year-old man, working as a radiologist, was admitted

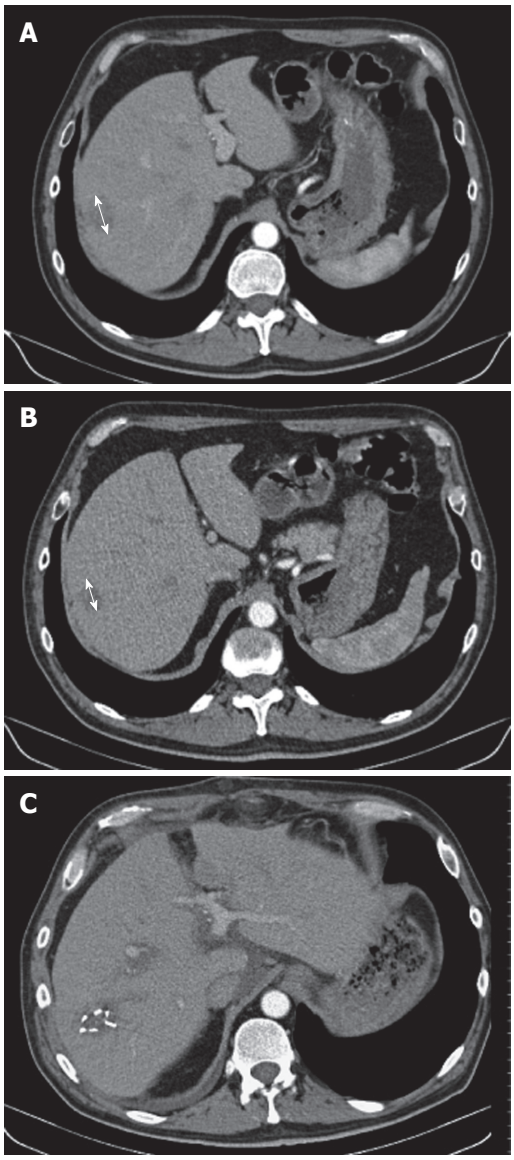

Figure 1 Computed tomography scan. A: A 4.5-cm hypervascular hepatic tumor (arrow) that occupied the sixth hepatic segment, without signs of underlying cirrhosis; B: After eight cycles of gemcitabine and oxaliplatin combined with sorafenib, we observed tumor shrinkage of > 40% (arrow), as compared to baseline assessment; C: Secondary surgery was performed: hemostatic clips were still visible on this postoperative CT scan.

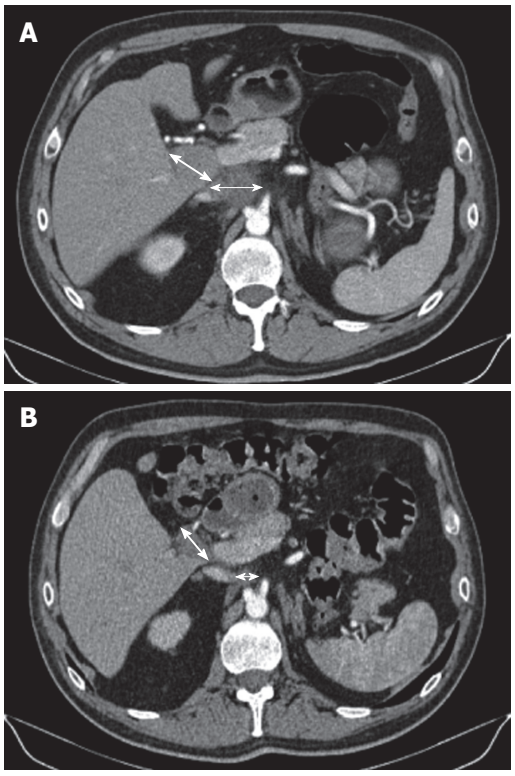

Figure 2 Computed tomography scan. A: Initially, there were two metastatic lymph nodes (MLNs): one celiac (thin arrow) measuring 4 cm and one measuring 3 cm located in the liver hilum (thick arrow); B: Computed tomography scan taken after the end of the eight cycles of gemcitabine plus oxaliplatin combined with sorafenib, showed that the MLNs (thin arrow for celiac node and thick arrow for hilar node) decreased in size dramatically with a more necrotic aspect, which allowed secondary surgery followed by radiotherapy.

for a liver mass that was detected by ultrasonography that was performed for abdominal pain, in December 2009. He had a history of cholecystectomy and post-transfusional hepatitis C, which was treated effectively by interferon and ribavirin therapy in 1998. Clinically, the ECOG performance status was 0, the patient weighed 94 kg, his height was 188 cm, and he had no symptoms except for moderate abdominal pain that was controlled with class Ⅱ analgesics. In January 2010, Abdominal computed tomography (CT) showed a 4.5-cm hypervascular hepatic tumor that occupied the sixth hepatic segment, without signs of underlying cirrhosis (Figure 1A). There were also two metastatic lymph nodes (MLNs): one celiac measuring 4 cm, and one measuring 3 cm located in the liver hilum (Figure 2A). Laboratory data showed normal blood cell and platelet counts, liver enzymes, bilirubin and albumin levels. α-fetoprotein (AFP) was markedly increased at 2572 ng/mL (upper limit of normal = 8). Esophagogastroduodenoscopy did not show gastroesophageal varices or other upper gastrointestinal tract abnormalities.

Fine needle echoendoscopic aspiration from celiac lymph nodes was performed and showed a typical trabecular liver carcinoma. Evaluation by a multidisciplinary team concluded that the primary tumor was theoretically resectable in this patient, who had an excellent performance status, without underlying cirrhosis, and who wished an aggressive therapeutic approach. However, because there were MLNs, initial surgery was rejected and intensive systemic therapy with sorafenib combined with gemcitabine plus oxaliplatin was initiated. He received four cycles of the GEMOX regimen (gemcitabine 1 g/m^2 on day 1 and oxaliplatin 100 mg/m^2 on day 2), as previously described[3]. This treatment was repeated every 2 wk and sorafenib was taken every day (400 mg twice daily). No grade 3/4 toxicities were observed. Grade 1 diarrhea, thrombopenia and neurotoxicity were reported, together with grade 2 asthenia after the fourth cycle. Efficacy assessment showed a decrease of AFP from 2572 to 97 ng/mL. CT scan showed tumor shrinkage of 25% in the liver (Figure 1B), and a slight decrease in MLN size, which were more necrotic (Figure 2B). A new multidisciplinary

evaluation was performed and it was decided to continue the same therapeutic schedule for more cycles. Second efficacy evaluation showed normalization of AFP levels (2.7 ng/mL) and tumor volume reduction of around 40% (Figure 2A), as compared to baseline assessment.

Surgery was then decided upon, which consisted of removing the primary tumor, together with satellite adenopathies. The sites were marked by surgical clips (Figure 1C) to allow for postoperative radiotherapy (RT).

Pathological examination of the specimens showed a well-differentiated HCC that measured 2.5 cm, without underlying cirrhosis (METAVIR score A1F2). All resection margins were negative, and only one lymph node of five removed was metastatic, which was located in the liver hilum and measured 2 cm, with a necrotic center. There were no postoperative complications except for exacerbation of oxaliplatin-induced neurosensory toxicity, as previously described[6,7]. Global status of the patient improved and postoperative RT of MLNs was performed, by delivering 46 Gy over 5 wk, without any side effects. Complementary adjuvant chemotherapy, with combined sorafenib and gemcitabine for 3 wk in four, was started for 3 mo, and is currently ongoing. Oxaliplatin was not reintroduced because of persistent grade 2 neurosensory toxicity.

DISCUSSION

The role of chemotherapy in HCC patients is still a matter of debate. There is no clear evidence for efficacy of any tested schedule in the adjuvant (or neoadjuvant) setting, as reviewed by a recent Cochrane study[8]. However, many oncologists are using systemic chemotherapy for their HCC patients in a palliative setting. Doxorubicin, which was considered as potential standard chemotherapy 20 years ago, seems to be toxic and does not convincingly improve survival over that with supportive care[9]. Recent studies have suggested that oxaliplatin and gemcitabine could be interesting agents, alone or in combination, even if there has been no phase Ⅲ trial to confirm their efficacy. The choice of gemcitabine-oxaliplatin combinations in this disease is based on: (1) synergy between the two drugs and their sequence dependency (gemcitabine followed by oxaliplatin is most active in preclinical models)[10]; (2) clinical activity of gemcitabine alone and of 5-fluorouracil/oxaliplatin combination in patients with HCC in phase Ⅱ trials[11,12]; and (3) lack of renal and hepatic oxaliplatin toxicity in cirrhotic patients, unlike doxorubicin. The GEMOX combination has thus been evaluated in many phase Ⅱ studies with promising results in term of response rate and survival[3-5,13]. Patients with complete response have been described by ourselves and others[14].

In the pivotal SHARP trial, response rate with sorafenib was particularly low (2.3%) and not significantly different from placebo[2]. To the best of our knowledge, only one patient receiving sorafenib and eligible for secondary surgery has been reported in the literature[15]. Sorafenib does not seem to be a candidate drug for tumor downstaging that allows secondary surgery. However, the antiangiogenic effect of sorafenib seems to modify HCC vascularization, by normalizing tumor vessels[16]. This vascular normalization has been described as potentially favoring chemotherapy efficacy through better delivery of the drugs to the tumor site[17]. Combination of sorafenib and GEMOX could thus lead to synergistic antitumor activity.

The toxicity of the GEMOX regimen has been well described over the past 10 years through 31 phase Ⅱ and two phase Ⅲ trials, mainly in pancreatic and biliary tract cancers. Grade 3 neutropenia, thrombocytopenia and neurotoxicity have been reported in 22%, 11% and 10%, respectively, and severe nausea has occurred in 16% of the cases[18]. These results are similar to those described in the phase Ⅱ trials that have evaluated this combination in HCC patients with underlying cirrhosis. The elevated liver enzymes and anaphylactic reactions (due to gemcitabine and oxaliplatin, respectively) did not seem more frequent in this population.

In the SHARP trial, diarrhea (grade 2 in 50%, grade 3 in 10%), weight loss, fatigue, hand-foot skin reaction (grade 2 in 30%, grade 3 in 8%), and hypophosphatemia were the most frequent toxicities associated with sorafenib. To date, no trial has assessed GEMOX plus sorafenib combination therapy.

Adjuvant RT after resection of MLNs from HCC is not common. To the best of our knowledge, only two Asian cases have been described in the literature[19,20]. However, three retrospective studies have demonstrated its role in local control of MLNs from HCC that is not eligible for surgery, but is present in patients with good performance status. RT doses range from 40 to 60 Gy, and could prolong overall survival[21-23]. For example, in Zeng's retrospective study on 125 patients, partial and complete responses were observed in 37.1% and 59.7% of patients, respectively. The median survival was 9.4 mo[23]. However, these results need to be confirmed. To avoid unnecessary toxicity, the RT dose chosen in the present case did not exceed 50 Gy[21].

In conclusion, this case is an example of successful multimodal aggressive treatment for advanced HCC, which combined for the first time, GEMOX and sorafenib followed by surgery and adjuvant radiotherapy and chemotherapy. GEMOX + sorafenib combination therapy was well tolerated with only grade 1/2 toxicity that was easily manageable, and gave an objective response, together with a dramatic decrease in AFP levels. These promising results need to be confirmed and GEMOX/sorafenib combination therapy is currently being evaluated in an ongoing randomized phase Ⅱ trial and compared to sorafenib alone.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 May 7; 17(17): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2011 May 7; 17(17): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, 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 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
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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
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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

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2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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World J Gastroenterol 2011 May 7; 17(17): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ISSN and EISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

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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.0000035706.28494.09]

Both personal authors and an organization as author

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]

No author given

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]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary 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

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

Conference paper

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

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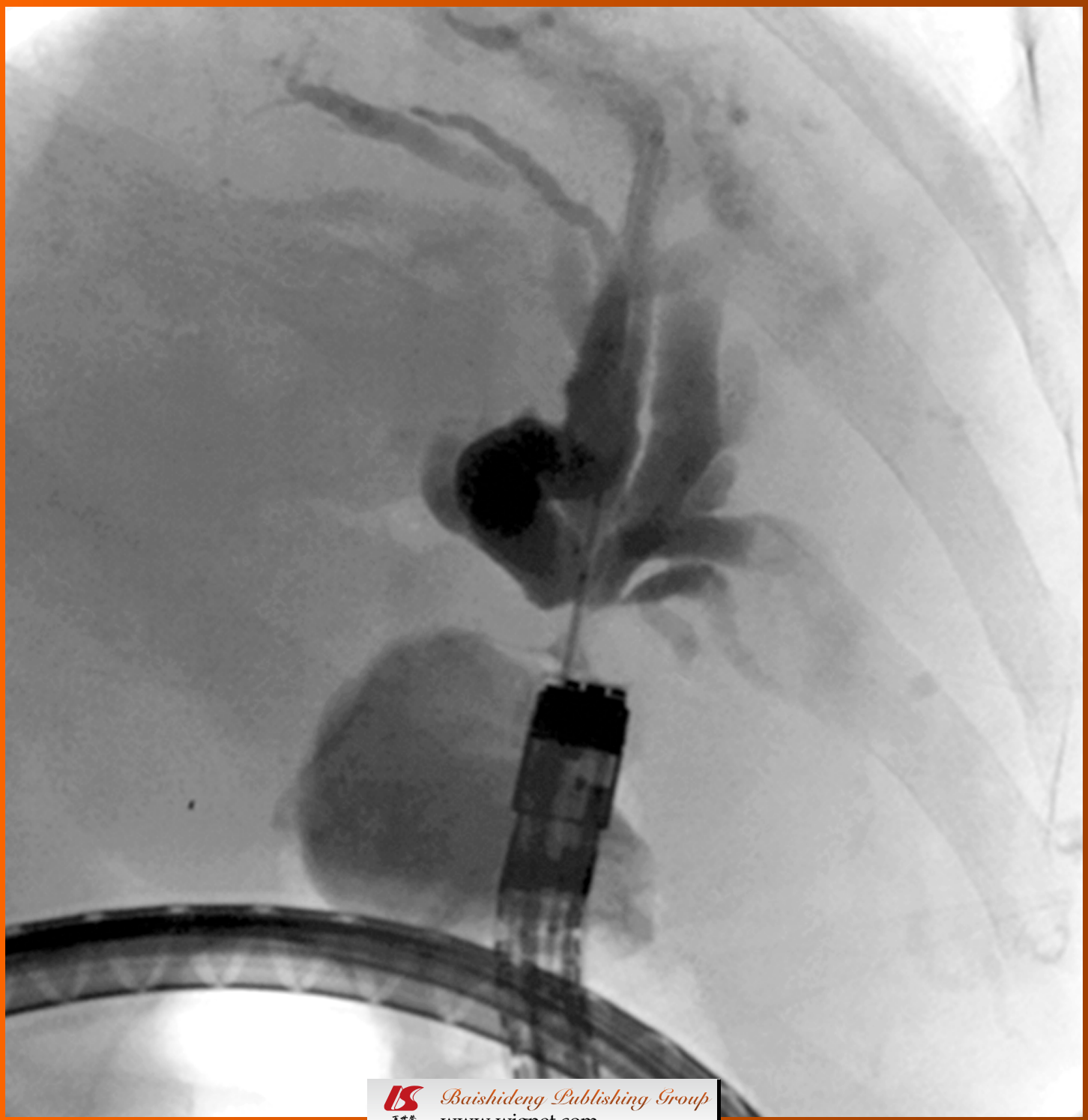
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2011 May 14; 17(18): 2259-2356



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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 18 May 14, 2011

EDITORIAL

2259 Recent advances in celiac disease
Freeman HJ, Chopra A, Clandinin MT, Thomson ABR

2273 Management of liver cirrhosis between primary care and specialists
Grattagliano I, Ubaldi E, Bonfrate L, Portincasa P

TOPIC HIGHLIGHT

2283 Gastroenterology training in Latin America
Cohen H, Saenz R, de Almeida Troncon LE, Lizarzabal M, Olano C

REVIEW

2288 Silybin and the liver: From basic research to clinical practice
Loguercio C, Festi D

ORIGINAL ARTICLE

2302 Double-balloon-enteroscopy-based endoscopic retrograde cholangiopancreatography in post-surgical patients
Raithel M, Dormann H, Naegel A, Boxberger F, Hahn EG, Neurath MF, Maiss J

2315 (-)-Epigallocatechin-3-gallate inhibits VEGF expression induced by IL-6 *via* Stat3 in gastric cancer
Zhu BH, Chen HY, Zhan WH, Wang CY, Cai SR, Wang Z, Zhang CH, He YL

2326 LBP and CD14 polymorphisms correlate with increased colorectal carcinoma risk in Han Chinese
Chen R, Luo FK, Wang YL, Tang JL, Liu YS

BRIEF ARTICLE

2332 Utility of pancreatography for diagnosing autoimmune pancreatitis
Takuma K, Kamisawa T, Tabata T, Inaba Y, Egawa N, Igarashi Y

2338 Prospective randomized controlled trial investigating the type of sutures used during hepatectomy
Harimoto N, Shirabe K, Abe T, Yukaya T, Tsujita E, Gion T, Kajiyama K, Nagaie T

2343 XPD Lys751Gln polymorphism and esophageal cancer risk: A meta-analysis involving 2288 cases and 4096 controls
Yuan L, Cui D, Zhao EJ, Jia CZ, Wang LD, Lu WQ

Contents

World Journal of Gastroenterology
Volume 17 Number 18 May 14, 2011

CASE REPORT

2349 Lapatinib-induced hepatitis: A case report
Peroukides S, Makatsoris T, Koutras A, Tsamandas A, Onyenadum A, Labropoulou-Karatza C, Kalofonos H

2353 Transarterial injection of H101 in combination with chemoembolization overcomes recurrent hepatocellular carcinoma
He Q, Liu Y, Zou Q, Guan YS

LETTERS TO THE EDITOR

2356 Prophylactic antibiotics for variceal hemorrhage: Clostridium difficile infection still can be a risk
Okano N, Iwata K

World Journal of Gastroenterology
Volume 17 Number 18 May 14, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Raithel M, Dormann H, Naegel A, Boxberger F, Hahn EG, Neurath MF, Maiss J. Double-balloon-enteroscopy-based endoscopic retrograde cholangiopancreaticography in post-surgical patients. *World J Gastroenterol* 2011; 17(18): 2302-2314 http://www.wjgnet.com/1007-9327/full/v17/i18/2302.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

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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
http://www.wjgnet.com

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.

PUBLICATION DATE
May 14, 2011

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

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SPECIAL STATEMENT
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2259

World J Gastroenterol 2011 May 14; 17(18): 2259-2272
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Recent advances in celiac disease

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Received: January 6, 2011 Revised: February 12, 2011
Accepted: February 19, 2011
Published online: May 14, 2011

Abstract

Celiac disease now affects about one person in a hundred in Europe and North America. In this review, we consider a number of important and exciting recent developments, such as clinical associations, HLA-DQ2 and HLA-DQ8 predispositions, the concept of potential celiac disease, the use of new imaging/endoscopy techniques, and the development of refractory disease. This review will be of use to all internists, pediatricians and gastroenterologists.

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Key words: Inflammation; Infection; Malabsorption; Pathophysiology; Physiology

Peer reviewers: Chew Thean Soon, (JOSH), BMedSci (Hons), MBChB (Hons), MRCP(UK), University of Manchester, 805 The Lock Building, 41 Whitworth Street, Manchester M1 5BE, United Kingdom; Rasmus Goll, MD, PhD, Department of Gastroenterology, Clinic of Internal Medicine, University Hospital of North Norway, Sykehusveien, Tromso, N-9038, Norway

Freeman HJ, Chopra A, Clandinin MT, Thomson ABR. Recent advances in celiac disease. *World J Gastroenterol* 2011; 17(18): 2259-2272 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2259.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2259

INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy characterized by intolerance to gluten. CD is usually characterized by various gastrointestinal (GI) symptoms (e.g. diarrhea, malabsorption, weight loss) associated with consumption of grains containing gluten (wheat, barley, rye). Although some CD patients may have primarily GI symptoms, CD may be detected due to associated extraintestinal disorders, even without GI symptoms, or due to screening for CD based on a positive family history. CD has a strong association with HLA-DQ2 and HLA-DQ8. Serological testing for antibodies to tissue transglutaminase (tTG) is usually positive (-95%) in the untreated patient. Endoscopic and histological damage seen in the proximal intestine is characteristic, but not diagnostic. As CD is defined to be a gluten-sensitive enteropathy, definitive diagnosis ultimately depends on a positive small bowel biopsy and demonstration of a response to a gluten-free diet (GFD)[1]. Common serological changes include the appearance of anti-tTG and other antibodies, e.g. endomysial antibodies (EMA). These antibodies have been reported in some, but not all studies, to decline or disappear in association with a clinical and/or histological response to a gluten-free diet.

The clinical spectrum of CD includes patients with classical gastrointestinal symptoms (e.g. diarrhea and weight loss), those who are detected on screening because of a family history of CD or having a CD-associated autoimmune condition, or those who have a predisposition for developing CD but at a particular time of testing, conceivably could have no symptoms, negative CD serology and a histologically normal small bowel biopsy. Now, a CD-specific quality of life instrument has also been developed and validated psychometrically[2], and may prove useful in everyday clinical practice.

EPIDEMIOLOGY

CD is highly prevalent in Caucasian populations and their descendants. The age of clinical onset (based on diagnosis) is often described by some experts as bimodal: the first peak is at 8 to 12 mo of age, and the second during the third to fourth decades of life[3]. Recent studies suggest, however, that CD should be considered as a disorder that has a risk of developing throughout life, even in the elderly. Overall, CD is twice as frequent among females as compared to males, possibly because the necessary HLA haplotypes, DQ2/DQ8, are more frequent in female than in male CD patients (i.e. 94% *vs* 85%)[4]. In addition, CD may be detected more frequently in females because females tend to seek medical care more often than males, usually at a younger age. With aging, however, this female predominant pattern disappears. In the elderly, the ratio of newly diagnosed males is equivalent to newly diagnosed females. In the few DQ2/DQ8 -negative CD patients, there is a male excess, and only inheritance of a paternal DQ2 haplotype leads to a daughter's predominance[4].

While it is estimated that CD affects up to 2% of Caucasians, the risk is higher in first-degree relatives of affected sibling pairs (17%), monozygotic twins (75%), and HLA-identical siblings (40%). Indeed, the single most important risk factor for celiac disease is having a first-degree relative with already-defined CD, particularly a sibling[5]. The estimated prevalence of CD in first-degree relatives living in Minneapolis, USA is 11% of all affected family members who carried at-risk genotypes (HLA-DQ2 in more than 90% of cases, and the remainder carrying HLA-DQ8). About half of these persons have clinically "silent disease", and yet, their small bowel biopsies may show severe architectural changes[6]. This apparent disconnection in some patients between severity of symptoms with CD and the severity of histological abnormalities (typically defined in the proximal small bowel) may reflect the variable extent of histological involvement further along the length of the small intestine.

The prevalence of CD appears to be increasing, particularly as the population ages[7]. CD in the elderly has been reviewed and also likely reflects increased recognition of undiagnosed CD in this age group[8-10]. The estimated prevalence of CD in individuals in the United Kingdom between 45 to 76 years of age is approximately 1.2%. About 20% of all newly diagnosed celiac cases are over 60 years of age. The clinical presentation in these older individuals is variable, ranging from "silent" disease, to vague abdominal complaints, to anemia. These limited symptoms could also lead to a delay in diagnosis.

Prolonged gluten exposure in undiagnosed CD is seen to increase the incidence of autoimmune diseases, such as diabetes and autoimmune thyroiditis. As far as gastrointestinal complications are concerned, it is important to rule out collagenous and lymphocytic colitis[11,12], which may be mistaken for non-compliance with a gluten-free diet (GFD). Eight percent of elderly persons in nursing homes or long-term care centers may have associated small bowel bacterial overgrowth, manifesting with malabsorption and diarrhea-like symptoms. Nutrient depletion in these individuals is common, and the incidence of osteopenic bone disease is increased. Finally, neurological disorders, such as dementia, are becoming increasingly recognized in elderly patients with CD[13].

Screening serological tests (IgA and IgG) appear to be age-independent in adults. However, the elderly seem to have an increased incidence of sero-negative CD. Interestingly, the fate of different celiac antibodies in genetically at-risk children on a normal diet has been assessed, and, remarkably, these appear to spontaneously disappear[14]. Specifically, antibodies to tTG and EMA were spontaneously lost in 49% and 45%, despite continuing gluten exposure. Although the procedural risk of endoscopy and biopsy may be marginally increased in the elderly, endoscopic biopsy remains crucial as the "gold standard" for diagnosis of CD in this age group as well as in all other age groups.

GENETICS

It is conceivable that CD could be a heterogeneous disorder, not only with differing clinical presentations, but also different degrees of pathological change in the small intestinal mucosa. Further morphometric and immunohistochemical studies from geographically and genetically diverse populations are needed to confirm observations on increased intraepithelial lymphocytes (IELs) in otherwise architecturally normal small intestine.

A small percentage of EMA-positive patients may have a small bowel biopsy in which the mucosa might be considered by some pathologists to be architecturally normal. From 409 children who were positive for celiac-related antibodies, 24 (5.9%) of the individuals were reported to have an architecturally normal small intestinal mucosa, 46% (11 of these 24 patients) had increased CD3+ intraepithelial lymphocytes, and 71% (17 of the 24) had an increased density of gamma-delta + cells[15]. In 17 of these 24 persons (70%), the number of lamina propria CD25+ cells was increased, and/or the expression of ICAM-1 and crypt HLA-DR was enhanced. Interestingly, in those persons with apparently normal jejunal histology, there appeared to be immunohistological evidence of immune activation in the epithelium, lamina propria and intestinal crypts. A GFD is usually not recommended in individuals with an architecturally-normal small bowel biopsy. Sophisticated immunohistochemical findings, such as these, raise the question as to whether definition of "normal" needs to be extended, and whether such subjects with abnormal antibodies in the intestinal biopsy may need further monitoring to determine if a GFD is indicated. In addition, duodenal mucosal tTG detection improves the sensitivity of diagnosis in CD for those with very mild histological changes, i.e. Marsh 1 lesions[16]. Thus, the definition of CD may conceivably extend to persons with an architecturally normal mucosal biopsy, but with abnormal CD-associated immunohistochemical changes. Additional studies are needed to confirm, evaluate and further elucidate these interesting observations.

Gluten has specific peptide sequences which show

HLA-DQ2 or HLA-DQ8 restrictive binding motifs across the various gluten proteins. About 40% of the heritability of CD includes the human leukocyte antigen HLA-DQ2 and HLA-DQ8 heterodimers. The HLA DQ2 and HLA-DQ8 molecules are necessary to develop CD, but are not, in themselves, sufficient for phenotypic expression of the disease. Indeed, HLA markers only explain an estimated 40% of the heritable risk for CD. Therefore, other non-HLA genes must also be involved. One of these may relate to genetic variants on chromosome 19, in the myosin IXB gene (i.e. MYO9B), and may potentially predict responsiveness to a GFD[17].

CD-associated HLA-DQ molecules bind and present gluten peptides to antigen-specific T-cells in the intestinal mucosa, and induce T-cell proliferation as well as cytokine secretion. Siblings who share HLA haplotypes have a greater likelihood of concordance with CD than the generally estimated risk for siblings. A small percentage of CD patients are DQ-2 negative, usually being DR4-positive for the class 2 antigen, DQ8. Carrying two copies of DQB1*02 is associated with an even greater risk for CD, but does not predict an earlier age of onset of disease or disease severity. This suggests that assessment of copy number of the DQB1*02 allele could permit stratification of risk[18].

In twin studies in which CD was diagnosed by small bowel biopsy and serology in one twin, monozygotic twin pairs had a high probability of being concordant with CD in the second twin: monozygotic and dizygotic co-twins had 70% and 9% cumulative probability of having symptomatic or "silent" forms of CD, respectively, within 5 years[19]. Under ACE (additive genetic, common and unshared environmental factors/models) with CD prevalences of 1/91 and 1/1000, heritability estimates were 87% and 57%, respectively[19].

The overall risk of siblings of children with CD developing CD was 10% in an Italian population, but the risk estimate ranged from 0.1% to 29% when HLA-DQ information of the proband, parents and sibling was considered[20]. The risk for the sibling developing CD was less than 1% for 40% of the sibs of the probands, 1% to 10% for 30% of the probands, and above 25% for the remaining 30% of the siblings. Thus, information about the risk for a second child to develop CD can be provided to parents with a child with CD. This antenatal estimate of the risk of CD in the child may be useful to provide early diagnosis and management, as well as providing focused and specific follow-up depending upon the risk stratification. Of importance, because CD is treated with a GFD and the resultant quality of life of the CD patient is high, the antenatal diagnosis of CD should not be used as a reason to consider termination of the pregnancy.

A double dose of DR3 (often with DQ2) is associated with an even higher risk of development of CD. HLA genotyping is thought to be useful to exclude CD in family members, or in persons in whom there is an increased risk of CD, such as those with Turner syndrome or Down's syndrome[21].

A strategy that combines gene expression profiling of intestinal biopsy specimens, linkage region information, and different bio-informatic tools for the selection of potential regulatory single-nucleotide polymorphisms (SNPs) has been used to search for novel candidate determinants of predisposition to CD in previously identified linkage regions[22]. Abnormalities in functional proteins have been observed in CD. By using genetic association analysis with a SNPs approach, the tight junction permeability barrier genes, KRD 3 (2SNPs) and MAGI 2 (2SNPs), were shown to be associated with CD in British and Dutch persons[23].

In addition to the involvement of HLA class I restricted CD8+ T-cells, the innate immune system may also be involved in CD. In the mucosa of untreated CD, there is an increase in activated CD8+ T-cells containing large granzyme-B (GrB)-positive granules, as well as cell surface expression of the Fas ligand (FasL). CD8+ T-cell cytotoxicity occurs in the mucosa of patients with active CD (through Fas and FasL-mediated killing of enterocytes). The gliadin interaction for this CD8+ T cell-mediated response (occurring through TCR/HLA class I) induces enterocyte apoptosis[24].

Variation among four closely linked genes on chromosome 4q27 represents a non-HLA genetic risk factor for CD, mapping to a region that contains IL2, IL21, TENR, and K1AA1109[25]. Also, multiple common variants for CD influencing immune gene expression have been defined with second generation genome wide association studies[26].

Genetic studies have also recently identified nine non-HLA loci that contribute to CD risk. Combining HLA and non-HLA risk genotypes increases the sensitivity of CD diagnosis by 6.2% compared with using only HLA for identification, with only a slight decrease in specificity[27]. There may be a quantitative relationship between the type and proportion of DQ heterodimers and the risk of CD[28].

PATHOGENESIS

Gliadins, derived from an alcohol soluble fraction of gluten, are storage proteins that are ingredients in wheat, barley and rye as well as other grains that contain gluten (albeit of less importance). Gliadins are characterized by a high content of glutamine and proline residues. Glutenins are insoluble in aqueous alcohol and are different in structure from the glutens. The early immune response in CD patients is directed towards several of these peptides, while the long-standing inflammatory response may be driven by gluten peptides deamidated or cross-linked by tTG and bound more tightly to HLA-DQ2 and HLA-DQ8. The tTG catalyzed modifications in gliadin are not restricted to single gliadin types or epitopes[29]. Prolamines in barley and rye are known as hordein and secalin, respectively. These barley and rye prolamines induce an mRNA interferon-gamma response in celiac mucosa[30]. The α-2 gliadin-33mer appears to cross the brush border membrane (BBM) of the jejunal enterocyte by a dose-dependent mechanism. Both the uncleaved as well as the degraded form of the 33mer translocate into the enterocyte[31]. Interferon-gamma enhances the trans-

location of the 33mer.

After passage across the BBM, gliadins trigger a Th-1 type-dependent inflammatory reaction. The effects of gliadin peptides and A-gliadin peptide P31-43 on cell lines and cultured small intestinal biopsies are mediated through epidermal growth factor receptor (EGFR) activation by interfering with EGFR endocytosis[32]. Gliadin has an immunogenic effect, but also directly affects cultured cells and intestinal preparations by way of separate peptides such as A-gliadin p31-43 (P31-43). The gliadin-induced delay of EGFR endocytosis in cultured intestinal biopsies suggests a role for EGFR activation in CD[32]. A 33 amino acid fragment of α-2 gliadin is an important trigger of the inflammatory process. In patients with active CD, there is transepithelial translocation of the attack 33mer, as well as incomplete degradation of the 33mer during intestinal transport.

In persons with active CD, there is a marked accumulation of polarized Th-1 cells that produce large amounts of interferon γ (IFNγ). T-bet, a member of the T-box family of transcription factors, is present in CD4+ and CD8+ mucosal T cells in patients with CD. Interleukin 21 (IL-21) is present in activated CD4+ T cells, as well as in natural killer T cells (NK T cells). IL-21 regulates the production of cytokines by T cell subsets. IL-21 increases the expression of Stat4 and T-bet, and stimulates the production of IFNγ in human T cells. In duodenal mucosal biopsies from patients with CD, there is enhanced IL-21 RNA and protein expression, and neutralization of IL-21 largely prevents peptic-tryptic and digest-enhanced IL-21 expression[33,34].

In persons with a genetic susceptibility to develop CD, gliadin interacts with the intestine to trigger disassembly of the inter-enterocyte tight junctions (TJs). The 2α-gliadin 20mer synthetic peptides of gliadin bind to the chemokine receptor, CXCR3. This binding induces MyD88-dependent zonulin release. In turn, zonulin release leads to increased intestinal permeability[35]. Increased intestinal permeability occurs prior to the onset of clinically apparent CD. Even on a GFD, the initially enhanced intestinal permeability does not necessarily return to normal.

Another important aspect related to the pathogenesis of CD may include the intestinal microflora that may be central to the clinical expression of the disease. In one recent report, enrichment in the mucosa-associated microbiota with rod-shaped bacteria in those that developed CD may have contributed to the so-called "epidemic" in Swedish children less than two years of age[36].

Finally, other recent studies related to CD pathogenesis have directly used intestinal biopsy specimens from CD and non-CD persons. For example, IL-15 receptor α mRNA expression is higher in duodenal biopsies from CD compared to non-CD persons, regardless of whether the CD subjects are or are not consuming gluten. IL-15 induces an intense immunological response in CD, with the production of nitrites and IFN gamma[37].

SEROLOGY

Serological markers of significance include EMA and tTG antibodies. The sensitivity of tTG is 98% and specificity 96%, whereas the EMA is 100% specific and sensitivity is greater than 90%[38]. Assays for tTG antibodies are largely based on the dominant antigen in the EMA test, however, tTG assays are more reliable and more reproducible, largely because the EMA is a qualitative assay and tTG assays are quantitative.

The antibodies to tTG and deamidated gliadin peptide (DGP) have been combined in a multiplex immunoassay of persons suspected as having CD, to potentially provide a complete antibody phenotype[39], and thereby to improve the performance characteristics of the serological testing. A meta-analysis has shown that the tTG antibody test out-performs the DGP antibody test, with a 5.2% greater sensitivity (93.0% *vs* 87.8%) and a 2.4% greater specificity (96.5% *vs* 94.1%), respectively[40].

DGP has been suggested to possibly be a better diagnostic test for CD before institution of a GFD than is the conventional gliadin antibody testing: the sensitivity, specificity, and accuracy of deamidated gliadin-IgA (74%, 95%, and 86%), deamidated gliadin-IgA (65%, 98%, and 84%), and deamidated gliadin-IgA + IgG (75%, 94%, and 86%), were superior to gliadin-IgA (63%, 90%, and 79%) ($P > 0.05$) and gliadin-IgG (42%, 90%, and 60%) ($P > 0.01$), and were similar to tTG-IgA (78%, 98%, and 90%)[39]. Further comparative evaluation with more modern serological assay methods would be useful, including tTG antibodies.

Because the small bowel biopsy in the person with CD does not have a pathognomic histological feature, serological testing may have an important supportive role in providing added information for the diagnosis of CD. Tissue transglutaminase (tTG) catalyzes the Ca^{2+}-dependant formation of cross links between protein-bound glutamine and glycine residues. The glutamine residue can be deamidated to glutamic acid by tTG, including specific glutamines in gluten-containing proteins. Deamidated gluten proteins have enhanced affinity for the HLA-DQ heterodimer of antigen-presenting cells. This activates T-lymphocytes and produces a T-helper type 1 response in the mucosa of celiac patients. The tTG-gluten complex is processed by B-cells, and presented to gluten-specific T cells, that give rise to tTG antibody T-helper type 2 response[41]. The tTG autoantibodies interact with extracellular membrane-bound transglutaminase, and thereby play an important role in proliferation of epithelial cells in persons with predisposition to CD.

The tTG is responsible for post-translational modification of proteins by introduction of lysine crosslinks, as well as deamidation. The IgA anti-tTG responses in CD and in dermatitis herpetiformis are focused on the region of tTG responsible for its transamidation and deamidation reactions, whereas the IgG response targets other regions of the enzyme[42].

The performance of serum anti-tTG may depend on clinical presentation of CD; e.g. classic symptomatic disease or silent asymptomatic disease. In patients estimated to have Marsh-Ⅲ A, B, or C degree of villous atrophy,

the sensitivity, specificity, positive and negative predictive values of the anti-tTG antibody test were 71%, 65%, 91%, and 30%, respectively[43]. The sensitivity was 90% for subjects with total villous atrophy, and only 42% for those with partial villous atrophy. In persons thought to have a high pretest probability of having CD based on symptoms such as weight loss, anemia, or diarrhea, 9.1% were anti-tTG negative[44], indicating that serological testing may miss a substantial number of cases of untreated CD that are antibody negative. Strongly positive tTG assay results without CD biopsy changes have also been recorded[45]. In the latter, it is not known if further biopsies at a later date will reveal the typical morphological changes of untreated CD.

The supply sources for EMA are limited to monkey esophagus or umbilical cord, and many assays are done "in-house" that may not be readily duplicated in other laboratories. EMA are considered to be highly sensitive and specific for serological changes seen in untreated CD. However, EMA assays are expensive, qualitative, and therefore subjective. EMA is increasingly being replaced by serological testing for antibodies to tTG, especially since the anti-tTG assay can be more precisely quantitated.

The EMA binding patterns and serum samples from CD patients are tTG-2 targeted, and the humoral response against tTG occurs at the level of the intestinal mucosa. tTG-2 targeted extracellular IgA deposits have been demonstrated by immunofluorescence in the small bowel mucosa in untreated celiac subjects, even when they are absent from the serum. In those subjects suspected of having CD but who are EMA and anti-tTG negative, finding the tTG-2 targeted antibody in the jejunal mucosa may help to make the diagnosis of CD[46].

Frozen sections of small bowel specimens were evaluated by immuno-fluorescence using rabbit antibody against human IgA. Although at best, semi-quantitative, these immunofluorescent deposits may be better initial markers for gluten sensitivity than small bowel mucosal IEL densities[47]. While architectural changes, such as villous atrophy, may lead to suspicion of untreated CD, tTG-2 specific IgA deposits may potentially be more useful. Further studies are needed.

Seronegative (EMA or tTG) CD occurs in less than 10% of celiacs, particularly in those with lesser degrees of villous atrophy. The presence of EMA in subjects with an architecturally-normal small bowel biopsy could indicate early developing CD. Serum and intestinal celiac anti-autobodies and intra-epithelial lymphocytes have been assessed as possible indicators of developing CD. Celiac autoantibody deposits have been recorded to provide a sensitivity and specificity of 93% and 93%, respectively, in detecting subsequent CD; this is compared to 59% and 57% for CD3+; 76% and 60% for gamma-delta+, and 88% and 71% for villous tip intra-epithelial lymphocytes[48].

Simple "in the office" anti-tTG tests have been developed commercially, and the blood drop-based assay for IgA anti-tTG was reported to have a sensitivity of 90% and a specificity of 95%[49]. The sensitivity and specificity of serum anti-tTG is laboratory-dependent, and assay results may differ for clinical compared to research laboratories. Because CD does not have a pathognomic histological feature, serology may have a supportive role in making the diagnosis. As CD is defined as a gluten-sensitive enteropathy, a clinical or serological response to a GFD is essential to establish a diagnosis of CD. Sometimes, re-biopsy after a GFD is necessary, or even further evaluation after a gluten challenge may be required.

A normal tTG level does not predict recovery of villous atrophy in celiac subjects on a GFD. For example, 16 of 48 (33%) subjects with CD on a GFD had persistent villous atrophy, but 7 of these 16 (44%) had a normal tTG[50]. In a multicenter, prospective study involving adult subjects attending one of several primary care practices, and in individuals not having symptoms or a condition known to be associated with CD, initial testing was done with anti-tTG. Those with elevated anti-tTG were tested for EMA (IgA), and then those in turn who were positive for EMA underwent an intestinal biopsy and HLA typing[51]. A positive anti-tTG was found in 3.1%, and the prevalence of CD in the serologically screened sample was 2.3%. When a similar study was performed in a university hospital, the prevalence of CD was 3.5%, and a negative HLA-DQ type excluded the diagnosis[52]. However, the "addition of HLA-DQ typing to TGA and EMA testing, and the addition of serological testing to HLA-DQ typing, provided the same measures of test performance as either testing strategy alone"[52].

Because the EMA and anti-tTG responses may remain elevated in CD on a GFD, it may be useful to measure soluble CD163, a scavenger receptor shed by tissue macrophages and correlated with the inflammatory lesion in CD. Those subjects with a more severe (Marsh grade 3) lesion had higher levels of CD163 than did those with a milder (Marsh grade 2, grade 1 or grade 0) lesion[53]. Further studies are needed.

There are three fatty acid binding proteins in the cytosol of the intestine: intestinal FABP (I-FABP), liver FABP (L-FABP), and ileal bile acid binding protein (I-BABP). These are present in increased amounts in the serum of persons with enterocyte damage from, for example, mesenteric thrombosis or necrotizing enteritis. Because I-FABP and L-FABP are found predominantly in the enterocytes in the upper portion of the jejunal villi, it is not surprising that their concentration is increased in the plasma of persons with CD. When measurements were made within one year of the introduction of a GFD, these initially increased I- and L-FABP levels fell to normal[54]. Together with following the patient's symptoms, quality of life, and celiac serology, assessing intestinal permeability may potentially prove to be a useful non-invasive test to follow the histological improvement of CD patients on a GFD.

The current standard for the assessment of adherence to a GFD in adult CD patients is largely based on a personal clinical evaluation. However, most serological assays appear to compare adequately in sensitivity and specificity to a thorough nutritional evaluation of the assessment of adherence to a GFD[55]. This is important, since only approximately 45%-80% of patients with CD adhere strictly to a GFD. This is thought to place them at increased risk

of developing metabolic bone disease, anemia, gastrointestinal symptoms, as well as impaired psychological well-being and quality of life.

MUCOSAL HISTOLOGY

Criteria for the diagnosis of CD include the initial demonstration of small bowel architectural changes including mucosal villous atrophy with crypt hyperplasia, along with increased intra-epithelial lymphocytosis. However, this may be a slowly developing process and the changes are not specific. In addition, some persons may suffer from CD symptoms before histological evidence can be documented. While some authors have suggested that an anti-tTG level can be defined which gives a positive predictive value of 100% for CD[56], it remains the standard of practice to always obtain a biopsy to determine if the histological changes of untreated CD are present before initiation of a GFD.

Some have noted that there may be significant differences between pathologists in mucosal biopsy interpretation[57]. The older Marsh classification, as modified by Oberhuber and colleagues, continues to be used by many pathologists. But for some, it may be considered complex because there are many different diagnostic categories. A simpler grading system has been proposed[57] based on three villous morphologies (A, non-atrophic; B1, atrophic, villous-ratio < 3:1; B2, atrophic, villi no longer detectable), and an intraepithelial lymphocyte count of > 25/100 enterocytes. Compared to the older classification system, this simpler classification schema was thought by the investigators to be superior.

However, the severity of villous atrophy based on histological analysis of biopsy specimens taken from the proximal intestine does not necessarily predict the severity of symptoms in CD for either children or adults. For example, when clinical symptoms of 18 CD patients with a good histological recovery were compared with 13 CD who had persistent small intestinal villous atrophy despite maintaining a GFD, symptoms could be absent despite the persistence of morphological abnormalities[58]. Other authors also noted the lack of association between the histological CD lesion and clinical manifestations[59]. Indeed, the lack of correlation between the degree of villous atrophy and symptoms was stressed in a further study of 499 CD patients, in which 44% had a classical presentation and 56% had atypical or silent CD[60]. These findings are not surprising, however, since the response to a GFD occurs initially in the most distal small bowel. Months to even years on a strict GFD may be needed before improvements in the proximal intestinal mucosa occur.

While duodenal biopsy represents the "gold standard" for the diagnosis of CD, capsule endoscopy (CE) has revealed that over a third of celiac patients have macroscopic mucosal changes extending beyond the duodenum, and in approximately 7%, the entire small bowel was involved[61]. As compared with duodenal biopsy for detecting changes in CD, the sensitivity of CE was reported to be 88%, specificity 91%, positive predictive value 97%, and negative predictive value 71%.

"Latent CD" is defined as abnormal celiac serology and a normal small bowel biopsy (Marsh stage 0). These so-called "latent CD" patients have an increased hazard (HR) ratio for death comparable to those with Marsh 1-2 and Marsh 3: 1.35; 95% CI, 1.14-1.58, median follow-up, 6.7 years; HR, 1.72; 95% CI, 1.64-1.79; median follow-up, 7.2 years; HR, 1.39; 95% CI, 1.33-1.45; median follow-up, 8.8 years, respectively[62]. This corresponded with excess mortality of 1.7 per 1000 person-years in "latent CD", 10.8 in Marsh 1-2, and 2.9 in Marsh 3 stage CD. This raises the possibility that it may be important to diagnose very early CD. However, this label of "latent CD" may differ from the original definition of latent CD (without serological studies) where abnormal small intestinal architectural changes were induced with a high gluten-containing diet (initially reported in dermatitis herpetiformis) and then normalized on a GFD.

Given that duodenal biopsy is still the "gold standard" for the diagnosis of CD, it is of interest to know that when only two duodenal biopsies are obtained, diagnosis of untreated CD is confirmed in 90%, however, increasing the number of biopsies to 3 increased detection to 95%, and to 4 biopsies, 100% respectively[63].

While some present with symptoms and a small bowel biopsy is done to exclude CD, others will present with positive serology and a duodenal biopsy is then obtained. Occasionally lesions may be patchy or detected only in the duodenum, resulting in potential for sampling error and a false-negative result. Confocal endomicroscopy (CEM) is a novel method that permits magnification *in vivo* of the gastrointestinal mucosa by up to 1000-fold. In persons with known CD, accuracy of CEM in diagnosing CD was reported to be excellent, with receiver operator characteristics under the curve of 0.946, sensitivity of 94%, and specificity of 92%[64]. CEM was also sensitive in the detection of histological changes following treatment with a GFD.

"Lymphocytic enteritis" (Marsh 1) may be associated with symptoms, yet serological markers of CD appeared to be of limited value in identifying these individuals. In 130 of 221 first-degree relatives of HLA-DQ2-positive patients with CD, relatives were positive also for HLA-DQ2, and 49% were Marsh 0, 25% Marsh 1, < 1% Marsh 2 and 10% Marsh 3. Only 17 of 221 relatives had positive serological markers for CD[65]. These authors argued that the higher number of symptomatic patients with lymphocytic enteritis (Marsh 1) supports HLA-DQ2 genotyping strategy followed by duodenal biopsy in relatives of CD patients. Further studies to confirm these observations are needed.

Anti-tTG levels have continued to be used in assessing initiation and maintenance of a GFD. It is believed that tTG levels might be followed to reduce the risk of complications and monitor histological changes in the upper small bowel[66].

CLINICAL PHENOTYPES

The ESPGHAN (European Society of Pediatric Gastro-

enterology, Hepatology and Nutrition) criteria distinguish between three different forms of CD so that classification might be more precise: the latent or *potential* form defined by the presence of anti-celiac antibodies; the silent form (*asymptomatic*) defined by the presence of anti-celiac antibodies and villous atrophy of the small intestine; and the symptomatic form defined by the presence of anti-celiac antibodies, villous atrophy and clinical symptoms.

The adult height of children with classical CD (e.g. symptomatic with diarrhea) is influenced by their compliance to a GFD. Children diagnosed with CD after 4 years of age show a slower and less complete catch-up growth. A delayed diagnosis of CD may be associated with a shorter adult height in men, but not in women[67].

While abdominal symptoms may respond quickly to a GFD, it may take up to a year or more after the introduction of a GFD for persons with CD to achieve normalization of their initially abnormal small bowel biopsy. Elderly patients respond more slowly than younger patients to a GFD.

"Gluten sensitivity" may be defined as symptoms, such as diarrhea, apparently induced by gluten-containing foods. These have been reported in the absence of changes in small intestinal histology. In persons with diarrhea-predominant irritable bowel syndrome (D-IBS), stool frequency and the gastrointestinal symptoms score return to normal values in 60% of D-IBS subjects who were positive for HLA-DQ2 and CD-associated serum IgG after six months on a GFD, compared to only 12% who were negative[68].

Among the complications of undiagnosed and, therefore, untreated CD are growth failure in children, infertility, anemia, osteoporosis, small intestinal non-Hodgkin lymphoma[69], and a 3.9-fold increased all-cause mortality rate[70]. Potentially, this may underscore the importance of diagnosing and treating even latent CD.

Celiac patients were reported to have a 5.4-fold higher risk of non-Hodgkin's lymphoma, but no increased risk of Hodgkin's or chronic lymphatic leukemia. A shared susceptibility amongst siblings is observed[69]. It remains controversial whether there is an increased risk of developing lymphoma in CD if the disease is asymptomatic[58,71].

There is a 5-fold increase in risk of lymphoproliferative malignancy in CD in comparison to the general population[72].

CLINICAL ASSOCIATIONS

The prevalence of autoimmune diseases (e.g. autoimmune thyroid disease) is increased in persons with CD, as compared with the healthy control population. Conversely, CD is increased in persons with autoimmune diseases. The cumulative risk of autoimmune disease in patients with CD is 8% at age 15, and 16% at age 30 years. Factors associated with an increased risk of autoimmune diseases associated with CD include a family history of autoimmune disorders, and a diagnosis of CD before the age of 36[73]. Once the diagnosis of CD has been made, patients who are adherent to a GFD have a 6% risk of developing autoimmune disease at 10 years, *vs* 16% in those who are not compliant with a GFD. Expressed differently, the incidence of autoimmune disease is 5.4 per thousand patient years during adherence to a GFD, *vs* 11.3 per thousand patient years during non-adherence.

Asymptomatic CD is also seen in children and adults with autoimmune hepatitis and autoimmune bile duct disease. CD may be associated with asymptomatic increases in transaminase values. Persons with autoimmune liver disease should be examined for possible CD. In persons with CD who have acute hepatitis, an autoimmune cause should be suspected[74].

More and more clinical associations have been suggested for CD. For example, 42% of CD patients had oral soft tissue lesions, as compared to only 2% of non-CD patients[75]. Recurrent aphthous stomatitis disappeared in 89% of patients after one year of a GFD.

Biopsy-defined CD has a 4-fold higher prevalence in those with irritable bowel syndrome[76]. Mental disorders, non-compliance with the GFD, active medical co-morbidities, and dissatisfaction with doctor/patient communication were associated with reduced CD Questionnaire scores[77].

Up to 10% of patients with CD have neurological symptoms ranging from polyneuropathy, epilepsy, myoclonus, multifocal leukoencephalopathy, dementia, chorea, migraine, memory/attention impairment and peripheral axonal and demyelinating neuropathies as well as acetylcholine-antibody positive myasthenia gravis[78]. Autoimmunity may act as a mechanism triggering neurological dysfunction[79], and anti-neuronal, anti-gliadin and tTG antibodies may contribute to neurological impairment through Apaf-1 activation with Bax and cytochrome C translocation, leading to impairment of mitochondrial-dependent apoptosis. There is no statistically significant association between CD and subsequent development of Parkinson's disease, Alzheimer's disease, hereditary ataxia, symptoms of ataxia, Huntington's disease, or spinal muscular atrophy[80].

Adult but not pediatric patients with CD have an increased risk of sepsis, particularly pneumococcus infection[81]. In CD there is an increased prevalence of splenic hypofunction[82].

Using community-based cohorts and a record-linkage database, the adjusted relative risk of cardiovascular disease in CD was 2.5 for new EMA positive *vs* EMA negative individuals[83]. This suggests that CD may be associated with an increased risk of cardiovascular outcome. There may also be an association between CD and eosinophilic esophagitis[84].

Aldolase B deficiency causes hereditary fructose intolerance, and this may be associated with CD[85]. In CD patients as compared with individuals with dyspepsia or healthy controls, serum ghrelin concentrations are higher, not correlated to the severity of duodenal histological lesions, and revert to normal during the institution of a GFD, despite persistent duodenal lymphocytic infiltration[86]. It is not clear if these alterations in ghrelin concentrations have any biological importance in CD.

In 18 CD patients, there was increased intestinal 5-HT-enterochromaffin cell numbers, higher peak plasma 5-HT levels and postprandial area under the curve of 5-HT levels after a high-carbohydrate meal, as well as increased platelet 5-HT stores[87]. The authors suggested that serotonin excess may mediate dyspeptic symptoms in untreated CD. Further evaluation is required.

A meta-analysis of serological or histological diagnosis of CD in unselected adults with dyspepsia showed that the numerically increased prevalence was not statistically significant[88]. Another systematic review and meta-analysis by the same group, examining 14 studies with 2,278 persons diagnosed with IBS, had an approximately 4% prevalence of CD. The OR for biopsy-proven CD in IBS cases *vs* controls was 4.34[89]. In children with CD, 40% had elevated transaminase values; of those with elevated transaminase values, 95% were cryptogenic and normalized on a GFD, and 5% had autoimmune hepatitis that required immunosuppression plus a GFD to normalize clinical and biochemical parameters[90].

TREATMENT

Gluten free diet

What is the definition of a GFD? Even "gluten-free" products may not be completely free of gluten. In 1998, the World Health Organization/Food and Agriculture Organizations Commission proposed that foods which are said to be "gluten-free" could not contain more than 200 ppm of gluten. Each individual with CD may have a unique threshold or tolerance to the amount of gluten in the diet. Daily gluten intake of less than 10 mg is unlikely to cause significant histological abnormalities in the intestine of patients with CD[91]. Patients adhering to a GFD report an improved health related quality of life.

A systematic review has revisited the complications and need for long term follow-up in CD[92]. A 16-question disease-specific symptom index has been validated in adults with CD[93]. Such an index might be used to monitor the response of a CD patient to a GFD.

The treatment of CD is the life-long use of a GFD. A primary goal in the care of patients with CD is to improve the quality of their lives, through a collaboration of the stakeholders[94]. Lanzini *et al*[95] assessed in 465 consecutive CD patients, the histological outcome after a GFD consumed for a median of 16 mo. While CD serology became negative in 83% of CD patients with Marsh Ⅲ lesions on a GFD, mucosal biopsy histology normalized in only 8%, improved except for increased intraepithelial lymphocytes in 65%, was unchanged in 26% and worsened in 1%. The authors concluded that "complete normalization of duodenal lesions is exceptionally rare in adult celiac patients despite adherence to GFD, symptoms disappearance and negative CD related serology".

Early diagnosis and treatment are important in CD, as some of the associated complications may be irreversible, unless the CD is treated[96]. Growth retardation, osteoporosis and abnormal dentition will remain permanent if not treated early. The prevalence of associated depression is up to 37%, similar to that of persons with other chronic conditions[97].

In children with CD, long-term consumption of oats may be well tolerated[98], although concern has been expressed regarding possible contamination of oats with other gluten-containing grains. Other investigators have demonstrated that transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in CD[99]. It has always been assumed that patients with CD must remain on a GFD for life. However, up to 10% of CD patients diagnosed in childhood were reported to develop long-term latency of their CD when returning to a gluten-containing diet[100]. In patients who have been on a GFD and have no symptoms, even if there were small bowel CD-like histological abnormalities which remained, some had no evidence of clinical relapse even though they had been on a gluten-containing "normal" diet for more than 2 years.

Individuals with villous atrophy but no symptoms are said to have "silent" CD. Almost half of CD patients may clinically tolerate a gluten-containing diet, yet continue to have mucosal abnormalities. Indeed, approximately 10% of CD patients diagnosed in childhood may develop clinical tolerance to gluten. In the United Kingdom, about one-third of CD subjects are under no active follow-up[101].

Alternatives to a gluten free diet

There may be poor compliance with the GFD because it is difficult and gluten-free products are expensive. For these reasons, new approaches have been taken in the treatment of CD. Some of these include orally administered endopeptidase, antagonists to S100B protein, IL-15 blockers, elemental diets and transamidation of wheat flour. Lactobacilli added to sourdough for fermentation are able to break down the proline-/glutamine-rich gluten peptide. This may play a role in the future treatment of CD[102]. Supplementation of nutrients may become essential depending upon the severity of malnutrition. One double–blind placebo-controlled multicenter trial showed a significant improvement in general well being after 6 mo of supplementation with vitamin B[103].

Prolyl-endopeptidases are able to digest ingested gluten. Oral therapy with prolyl-endopeptidases, exogenous protease enzymes, represents a new approach to managing CD[104]. Bacterial prolyl-endopeptidase from *Flavobacterium meningosepticum* removes gluten toxicity by cleaving it into small fragments which lack T-cell stimulatory properties[105]. After prolonged exposure to high concentrations of bacterial prolyl-endopeptidase, the amount of immunostimulatory gliadin peptides reaching the local immune system in CD is decreased[106]. The prolyl-endoprotease from *Aspergillus niger* (AN-PEP) is a member of the serine peptidase family, and this degrades gluten peptides rapidly[107]. This AN-PEP is capable of accelerating the degradation of gluten in a gastrointestinal model that closely mimics *in-vivo* digestion[108]. The

pH optimum of the enzyme is compatible with that found in the stomach, and the enzyme is resistant to degradation by pepsin.

The gluten proteins may be purposely modified to abolish their capacity to stimulate the interferon gamma from CD4+ T-cells[109]. Another approach to the therapy of CD is the designing of non-toxic wheat, rye or barley based on their protein homology[110,111].

Other therapeutic approaches would include the binding of gluten to HLA-DQ2 or HLA-DQ8, or blocking the gluten-reactive T cells by immunotherapy (e.g. vaccination). For example, transamidation of wheat flour with a food-grade enzyme and an appropriate amine donor (microbial transglutaminase and lysine methyl ester) can be used to block the T-cell mediated gliadin activity[99]. Gluten contains many immunogenic peptides, but there may be weak varieties with a natural low number of T-cell-stimulatory epitopes[111].

Polymeric binders reduce the deleterious effects of gliadin on intestinal epithelium in cultured cells and transgenic mice[112]. These binders have a strong affinity for gliadin, inhibiting cytoskeleton disorganization and ultrastructural changes in intestinal epithelial cells. Their beneficial use in humans remains to be established.

Antigen-presenting cells include dendritic cells, macrophages and B-cells. A unique subset of dendritic cells appears to be responsible for local activation of gluten-reactive T-cells in the celiac lesion[113]. Enteric glial cells (EGC) release neurotropic factors and are activated by inflammatory insults. EGC-derived S100B protein released in astroglial cells is increased in the duodenum of patients with CD, with increased S100B messenger RNA and protein expression, increased iNOS protein expression, and increased nitrite production in treated CD[114]. This does not occur in those CD patients on a GFD, or in non-CD control subjects. Products derived to block EGC-derived S100B protein may have a therapeutic role.

IL-15 has proinflammatory and anti-apoptotic properties. IL-15 is over-expressed in the enterocytes and lamina propria mononuclear cells of untreated CD, where its level reportedly correlates with the degree of mucosal damage[115]. IL-15 also promotes IEL survival. Blocking IL-15 and suppressing uncontrolled IEL activation and survival has the potential to provide a new therapeutic approach to prevent tissue damage in CD. In the intestinal mucosa of CD patients, IL-15 impairs Smad3-dependant TGF-beta signaling in human T-lymphocytes downstream from Smad3 nuclear translocation[116]. There is upregulation of phosphor-c-jun. This provides further support to the suggestion of the potential therapeutic effect of blocking IL-15.

Intestinal permeability is increased in patients with CD, and is associated with alterations in tight junction proteins (e.g. zonulin). Addition of zonulin may prevent T-cell mediated stimulation in CD. In a double-blind randomized placebo-controlled study of milligram doses of AT-1001, an inhibitor of paracellular permeability derived from *Vibrio cholera*, prevented the expected increases in intestinal permeability in subjects with CD challenged with gluten[117]. AT-1001 use is also associated with a diminution in the anticipated rise of interferon-gamma levels.

REFRACTORY DISEASE

Recurrent symptoms sometimes develop in biopsy-proven CD patients on a GFD. The most common cause of non-responsive CD, which occurs in about 30% of CD patients, is non-adherence to a GFD. A Celiac Dietary Adherence Test (CDAT) consistency in a 7-item questionnaire was developed using a logistic regression, and validated against transglulaminase serology for the assessment of adherence to a GFD[118]. Usually, poor compliance to a GFD is thought to be responsible, although compliance may sometimes be difficult to establish. Intentional dietary indiscretion may be evident, but sometimes, there is limited awareness of gluten-containing substances. Gluten is ubiquitous, being documented in pill capsules and other materials, such as communion wafers.

Other causes, detailed elsewhere[119], may be responsible for recurrent symptoms even though the CD patient appears to be following a strict GFD. Some causes include associated primary or secondary pancreatic insufficiency, small intestinal bacterial overgrowth, collagenous sprue, or lymphocytic or collagenous colitis. Rarely, a complication may be responsible (e.g. lymphoma, carcinoma).

Sometimes, no initial response to a GFD ever occurs and symptoms persist. In these subjects, biopsies may be abnormal, but the gluten-dependent nature of the small bowel abnormalities was never documented. This has been labeled "unclassified sprue" or "sprue-like intestinal disease". Some of these persons may eventually prove to have a lymphoma. As many as approximately half of patients with CD on a GFD for more than two years may be able to tolerate a gluten challenge, even though they have mucosal abnormalities[100]. About 10% of CD patients diagnosed in childhood may develop temporary tolerance to gluten. However, because of the continuing mucosal abnormalities, they remain at risk of developing the complications of CD. Indeed, adolescents who do not adhere to a GFD have a lower quality of life[120].

Immunohistochemical labeling has helped to define an abnormal prognostic profile of intra-epithelial lymphocytes in the small bowel mucosa, so-called "refractory celiac disease, type 2 (RCD 2)" characterized by an aberrant clonal IEL population with loss of IEL antigens. About half of RCD 2 patients develop an enteropathy-associated T-cell lymphoma (EATL) within 5 years, and a particular HLA-DQ subtype, DQ2, if homozygous, predisposes to RCD 2[121].

Complete duodenal mucosal recovery in CD may be limited and may require prolonged periods of a GFD. In one study, remission was seen in 65% or no histological improvement was seen in 26% of patients[95]. This might be anticipated, however, if only duodenal biopsies are being taken since the proximal small intestine is most severely affected and varying periods of time are required for recovery to be significant. An apparent failure to histologically respond to a GFD may only reflect that duo-

denal, rather than more distal small intestinal biopsies were repeated.

Refractory CD has been defined as persistent or recurrent villous atrophy with crypt hyperplasia and increased intraepithelial lymphocytes (IELs) despite a strict GFD for greater than 12 mo (or if severe persisting symptoms necessitate intervention independent of the duration of the GFD)[122]. In assessing the GFD response, the site of re-biopsy and duration on a strict GFD are crucial to the definition of refractory disease. In some, as suggested by this definition, the opportunity for re-evaluation is very limited because of rapid progression of the intestinal disease.

RCD is usually manifested by recurrence of symptoms and intestinal abnormalities, despite adherence to a GFD. RCD can be further defined from a prognostic perspective by the histological appearance of monoclonal or polyclonal intraepithelial lymphocytes (RCD type 2) *vs* normal lymphocytes (type 1)[123]. These changes and the development of an EATL were shown to be adverse factors in the prognosis of CD, particularly in the first two years after CD has been deemed to be refractory[124].

Refractory celiac disease (RCD) type 2 but not type 1 shortens the sufferer's life expectancy[123].

Corticosteroids may improve clinical symptoms in some patients with RCD. Unfortunately, the histological response to steroids has not been consistent[123]. Patients with RCD 1 may benefit from immunosuppressive therapy, whereas those with RCD 2 may respond to Cladribine or to stem cell transplantation. Treatment with cladribine and anti-CD-52 has been shown to be associated with histological improvement. Azathioprine and anti-tumor necrosis factor-α have shown only limited success. The development of an overt lymphoma within 8 wk of treatment was seen in 3 out of 4 of these patients, thereby preventing further use. Alternative strategies that have been suggested include stem cell transplantation to replace the abnormal intra-epithelial lymphocyte population, and the blocking of IL-15[125-129].

CONCLUSION

Celiac disease is being increasingly diagnosed because of the recognition that the disease may be present without significant intestinal symptoms, may be associated with other autoimmune disorders and may be suspected from serological screening. Definition of the disease includes an intestinal biopsy before treatment with a GFD along with documentation of a definitive GFD response. In some patients, this may necessitate further intestinal biopsy after a period on a GFD. Serological testing may be useful in providing additional evidence that CD is present and may be useful in some patients to assess GFD compliance. Recent studies focused on the genetic basis and pathogenesis of CD have emerged to improve understanding of the complex molecular alterations that occur with CD.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2273

World J Gastroenterol 2011 May 14; 17(18): 2273-2282
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Management of liver cirrhosis between primary care and specialists

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Received: January 21, 2011 Revised: February 21, 2011
Accepted: February 28, 2011
Published online: May 14, 2011

Abstract

This article discusses a practical, evidence-based approach to the diagnosis and management of liver cirrhosis by focusing on etiology, severity, presence of complications, and potential home-managed treatments. Relevant literature from 1985 to 2010 (PubMed) was reviewed. The search criteria were peer-reviewed full papers published in English using the following MESH headings alone or in combination: "ascites", "liver fibrosis", "cirrhosis", "chronic hepatitis", "chronic liver disease", "decompensated cirrhosis", "hepatic encephalopathy", "hypertransaminasemia", "liver transplantation" and "portal hypertension". Forty-nine papers were selected based on the highest quality of evidence for each section and type (original, randomized controlled trial, guideline, and review article), with respect to specialist setting (Gastroenterology, Hepatology, and Internal Medicine) and primary care. Liver cirrhosis from any cause represents an emerging health issue due to the increasing prevalence of the disease and its complications worldwide. Primary care physicians play a key role in early identification of risk factors, in the management of patients for improving quality and length of life, and for preventing complications. Specialists, by contrast, should guide specific treatments, especially in the case of complications and for selecting patient candidates for liver transplantation. An integrated approach between specialists and primary care physicians is essential for providing better outcomes and appropriate home care for patients with liver cirrhosis.

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Key words: Ascites; Family medicine; Hepatic encephalopathy; Hypertransaminasemia; Portal hypertension

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Grattagliano I, Ubaldi E, Bonfrate L, Portincasa P. Management of liver cirrhosis between primary care and specialists. *World J Gastroenterol* 2011; 17(18): 2273-2282 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2273.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2273

INTRODUCTION

Liver cirrhosis is defined in histology as a bridging fibrosis-a late stage of hepatic fibrosis-leading to deranged liver architecture and regenerative nodules. Liver cirrhosis is considered the end stage of a variety of chronic liver diseases, and is irreversible in its advanced stages[1]. Cirrhosis is characterized by poor life expectancy and is a leading cause of morbidity and mortality: in the United States liver cirrhosis is the 12th most common cause of death (9.5/100 000 individuals), while in Italy the incidence of liver cirrhosis is over 26 000 new cases each year, with a prevalence over 120 000 cases (7000 below 45 years), and 20 deaths/100 000 individuals[2,3]. Figures are likely to be even higher in Asia and Africa. Liver cirrhosis carries the

risk of life-threatening complications, partly due to a number of co-morbidities. Medical treatments that may halt the progression of compensated cirrhosis to decompensated cirrhosis are currently being developed[1]. Liver transplantation, however, is the only option in a selected subgroup of patients with end-stage disease. Because of the increasing prevalence of chronic viral hepatitis and (alcoholic-nonalcoholic) steatohepatitis and their high risk evolution toward liver cirrhosis and end-stage liver disease, preventive programs and early management of these conditions are considered an emerging health issue. It is essential that primary care physicians (PCPs) be optimally trained to identify patients with chronic liver disease as early as possible, and to properly manage those with liver cirrhosis[4]. A close interaction is therefore required between PCPs and specialists (i.e. gastroenterologists, hepatologists, and internists) who have a fundamental role as consultants and guides for specific treatments, i.e. in the case of complications and the management of patients approaching liver transplantation.

This article is based on a PubMed search to provide an updated view for comprehensive management of several aspects of liver cirrhosis in different settings.

DATA SOURCES

Full papers were searched on Medline (http://www.ncbi.nlm.nih.gov/PubMed) for guidelines, randomized controlled trials (RCTs), and authored review articles published in English-language journals in the past 25 years. The following MESH headings were used: "ascites", "liver fibrosis", "cirrhosis", "chronic hepatitis", "chronic liver disease", "decompensated cirrhosis", "hepatic encephalopathy", "hypertransaminasemia", "liver transplantation", and "portal hypertension". The reference list was updated as of November 2010. Authors independently assessed articles for relevance and study quality. For each section, evidence levels were scored as follows: (1) LEVEL I (at least one properly conducted RCT, systematic review, or meta-analysis); (2) LEVEL Ⅱ (other comparison trials, non-randomized, cohort, case-control, or epidemiologic studies, and preferably more than one study); and (3) LEVEL Ⅲ (expert opinion or consensus statements).

APPROACH TO PATIENTS WITH LIVER CIRRHOSIS

The clinical presentation of liver cirrhosis is often asymptomatic until complications appear. The presence of liver cirrhosis should be suspected in any patient with chronic liver disease and abnormal aminotransferases and/or alkaline phosphatase. Chronic liver disease stigmata should be searched for, and include vascular spiders, palmar erythema, and muscle wasting. Also, a palpable left lobe of the liver, hepatomegaly and splenomegaly are suggestive for liver cirrhosis. The diagnosis becomes much easier in the presence of signs of decompensation, namely jaundice, ascites, and asterixis. Additional laboratory tests include those exploring liver synthetic function, such as serum albumin and prothrombin time, while serum bilirubin investigates the ability of the liver to conjugate and excrete bilirubin. A low platelet count is suggestive of portal hypertension and hypersplenism. An AST/ALT ratio above 1 is indicative of liver cirrhosis, but its absence does not exclude cirrhosis (i.e. low specificity). The imaging studies include abdominal ultrasound, CT scan or magnetic resonance and might reveal a nodular liver and splenomegaly. The differential diagnosis of advanced chronic hepatitis relies on liver biopsy, which is still the gold standard for end-stage chronic liver disease. Percutaneous liver biopsy is not necessary in the presence of decompensated cirrhosis or when imaging studies have confirmed the presence of cirrhosis. Thus, liver biopsy is reserved for selected patients and can also be performed in out clinic settings[5,6]. Histology provides information on etiology, disease stage and grade of inflammation. Although the ultimate decision is not currently taken by PCPs, they should repeatedly check the patient with blood tests before referral for liver biopsy (at least two times and at least 2-3 mo apart). If abnormalities persist in spite of second step analyses and a liver ultrasonography has been inconclusive, the decision to perform a liver biopsy must be taken on an individual basis and rely on the patient's age and general health status, as well as the need for prognostic information (LEVEL Ⅲ)[7]. According to the American Association for the Study of Liver Disease (AASLD), liver biopsy has a major role in diagnosis, assessment of prognosis, assistance in therapeutic decisions, and reinforcement of the patient's compliance (LEVEL Ⅱ)[5]. Biopsy, however, is a costly procedure which is not free of potential side effects and risks, and is often refused by the patient. A French survey, which interviewed over one thousand PCPs, concluded that liver biopsy may be refused by up to 59% of patients with chronic hepatitis C and that 22% of PCPs share a similar concern[8].

Novel non-invasive methods might provide preliminary information with good diagnostic accuracy for further selection of patients at risk for progressive liver disease. For example, tests might help to evaluate the presence and extent of liver fibrosis, and to differentiate cirrhosis from chronic hepatitis (positive predictive values exceed 85%-90%)[9]. Such policy may be helpful in the primary care setting. Transient elastography (FibroScan®), for example, assesses liver stiffness, with some limitations in the case of morbid obesity, small intercostals spaces, and ascites[10]. Ongoing liver fibrosis is also predicted by using specific algorithms of surrogate serum markers or by the application of standardized procedures (e.g. APRI: the aspartate transaminase to platelets ratio index; FibroTest: haptoglobin, α2-macroglobulin, apolipoprotein A1, γGT, bilirubin: Hepascore: bilirubin, γGT, hyaluronic acid, α2-macroglobulin, age, gender; BARD: Body mass index (BMI), AST/ALT ratio, diabetes). A novel technique based upon ultrasound-based elastography (Fibroscan, Echosens, Paris, France) can assess mean hepatic tissue stiffness[11]. Results are expressed in kilopascals (kPa) and

Table 1 Diagnostic tests, suggested etiology, and current treatment for the most frequent forms of liver cirrhosis in adult patients

Abnormal test(s)	Etiology	Treatment
γGT (high), MCV (high)	Alcohol	Abstinence
HBsAg, HBV-DNA, HBc-IgM, HDV-RNA (positivity)	HBV + Delta virus infection	Interferon α-2b, nucleoside (Lamivudine, Telbivudine, Entecavir) and nucleotide (Adefovir, Tenofovir) analogues
HCV-RNA (positivity)	HCV infection	Interferon plus ribavirin
γGT (high), alkaline phosphatase (high), AMA (positivity)	Primary biliary cirrhosis	Ursodeoxycholate
ANA, ASMA, LKM (positivity)	Autoimmune hepatitis	Prednisone, azathioprine
Ferritin (high), transferring saturation index (> 45%), liver iron content (high), *HFE* gene mutation for hereditary hemochromatosis (C282Y, H63D)	Hemochromatosis	Phlebotomy, deferoxamine
Ceruloplasmin (low), serum (low) and 24 h urine copper excretion (high)	Wilson's disease	D-penicillamine, zinc
HDL-cholesterol (low), glucose (high), triglycerides (high)	NAFLD/NASH	Low caloric diet, exercise, drugs lowering insulin-resistance

AMA: Anti-mitochondrial antibody; ANA: Antinuclear antibody; ASMA: Anti-smooth-muscle antibody; γGT: γ- glutamyltransferase; HBV-DNA: Hepatitis B virus DNA; HCV-RNA: Hepatitis C virus RNA; HBsAg: Hepatitis B surface antigen; HDL: High density lipoprotein; HDV-RNA: Hepatitis delta virus RNA; LKM: Liver kidney microsomes; MCV: Mean corpuscular volume; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

the harder or stiffer the tissue, the faster a shear wave propagates, as a marker of hepatic fibrosis. Similar results have been reported with magnetic resonance elastography (MRE)[12]. Likely, the combination of elastography with one of these indices will also help specialists to better select patients suitable for liver biopsy[9,10].

Life expectancy and quality of life in patients with advanced cirrhosis remains poor, despite diagnostic advancement. Patients experience fatigue, pruritus, ascites, bleeding and encephalopathy. Dyspepsia and malnutrition are common. Whereas liver transplantation has changed life expectation for a number of patients, many transplantable patients still die due to long waiting lists. Targeted therapy is crucial in slowing or even halting disease progression and to provide standard medical care. PCPs should identify and address alcohol abusers early, while conditions like nonalcoholic steatohepatitis (NASH), B and C hepatitis, autoimmune disorders, and hemochromatosis should be appropriately counseled and treated. Attention should be given to active immunization, nutrition, and general healthcare.

MANAGEMENT OF PERSISTENT ASYMPTOMATIC ELEVATION OF SERUM TRANSAMINASES

Measurement of serum ALT is part of standard laboratory tests in asymptomatic outpatients, and is a sensitive screening tool for chronic liver disease[13]. Between one and four percent of asymptomatic subjects may have elevated ALT (LEVEL Ⅲ)[7,14,15]. In a recent survey in the Mediterranean area, the most likely cause of elevated serum ALT was an excessive alcohol intake (45.6%), nonalcoholic fatty liver disease (NAFLD) (24%), and HCV infection (18.6%)[14].

Over 20% of subjects with elevated ALT show signs suggestive of relevant chronic liver disease[2]. PCPs are required to carefully investigate most common causes of elevated ALT and for early identification of treatable chronic liver diseases[16,17]. Patient histories should focus on the use of medications, herbal extracts, and alcohol consumption. The presence of diabetes and thyroid disease (hypothyroidism) must be considered. The problem, however, may be underestimated as about 38% of patients with occasional ALT elevation show normal values at next measurement[16]. Despite the very high number of subjects showing such liver test abnormality in family practice, only a few will need referral, i.e. those patients with doubtful diagnosis after initial evaluation and patients with established diagnosis requiring therapy (LEVEL Ⅲ)[18].

THE IMPORTANCE OF IDENTIFYING ETIOLOGY

The identification of the cause underlying liver cirrhosis is essential in starting preventive measures and designing specific intervention (LEVEL Ⅰ). Table 1 shows the most appropriate tests for etiologic diagnosis of cirrhosis. Anti-mitochondrial antibodies are specific for primary biliary cirrhosis, HBV-DNA or HCV-RNA positivity for hepatitis B or C, low serum ceruloplasmin levels for Wilson's disease, and high serum ferritin and transferrin saturation index for hereditary hemochromatosis. Of note, liver cirrhosis may result from coexisting etiologic factors (i.e. alcohol and viral infection, obesity and virus, *etc*.).

HOW TO SCORE AND DEFINE PROGNOSIS

Once the diagnosis of liver cirrhosis has been formulated, a further important step is to score the disease. However, neither physical findings nor transaminases are helpful for defining prognosis or scoring the disease. Other laboratory tests (bilirubin, albumin, and prothrombin time), combined with the presence and severity of encephalopathy and ascites, are included in the Child-Pugh score (Table 2), the traditional scale used by many clinicians for assessing the liver disease severity (LEVEL Ⅰ). Another scoring system is the model for end-stage liver disease (MELD, http://www.mdcalc.com/meld) which provides robust information on mortality in cirrhosis, and is used for prioritizing candidates for transplantation[19] (LEVEL Ⅰ). Both scores

Table 2 Child-Pugh scoring system for liver cirrhosis and related indication priority for transplantation[20]

Score	1	2	3
Bilirubin (mg/dL)	< 2	2-3	> 3
Prothrombin time (INR)	< 4 sec. (< 1.7)	4-6 sec. (1.7-2.3)	> 6 sec. (> 2.3)
Albumin (g/dL)	> 3.5	3.5-2.8	< 2.8
Ascites	Absent	Mild	Severe
Encephalopathy	Absent	Mild	Severe

The Child-Pugh score is given by the sum of the score (1 to 3) of each of the five parameters. A score of 6 or lower defines the patient as class A, 7 to 9 as class B, and 10 or higher as class C.

can also be easily applied in primary care. Regardless of the cause, once decompensation has occurred, mortality without transplantation is 85% over 5 years[1]. In general, one-year survival rates for patients with Child-Pugh score A, B and C are 100%, 80% and 45%, respectively[20]. MELD score provides a more accurate prediction[21]. The hepatic clearance of exogenous administered substances, which provides an indication of residual liver functional mass[22,23], are easy to perform and may also meet future applications in family practice.

SELECTION FOR LIVER TRANSPLANTATION

Liver transplantation is considered as a viable treatment option for patients with acute liver failure and end-stage liver disease. In liver cirrhosis, transplantation is generally considered when a patient has suffered from either a complication of portal hypertension or a manifestation of compromised hepatic synthetic function[24]. However, given the high costs, mortality rate, and the paucity of donor organs, transplantation is currently justified only in the case of long-term prognosis, and psychological, intellectual, financial and family support. Accordingly, patients may be considered as current, future or inappropriate candidates. Selection consists of a search for contraindications and PCPs are actively involved in this process (i.e. alcohol and drug use)[25]. Currently, patients are generally put on a waiting list once Child-Pugh class B or a MELD score of over 13 is reached[21]. Onset of complications may anticipate referral, but severely decompensated or debilitated patients are generally discarded. Current indications and relative and absolute contraindications to liver transplantation are reported in Table 3.

TREATMENTS TO BE SHARED BETWEEN PCPs AND SPECIALISTS

Assistance is based on disease stage, complications and grade of self-sufficiency. Stable (compensated) patients are generally self-sufficient and a six month check (blood tests and liver ultrasonography) is indicated. Complicated and decompensated forms require an integrated approach with referral centers. Home care reduces costs[26] and should focus on a chronic care model of patient education and

Table 3 Current indications and contraindications to orthotopic liver transplantation in adult patients with liver cirrhosis

Indications	Contraindications
Advanced chronic liver failure	Relative
Child-Pugh score > 7	HIV seropositivity
Qualifying MELD score	Methadone dependence
	Stage 3 hepatocellular carcinoma
Acute liver failure	Absolute
Drug, toxins or virus induced fulminant hepatitis	Extrahepatic malignant disease
	AIDS
	Cholangiocarcinoma
	Severe, uncontrolled systemic infection
	Multiorgan failure
	Advanced cardiopulmonary disease
	Active substance abuse
General	
No alternative available treatment	
No absolute contraindications	
Willingness to comply with follow-up care and family assistance	

AIDS: Acquired immunodeficiency syndrome; HIV: Human immunodeficiency virus; MELD: Model for end stage liver disease.

on empowering both the patient and the family to take responsibility for the care (Table 4)[17]. Several cirrhotic patients can benefit from treatments aimed to slow disease progression (Table 1)[27-31]. In particular, nucleoside (Lamivudine, Telbivudine, Entecavir) and nucleotide (Adefovir, Tenofovir) analogues have shown to be safe and effective in reducing the risk of decompensation and disease progression in patients with HBV infection, while interferon plus ribavirin is a therapeutic option for under-compensated liver cirrhotic patients with HCV infection.

SPECIFIC PROBLEMS

Monitoring alcohol and drug abuse

Alcohol abuse causes 25% of liver cirrhosis and contributes to another 25%-50% of cases. PCPs play a key role in the application of long-term detoxification programs, counseling, support, and monitoring. This step is crucial, since recovered abusers are considered for antiviral therapy or transplantation only after six months of continuous abstinence (LEVEL Ⅲ).

Ascites

Ascites is the most common complication and cause of hospitalization of cirrhotic patients, but it is also the complication which can be better treated at home. Portal hypertension, reduced albumin synthesis, decreased plasma oncotic pressure, and sodium retention are all determining factors. Paracentesis usually removes a transudative fluid (i.e. albumin < 1 g/dL; serum/ascites albumin gradient > 1.1). Patients exhibiting abdominal pain, tense ascites and fever may have a spontaneous bacterial peritonitis (SBP), a condition characterized by an ascitic granulocyte count exceeding

Table 4 Standard objectives for an efficient out clinic care of cirrhotic patients

1	Early diagnosis of chronic liver disease. Identification of etiology
2	Identification of patients with chronic liver disease at risk of cirrhosis
3	Evaluation of patient's general health status
4	Act on etiologic factors and on factors favoring disease progression. Identify treatment end-points and place the patient within his family and social setting
5	Promote family and cohabitants' participation to primary prevention for infective forms (health education), secondary prevention for inherited or metabolic disorders, support and surveillance for toxic forms (alcohol)
6	Suggest health-dietetic measures and therapeutic remedies
7	Check parameters of effectiveness and control side effects of specific treatments (antiviral, phlebotomy, immune-depressants, β-blockers, *etc.*)
8	Identify and treat associated conditions (diabetes, osteoporosis, malnutrition, *etc.*)
9	Avoid administration of hepatotoxic drugs, drugs promoting renal sodium retention and central nervous system depressants
10	Promote vaccination against flu and pneumonia, including transplanted patients, and against hepatitis A and B virus
11	Supervise for complications by promoting clinical, biochemical and instrumental follow-up
12	Assist specialists in identifying candidates for liver transplantation
13	Assist the patient requiring legal problems

250/mm^3. SBP can precipitate cirrhosis towards renal and liver failure. Therapy includes high doses of albumin to prevent renal failure and intravenous cefotaxime at doses of 2 g twice a day (LEVEL Ⅱ). Long term prophylaxis of SBP recurrence with norfloxacin is indicated in survived patients (LEVEL Ⅰ). Ascites is considered refractory if it persists despite the use of diuretic drugs at the maximum tolerable dose. Although some studies indicate the utility of bed rest as a remedy, no controlled trials have been performed in support to this practice. Therefore, initial treatment is dietary salt restriction[32,33] (LEVEL Ⅰ). Therapy starts with spironolactone at doses ranging from 100 to 400 mg/d. Furosemide may be added (40 to 160 mg/d) when spironolactone does not successfully improve fluid retention (LEVEL Ⅰ). Weight should be monitored daily and electrolytes should be frequently monitored. Albumin infusion is required to prevent post-paracentesis circulatory dysfunction[34] following large volume paracentesis[35]. Such treatments can be managed by PCPs or in an integrated care system with consultant specialists. Preventive measures include the avoidance of NSAIDs, since they promote sodium retention. In the case of recurrent or refractory ascites, before considering the patient for a transjugular intrahepatic portosystemic shunt (TIPS), large volume paracentesis is feasible at home. Paracentesis is safe and rarely precipitates hepatorenal syndrome (LEVEL Ⅱ). Patients with SBP or refractory ascites have a more advanced disease with a poorer prognosis, and so require hospitalization. Patients and their family have to be taught the importance of a daily body weight check, and to refer FD when it increases by 2-4 kg over a brief period of observation.

Hepatorenal syndrome (HRS) is a life-threatening complication in patients with refractory ascites. Diagnosis includes the following criteria: advanced chronic liver failure with portal hypertension; serum creatinine exceeding 1.5 mg/dL or a 24-h creatinine clearance of less than 40 mL/min; absence of shock, ongoing bacterial infection, or recent treatment with nephrotoxic drugs; no sustained improvement in renal function following diuretic withdrawal and the expansion of plasma volume with 1.5 L saline; less than 500 mg/dL proteinuria and no ultrasonographic evidence of obstructive uropathy or parenchymal kidney disease[36]. While awaiting transplantation, patients with HRS, eligible for transplantation, may improve with medications, namely albumin, terlipressin, and vasoactive drugs or TIPS[37].

Portal hypertension

Active variceal hemorrhage accounts for about one-third of all deaths related to cirrhosis. Steps related to the prevention and treatment of variceal hemorrhage includes: prediction of patients at risk, prophylaxis against a first bleed, treatment of an active bleed, and prevention of rebleeding. Diagnosing and treating portal hypertension is a way to prevent esophageal variceal bleeding, and PCPs may play an active role in this respect. Varices appearance should be checked by upper endoscopy every 2-3 years, with a follow-up after 2 years for low-risk bleeding or every year for high-risk bleeding. Non-selective β-blockers are effective in reducing the risk of bleeding by reducing the resting heart rate by 25% (LEVEL Ⅰ). Endoscopic band ligation is indicated for patients susceptible of high-risk bleeding and for those who have already bled[38] (LEVEL Ⅰ). TIPS is an alternative option for patients with previously failed treatments[39] (LEVEL Ⅱ). A recent study has shown that early use of TIPS is associated with significant reductions in treatment failure and mortality[40].

Hepatic encephalopathy

Hepatic encephalopathy is a chronically debilitating complication of hepatic cirrhosis and encompasses a wide spectrum of potentially reversible neuropsychiatric abnormalities seen in patients with liver dysfunction. This condition is deemed as the onset of brain dysfunction due to metabolic abnormalities, which occurs as a consequence of liver failure. Hepatic encephalopathy is mainly caused by a reduced clearance of gut-deriving neurotoxins, and is a potentially reversible condition ranging from subtle personality changes to coma, with flapping tremor as a frequent initial finding. PCPs should search for acid-base and electrolyte disturbances, constipation, infections, gastrointestinal bleeding, and inappropriate use of sedative medications. Treatment consists of identifying and correcting the precipitating factors, colon cleansing and acidification with lactulose (LEVEL Ⅱ). Dietary protein restriction is no longer advocated since it may facilitate malnutrition and the appearance of complications. Rifaximin, a minimally absorbed oral antibiotic, has an antimicrobial effect against enteric bacteria and has received approval from the United States Food and Drug Administration for reducing the risk of overt hepatic encephalopathy recurrence. In a randomized, double-blind, placebo-controlled trial, six-month rifaximin therapy at a dose of 550 mg twice daily was compared with a placebo

in patients with chronic liver disease who were in remission from recurrent hepatic encephalopathy. Rifaximin maintained remission more effectively than the placebo and also significantly reduced the risk of hospitalization for hepatic encephalopathy[41] (LEVEL Ⅰ). Venous infusion of branched-chain amino acids or flumazenil may be effective in the case of comas (LEVEL Ⅱ). Patients may be managed at home; admission to hospital is reserved for those who are non-responsive after 12 h treatment.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major complication of liver cirrhosis representing an increased cause of mortality; liver transplantation and cost management in most developed countries. As a consequence, screening for HCC is one of the most important tasks in patients with liver cirrhosis. American and European guidelines currently recommend at least one imaging screening/year for HCC (ultrasonography, triphasic CT). Serum alpha-fetoprotein has poor sensitivity and therefore is recommended only as an adjunctive screening marker[37,42]. Once HCC is detected, many treatment options are available, mainly depending on tumor size and number, and local expertise. Surgical resection can be effective; unfortunately most patients do not tolerate liver resection or have microscopic lesions, and so the best option for a cure remains liver transplantation. The Milan criteria are used as a guideline worldwide[43,44], and suggest that a four-year survival rate of 75 percent is achieved if liver transplantation is performed for either a single lesion of less than 5 cm in diameter, or up to 3 lesions with none larger than 3 cm. Outcomes are similar to the expected survival rates for patients undergoing transplantation for cirrhosis without HCC (LEVEL Ⅰ). Alternative treatments for patients who do not meet the criteria for resection or transplantation are ultrasound guided radiofrequency ablation, chemoembolization and alcohol ablation. These options are considered as a form of "bridging therapy" because it reduces tumor burden and delays tumor progression[45], and do not preclude future liver transplantation, if a donor organ becomes available.

Infections

Sepsis represents a high risk factor for mortality in cirrhotic patients which often do not present the typical signs and symptoms of infection (i.e. absence of leukocytosis due to severe leukopenia or even absence of fever). The active search for infections is important (cultures, X-ray, paracentesis, *etc.*). Most common infections concern the urinary tract (25%-55%), spontaneous bacterial peritonitis (10%-30%), and respiratory tract infection (20%). First line antibiotics include quinolones and cephalosporins[46] (LEVEL Ⅲ). Hospitalization is required for poor general health and/or the appearance of organ dysfunction.

SYSTEMIC PROBLEMS

Malnutrition

Malnutrition represents a negative prognostic factor for cirrhosis and consists of muscle wasting, hypoalbuminemia, decreased resistance to infections, and variceal bleeding. Causes include poor oral nutritional intake, malabsorption, ongoing alcohol use, chronic nausea, and early satiety due to abdominal compression from ascites. Nutritional status should be monitored in all cirrhotic patients; multivitamin supplementation is often indicated[47]. Nutritional support should be reserved only for severely malnourished patients scheduled for transplantation[48]. Oral supplementation with a branched chain amino acids has some utility by improving event-free survival in patients with decompensated liver cirrhosis[49]. Dental care is particularly important to allow adequate mastication.

Osteoporosis

In individuals with chronic liver disease, metabolic bone disease (hepatic osteodystrophy), is a potential complication of long-standing hepatic disease. It is therefore essential to prevent the development of fractures in individuals with advanced hepatic disease and those that have undergone liver transplantation[50]. In end-stage cirrhosis, vitamin D deficiency, hypoparathyroidism, and hypogonadism contribute to reduced bone formation. Osteopenia may occur early in patients with cholestasis or in those put on antiviral drugs[51]. This is also the case in patients after orthotopic liver transplantation[52]. Bisphosphonates, together with calcium and vitamin D_3, are effective in improving bone mineral density[53] (LEVEL Ⅱ).

Diabetes

Diabetes and cirrhosis are strictly interrelated, the first occurring with increased frequency in patients with NASH, hepatitis C or hemochromatosis. In a multivariate analysis, diabetes was an independent negative factor for liver disease evolution[54]. No controlled studies have tested the benefit of different regimens for cirrhotic patients with diabetes. Diet remains the first line remedy to control hyperglycemia. In the case of dietary failure, metformin is generally the first choice. Sulphonylureas can be used, but mindful of the risk of hypoglycemia. Glitazones are a new alternative, although no studies in liver cirrhosis have been performed. In any case, oral anti-diabetic drugs are not indicated in decompensated patients. Insulin represents the best approach, although this requires good self-monitoring (LEVEL Ⅲ).

PREVENTION

Primary prevention

The role of PCPs is important for this issue. The most attractive form of protection for liver cirrhosis is to prevent or slow the evolution of several risk factors triggering the hepatitis-fibrosis sequence. Mass infant vaccination has proven extremely effective in preventing hepatitis B infection. Screening blood donors effectively reduces hepatitis C transmission (LEVEL Ⅰ).

Secondary prevention

This step aims at preventing the appearance of cirrhosis

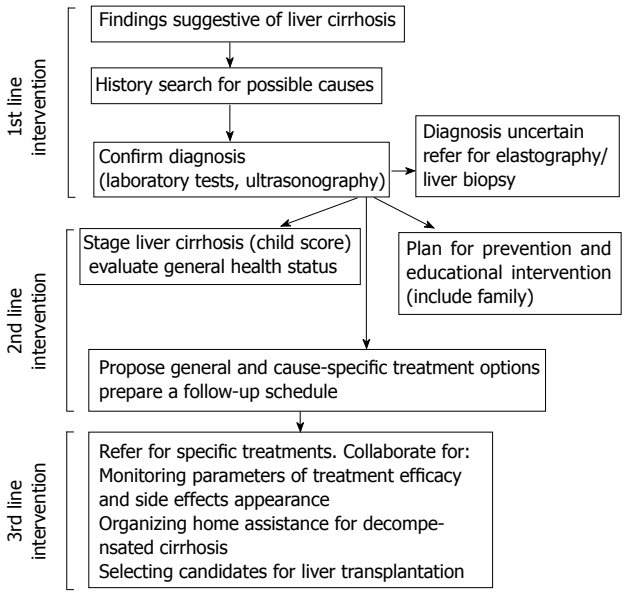

Figure 1 Algorithm for the management of patients with (or with suspected) liver cirrhosis in General Practice.

in patients with chronic liver disease and includes etiologic treatment for viral hepatitis, alcohol abstinence, phlebotomy in hemochromatosis, weight loss and improving insulin resistance in NASH patients[1]. Early detection of HCC by six-monthly ultrasonography and blood alpha-fetoprotein measurement may allow successful liver transplantation or mini-invasive treatments (LEVEL Ⅰ).

Prevention of infections

Vaccine immunization against hepatitis A and B, pneumococcus and influenza is important in preventing general status deterioration. SBP recurrence can be reduced by antibiotic prophylaxis (once-daily 400 mg norfloxacin or once-weekly 750 mg of ciprofloxacin)[55].

THE ROLE OF PCPs FOR OPTIMIZING CARE

The incidence of liver cirrhosis is expected to increase in the near future. Beside B and C viral infection and alcoholic cirrhosis, nonalcoholic liver steatosis (non-alcoholic fatty liver disease, NAFLD) is considered the hepatic manifestation of a new epidemic: the metabolic syndrome. This frequent condition is a cluster of risk factors for coronary heart disease and type 2 diabetes mellitus that includes visceral obesity, elevated blood pressure, insulin resistance, and dyslipidemia[56,57]. The onset of NAFLD represents a bridging condition between cardiovascular risk and potentially evolutive forms of liver diseases, namely steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma[58,59]. The PCPs are therefore asked to play a key role in programs involving prevention, treatment, surveillance, and home care of populations at risk (Figure 1)[4,60]. Referral of patients to specialists at least once is a good practice, since integrated management between PCPs and specialists is indeed associated with better outcomes[61]. Active cooperation is required for etiologic treatments, screening for complications and approaches to liver failure. However, appropriate timing for referral varies on an individual basis according to liver function and general health status, and this should include patient age, level of test abnormality, need for prognosis, and therapeutic decision. The need for a multidisciplinary approach should be considered, which includes feedback from dietitians, psychologists, and physical activity supervisors[62]. This integrated approach optimizes therapy adhesion, but necessitates the regular updating of health personnel[60].

Table 5 Features of home assistance in patients with liver cirrhosis

Advantages
Decreased number of hospitalization and re-admissions
Decreased costs of treatments
Assist the patient within his familiar comfort
Criteria of eligibility
Identification of a clinical status allowing home stay
Identification of priority criteria
Presence of a valid family support or of an active aid system
Selection criteria
Use the Karnofsky Performance Status[1] for patients with decompensated liver cirrhosis and limited self-sufficiency (set to < 50%)

[1]The Karnofsky Performance Scale Index allows patients to be classified as to their functional impairment. This scale is often used in the primary care setting to assess the prognosis in individual patients and to decide a treatment; the lower the score, the worse the survival.

PCPs can manage cirrhotic patients by checking therapy effectiveness and side effects. With the exclusion of major digestive bleeding, even severely decompensated patients or those in an irreversible coma or advanced HCC can be home managed (with the assistance of specialists and specialized nurses). Hepatic encephalopathy can be treated with lactulose (oral or rectal enema) and the minimally absorbed rifaximin, by controlling electrolytes, and treating infections. Ascites can be controlled with diuretics, albumin infusion or paracentesis. Albumin boosts the efficacy of diuretics, and reduces the number of hospital admissions[35]. Home care of cirrhotic patients should be encouraged since it allows a saving of up to two/third of the normal cost (Table 5) (LEVEL Ⅱ-Ⅲ).

CONCLUSION

Liver cirrhosis has an increasing prevalence worldwide, which matches the increasing diffusion of viral hepatitis infection, and metabolic steatohepatitis and fibrosis. Managing cirrhotic patients at home is challenging but cost-effective, although this policy requires active collaboration between PCPs and specialists, as well as nurses and paramedical staff. A set of conclusive key messages for practice are reported in Table 6.

Table 6 Key messages for best management of cirrhotic patients

Statement	Evidence level
1 A compensated liver cirrhosis is suspected with abnormal liver function tests, low platelets count, and prolonged prothrombin time[63]	Ⅲ
2 Ultrasonography is a reliable, non-invasive, fast, and cost-effective test working as a first-line tool for diagnosing liver cirrhosis[64]	Ⅱ-Ⅲ
3 Child-Pugh and MELD scores assess the prognosis of liver cirrhosis[19,20]	Ⅰ
4 First-line treatment of patients with cirrhotic ascites includes diuretics and sodium restriction. Anti-aldosterone drugs are given with loop diuretics to increase diuretic response or when renal perfusion is impaired. Dietary salt intake should be restricted to approximately 88 mmol/day (2000 mg/d). Marked salt restriction can expose the risk of hyponatremia[32,37]	Ⅰ
5 Removal of less than 5 liters of fluid does not appear to have a hemodynamic consequence. For larger paracentesis, albumin (6 to 8 g/L of fluid removed) can be administered. Albumin is indicated in patients with PBS to prevent renal failure, and in patients with hepatorenal syndrome. Albumin can be also used to treat refractory ascites. Its infusion at home is safe and cost-effective[37,65]	Ⅱ
6 β-blockers (e.g. propranolol or nadolol) are recommended for prophylaxis of variceal bleeding at a dosage titrated to a 25 percent reduction in pulse rate[66]	Ⅰ
7 Liver transplantation is the only definitive care for patients with major complications (ascites, bleeding, HCC) and/or MELD above 13[1]	Ⅰ
8 Osteoporosis is an important systemic complication of end-stage liver cirrhosis. Management includes vitamin D and bisphosphonates[53]	Ⅱ
9 Malnutrition is a negative and independent predictor of survival in patients with liver cirrhosis[67]	Ⅱ
10 An integrated assistance of patients with liver cirrhosis has a better outcome than the management by generalists/specialists alone[61]	Ⅱ

LEVEL Ⅰ: At least one properly conducted RCT, systematic review, or meta-analysis; LEVEL Ⅱ: Other comparison trials, non-randomized, cohort, case-control, or epidemiologic studies, and preferably more than one study; LEVEL Ⅲ: Expert opinion or consensus statements (see text for details); MELD: Model for end-stage liver disease.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2283

World J Gastroenterol 2011 May 14; 17(18): 2283-2287
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Gastroenterology training in Latin America

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Received: August 20, 2010 Revised: December 27, 2010
Accepted: January 3, 2011
Published online: May 14, 2011

Abstract

Latin America is characterized by ethnic, geographical, cultural, and economic diversity; therefore, training in gastroenterology in the region must be considered in this context. The continent's medical education is characterized by a lack of standards and the volume of research continues to be relatively small. There is a multiplicity of events in general gastroenterology and in sub-disciplines, both at regional and local levels, which ensure that many colleagues have access to information. Medical education programs must be based on a clinical vision and be considered in close contact with the patients. The programs should be properly supervised, appropriately defined, and evaluated on a regular basis. The disparity between the patients' needs, the scarce resources available, and the pressures exerted by the health systems on doctors are frequent cited by those complaining of poor professionalism. Teaching development can play a critical role in ensuring the quality of teaching and learning in universities. Continuing professional development programs activities must be planned on the basis of the doctors' needs, with clearly defined objectives and using proper learning methodologies designed for adults. They must be evaluated and accredited by a competent body, so that they may become the basis of a professional regulatory system. The specialty has made progress in the last decades, offering doctors various possibilities for professional development. The world gastroenterology organization has contributed to the speciality through three distinctive, but closely inter-related, programs: Training Centers, Train-the-Trainers, and Global Guidelines, in which Latin America is deeply involved.

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Key words: Training; Gastroenterology; Latin America

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Cohen H, Saenz R, de Almeida Troncon LE, Lizarzabal M, Olano C. Gastroenterology training in Latin America. *World J Gastroenterol* 2011; 17(18): 2283-2287 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2283.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2283

INTRODUCTION

Latin America is home to over 50 countries and a population close to 600 million people. The two main languages are Spanish and Portuguese, but there is also long list of native languages. In the last 60 years, the economy of Latin America and the Caribbean grew by 4%, while the population increased by 2.1% annually. Although unemployment has dropped in recent years, it still averages 7.5%. Although the number of poor people in the overall population has dropped by more than 9% between 2002 and 2007, it is still

around 35%, which implies that 210 million people live in poverty in the region, i.e. prior to 1980, 39%of the population were poor. More positive results have been obtained for people living in extreme poverty; it is estimated that in 2007, 12.7% of the population lived in indigence, versus 18.6% in 1980; however, in absolute terms, the number of people affected increased from 62 million to 76 million in the same period. The mortality rate of children under five years of age is 27 per 1000 (27‰), that is one third of the average observed in developing countries (81‰). However, some nations like Bolivia (61‰) and Haiti (72‰) lag behind in this regard. With regard to income distribution, the region has experienced some modest progress. One of the elements that have been of concern to the regional economic authorities in 2007 and 2008 has been the rise of inflation in the region. According to ECLAC's (Economic Commission for Latin America and the Caribbean) estimates, since early 2006, and with greater impetus in 2007, consumer prices rose with increasing speed in most economies in the region, with annual increases of 7% to 30% in the various countries and an average close to 16%[1].

Most countries in Latin America and the Caribbean have succeeded in enforcing universal primary education, and they are experiencing an expansion of pre-school, secondary, and tertiary education.

In 2006, there were 16 million students enrolled in further education in Latin America and the Caribbean. The average rate of enrollment in universities went from 21% to 31% between 1999 and 2006, but varied from one country to another (3% in Belize to 88% in Cuba)[2].

To summarize, Latin America is characterized by ethnic, geographical, cultural, and economic diversity; therefore, training in gastroenterology in the region must be considered in the framework of this context.

CURRENT STATUS OF MEDICAL EDUCATION IN GASTROENTEROLOGY IN LATIN AMERICA

The continent's medical education is typically characterized by the following features: a large number of medical schools in some countries, most of which grant no local accreditations; teaching is done by various agencies in the country, with no common programs and without previously agreed requirements for the gastroenterologist's (GE) or the endoscopist's training. In sum, a lack of standards is its main feature.

Current status of scientific research in gastroenterology in Latin America

Despite the efforts of several groups, especially those working in Mexico, Brazil, Peru, Chile, and Argentina, the volume of research continues to be relatively small, highlighting some contributions in the field of *Helicobacter pylori* and Celiac Disease, among others. There is still much to learn and report about the continent, especially with regard to epidemiology.

Table 1 Number of citable papers in all gastroenterology fields indexed at the SCOPUS database originating from three South American countries

Year	Argentina	Brazil	Colombia
2000	20	100	3
2008	28	220	46

Data were extracted from SCImago-Journal and Country Rank (retrieved on July 13, 2010, from http://www.scimagojr.com).

Nevertheless, continued investment in scientific research by government agencies in some countries, such as CONICET (Argentina), CNPq (Brazil) and COLCIENCIAS (Colombia) has been associated in recent years with a remarkable increase in the output of indexed papers in all fields, including gastroenterology, coming from these countries (Table 1).

Scientific events in gastroenterology in Latin America

There is such a multiplicity of events, that they might even be considered excessive. There is usually a succession of general gastroenterology events as well as others that deal with sub-disciplines, both at regional and local levels. Although this results in an undesirable fragmentation of the field, the positive consequence is that these events ensure that many colleagues have access to information, with the caveat that such information may not always be necessarily reliable or of good quality. It is important to address the role of the pharmaceutical industry and medical equipment in the continuing professional development programs (CPDPs).

Needs: Medical education programs must be based on a clinical vision and be considered in close contact with the patients. They should require previous training in Internal Medicine (via a two-year internship or a full postgraduate fellowship).

Proper supervision of the programs must be ensured. It is essential for each institution that the courses be accredited and to have links with the School of Medicine, to ensure that the premises are adequate, and that there is the necessary equipment and clinical support.

It is important to guarantee that the gastroenterologist's (GE's) training course is of an adequate duration (two to three years) with a minimum dedication of six hours a day. The recommendation is to prioritize the training of trainers that have no teaching background.

Moreover, it is essential for the programs to be appropriately defined and evaluated on a regular basis. The programs should guarantee the GE's training concerning the development of their skills and attitudes. The importance of electronic methods (e-teaching, e-learning, the use of the internet) must be emphasized. The syllabus should also contemplate the teaching of basic administration skills.

The disparity between the patients' needs, the scarce resources available, and the pressures exerted by the health systems on doctors are frequently cited by those that complain about their poor professionalism.

Teaching development can play a critical rol in ensuring the quality of teaching and learning in universities. "*A good university teacher is not the one who prepares their students to pass an exam, it is the one who obtains a students valuation of learning and a critical thought. He is also the one who encourages them to solve problems with creativity and curiosity and with ethical commitment as well as with a desire of improving their knowledge in a specific subject.*"[3].

Briefly put, the CPDP activities must be planned on the basis of the doctors' needs, with clearly defined objectives, using proper learning methodologies designed for adults. Andragogy, defined as "the art and science of helping adults learn", is based on five assumptions about how adults learn and their atitude towards, and motivation for, learning: (1) adults are independent and self directing; (2) they have accumulated a great deal of experience, which is a rich resource for learning; (3) they value learning that integrates with the demands of their everday life; (4) they are more interested in immediate, problem centred approaches then in subject centred ones; and (5) they are more motivated to learn by internal drives than by external ones.

The CPDP activities must also be evaluated and accredited by a competent body, so they may become the basis of a professional regulatory system (recertification or others). One of the greatest challenges is to balance the needs of professional employers and health care systems with those of the patients.

WHAT SHOULD THE FUTURE GASTROENTEROLOGIST LEARN?

Future specialists naturally aim at following their vocation, working with dignity and ethics, applying their knowledge, maintaining an ongoing training, and at times, they may seek academic development (teaching or research). The specialty has made progress in the last decades, offering the doctors various possibilities for professional development; it is no longer the outlook faced by the clinical gastroenterologist 50 years ago, or by more recent endoscopists. Today's GE can choose to develop an in-depth knowledge on certain sub-specialties, such as nutrition, hepatology, transplantations, interventional endoscopy, capsule endoscopy, NOTES (Natural Orifice Translumenal Endoscopic Surgery), motility, etc. In sum, the Latin American GE's training must be comprehensive, taking into account both society's interests, as well as the doctors' legitimate objectives.

SOME EXAMPLES ON THE TRAINING OF GASTROENTEROLOGISTS IN LATIN AMERICA

In Argentina, there are no unified criteria to be met to graduate as a gastroenterologist. The courses are dictated by the Argentine Society of Gastroenterology in partnership with the University of Buenos Aires, or by private or state-run schools of medicine in the interior of the country. Overall, there are eight courses that train GEs lasting for two to three years. Entry to the courses requires the completion of two years of internal medicine. There is no single accreditation body, nor are there any unified requirements for the training of endoscopists or liver experts[4].

In Uruguay, the specialists' training depends on the state School of Medicine's School of Graduates. It does not request a post-graduate degree in internal medicine. The gastroenterology course takes three years on a part-time basis, and it includes theoretical and hands-on activities that also include liver diseases. A degree in endoscopy has been recently created.

In Chile, gastroenterology is viewed as part of internal medicine. A GE is an internist that must go through three additional years in the specialty. There are several university programs in gastroenterology. The universities are recognized by the National Medical Certification Board.

Both Argentina and Chile have implemented mechanisms to regularly renew accreditation to specialists.

In Brazil, specialist training in gastroenterology has been regulated since 1977 by federal legislation, which established a two-year program in accredited institutions for candidates who have already completed a previous two-year basic training in Internal Medicine. Thus, the typical training takes four years, but most programs affiliated to university hospitals offer an additional elective year in sub-specialties, such as endoscopy or hepatology. After completing the specialist training, gastroenterologists are eligible to apply for certification, conferring the specialist title, which is provided by the Brazilian national gastroenterology society, known as Federation of Gastroenterology (FBG), to candidates passing the relevant examinations. Accreditation of institutions is provided by a federal agency, the National Commission for Medical Residency, on the basis of the characteristics of both the institution (infrastructure, number of hospital beds, average of outpatients visits, certified personnel, *etc*) and the program quality (balance between inpatients and outpatients activities, supervision, hours of endoscopy training, *etc*).

CME (Continuous Medical Education) in Brazil has been a compulsory requirement for the renewal of the specialist title since 2005, following the creation of the professional update certificate CAP (acronym for Certificado de *Atualização* Professional). Starting in January 2011, the titles will be renewed only to those who obtained their CAP or acquired a minimum of 100 CME credits in the previous five years. Those who fail have to sit an additional exam to keep their title. The national societies of each specialty, such as the Brazilian FBG, participate in the National Accreditation Committee (CNA) with other organizations, such as the Brazilian Medical Association or the Medical Federal Board. The CNA evaluates organizers' applications for activities that intended to grant CME credits and validates the credits already obtained by doctors. The national societies are requested to organize activities to provide a minimum of 40 credits a year. This is followed strictly by the FBG, which has the organization of CPDP meetings and distance learning activities as one of its main aims. Regarding the Medical Schools and the University hospitals, de Almeida Troncon *et al*[5] thinks that they do not devote enough time

and effort to comply with that requirement. His concerns about the support of the CME activities by the biomedical industry are based on the lack of independence of the programs. Lack of evaluation of the activities is another weakness of the program in Brazil, where the cognitive aspects are emphasized over the acquisition of skills and attitudes. Evidence that the CME activities in Brazil are positive for the quality of medical work is still scarce. Troncon suggests that new ways of implementing CME have to be found: funding must come from the Medical Schools and the working institutions, which should adopt the principles of adult learning, especially in identifying the needs of learners, developing the objectives of the activities, and finally designing the curricula. Finally, he stresses that the evaluation is a key issue in that process.

Venezuela: There are approximately seventy students within the sixteen post-graduate programs in gastroenterology in Venezuela. All of them differ in curriculum, academic and research structure, and even in their own graduation profile. The group led by Lizarzábal *et al*[6] tested their individual and global quality by conducting a users-satisfaction survey, questioning students and program directors. The sample included 46 students who answered anonymously. The students' results showed that 13% of the programs were considered to be of excellent quality (A) and 8.7% of the programs were graded as B (good). An important group (71.7%) was graded as C (bad) and 6.5% as D (very bad). Users' perception differed from the perception of directors, who evaluated the quality of more than half (57%) as A-B, while only 21.7% were graded A-B by the users.

In view of the above results, the Society of Gastroenterology of Venezuela made some recommendations to improve the quality of the postgraduate teaching programs, which are summarized as follows: (1) Implementing structured and explicit curricular designs in 100% of the Venezuelan Gastroenterology Postgraduate Programs; the programs that already have such designs (60%) will be asked to apply consistently unified criteria; (2) Request that 100% of the programs be accredited by the National Universities Council (CNU) before pursuing certification and re-certification; (3) Strengthen a culture of research; (4) Develop evaluation strategies to allow monitoring of the service provided; (5) Improve proficiency of the human resources available. Most of the staff lack the academic training needed to implement an adequate curricular design, evaluation, research, and learning strategies; and (6) Plan and reach consensus on education in Gastroenterology with a broad participation of the stakeholders. The profile and training of the Venezuelan gastroenterologist is still to be defined.

CONTRIBUTIONS OF THE WGO (WORLD GASTROENTEROLOGY ORGANIZATION) TO THE SPECIALITY IN LATIN AMERICA

The current objectives of WGO are enshrined in its mission statement: "to promote, to the general public and health care professionals alike, an awareness of the worldwide prevalence and optimal care of digestive disorders through the provision of high quality, accessible and independent education and training", which signals the commitment of WGO to address two challenges: firstly, providing the gastroenterologist of the future with an optimal training and, secondly, bringing the benefits of digestive health care to those who currently struggle or, indeed, fail to achieve access to it. The primary emphasis of WGO, therefore, is on education and training. These objectives are achieved through three distinctive, though closely interrelated, programs: Training Centers, Train-the-Trainers, and Global Guidelines.

Training Centers most directly address the issue of training specialists in gastroenterology or individuals with additional expertise in gastroenterology to serve previously underserved areas. Each centre represents a direct collaboration between local experts, international faculties, and national and regional societies from Europe and North America to deliver regionally relevant training to those who have limited, or in some cases, no access to such opportunities. The centers in Latin America: La Paz, Bolivia; La Plata, Argentina; Santiago, Chile; Mexico City, Mexico; San José, Costa Rica; and Bogota, Colombia, provide training of variable duration to several hundred young and aspiring gastroenterologists and digestive surgeons from underserved nations in the region. The newest center was inaugurated two years ago in Ribeirao Preto, State of Sao Paulo, Brazil, and is dedicated solely to training in gastrointestinal motility techniques. The centers offer an in-depth view of the various aspects of the field (clinical, endoscopic, and motility). On the other hand, the Inter American Association of Gastroenterology (Asociación Interamericana de Gastroenterología: AIGE) has created scholarships to facilitate the access of six gastroenterologists to the WGO's teaching centers annually.

Finally, it is an important aim for WGO to create an electronic network among all its teaching centers that will include the seven in Latin America. This network should be accessible to all who seek to train in our specialty; thereby, ensuring the highest standards of care for those who suffer from digestive disorders through the world.

Likewise, the WGO has already organized four courses called "Train the Trainers" (TTT) in South America (Uruguay, Brazil, Chile, and Peru), in an attempt to remedy a global problem, i.e. the local faculties' lack of training on educational methodology to teach at university level. Train-the-Trainers courses are uniquely devoted to bringing the very latest in educational techniques to those who will train the gastroenterologists of the future, including those who teach and train at the Training Centers. The TTTs are developed in several modules aimed at teaching educational skills to faculties, in a user-friendly manner, in an informal and friendly atmosphere.

Even if gastroenterologists should nowadays be fluent in English, language can be an issue in such TTT courses in Latin America. The WGO has decided to begin TTTs in Spanish in 2011.

Another activity pushed forward by the WGO, and in which Latin America has been deeply involved, is the devel-

opment of the Clinical Guidelines (CG), with one peculiarity: they are the only ones to consider the availability of resources globally, through the so-called cascade mechanism that enables a professional to adapt the situation to a specific patient, in the patient's own context. To make guidelines more applicable to different resource environments, the concept of "cascades" has been developed. A cascade is a collection of related diagnostic and treatment options arranged hierarchically in terms of conditions and available resources. Whilst guidelines should continue to summarize best known practice, they could also include alternatives for clinicians with limited funding. These alternatives are usually on the basis of cost, but could also take account of local availability, technology, and infrastructure[7].

The complete texts of all the CGs are fully available at the site of the WGO (www.worldgastroenterology.org) in six languages, including Spanish and Portuguese. Outstanding Latin American gastroenterologists were involved in the development of several CGs, such as the one on Celiac Disease or *Helicobacter pylori* in the developing world.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2288

World J Gastroenterol 2011 May 14; 17(18): 2288-2301
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Silybin and the liver: From basic research to clinical practice

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Received: November 18, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: May 14, 2011

Abstract

Herbal products are increasingly used, mainly in chronic liver disease. Extracts of milk thistle, Silymarin and silybin, are the most prescribed natural compounds, with different indications, but with no definitive results in terms of clinical efficacy. This review analyzes the available studies on the effects of the purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease. We searched PUBMED for articles pertaining to the *in vitro* and *in vivo* effects of silybin, its antifibrotic, anti-inflammatory, and antioxidant properties, as well as its metabolic effects, combined with the authors' own knowledge of the literature. Results indicate that the bioavailability of silybin phytosome is higher than that of silymarin and is less influenced by liver damage; silybin does not show significant interactions with other drugs and at doses < 10 g/d has no significant side effects. Experimental studies have clearly demonstrated the antifibrotic, antioxidant and metabolic effects of silybin; previous human studies were insufficient for confirming the clinical efficacy in chronic liver disease, while ongoing clinical trials are promising. On the basis of literature data, silybin seems a promising drug for chronic liver disease.

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Key words: Milk thistle; Silymarin; Liver disease; Hepatic fibrosis; Hepatic inflammation; Radical species

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Loguercio C, Festi D. Silybin and the liver: From basic research to clinical practice. *World J Gastroenterol* 2011; 17(18): 2288-2301 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2288.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2288

INTRODUCTION

The terms milk thistle, flavonoids, silymarin, and silybin are generally used interchangeably; however, each of these compounds has specific characteristics and actions, with an intrinsic beneficial or toxic effect. In the last 10 years, about 12 000 papers have been published on these substances, used as antioxidants or chemopreventives and anticancer agents, and especially as hepatoprotectants. This publication volume indicates that scientific interest in these molecules, or classes of molecules, is high worldwide. In the US and Europe, about 65% of patients with liver disease take herbal preparations; in Europe, the cost of the use of silymarin reaches $180 million in Germany alone. Despite the wealth of literature, no firm clinical evidence exists to recommend the use of these substances in clinical practice[1-10]. This discrepancy is attributable to various factors, such as quality of clinical trials, heterogeneity of diagnoses, lack of standardized preparations, and frequently inconsistent dosing and outcome parameters. At a time when the use of herbal products is increasing, whether driven by individual choice or industry promotion, in our opinion it is necessary to focus more intently on these compounds that may have beneficial, placebo, or toxic effects.

This review analyzes studies of the effects of the

purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease.

DEFINITION AND CHARACTERISTICS OF SILYBIN

As reported, silybin and silymarin are not synonymous[1,3,6]. Silymarin is a complex of at least seven flavonolignans that are the most common class of compounds present in milk thistle extract, and one flavonoid, taxifolin. The relative abundance of each compound may vary depending on the source of botanical material, supplier, and extraction processes. Silybin represents about 50% to 70% of the silymarin extract. Silybin can be resolved into two 1:1 diastereoisomers, silybin A and silybin B. In addition, silybin may be present as isosilybin, a 1:1 mixture of two diastereoisomeric compounds, isosilybin A and isosilybin B[11-17]. The concentrations of silybin in the main pharmaceutical products containing silymarin present in the US and other countries range from 20% to 40%[16].

PHARMACOKINETICS AND PHARMACODYNAMIC ASPECTS

Flavonolignans are known for their poor and erratic bioavailability; for example, silymarin absorption rate levels vary between 20% and 50%. Silybin has been separated commercially as a pure substance[11-16], and the study of silybin pharmacokinetics properties using an HPLC method has shown that the concentration-response relationship is linear over a concentration range of 0.5-100 μg/mL[16]. After administration to rats, the disposition of silybin in the plasma and bile fluid is due to rapid distribution and equilibrium between the blood and hepatobiliary system, and the bile levels of unconjugated and total silybin are greater than those in plasma[18-22].

Similarly to other flavolignans, limiting factors for the use of silybin are its low solubility in water, low bioavailability, and poor intestinal absorption. To counteract this aspect, different more soluble derivatives of silybin have been synthesized, such as silybin bis-hemisuccinate, β-cyclodextrin complex, silybin-N-methyl-glucamine, silybin 11-O-phosphate, and silybin-phosphatidylcholine. Another strategy for improving silybin solubility is represented by the enzymatic synthesis of its β-glycosides, such as silybin β-galactoside, silybin β-glucoside, silybin β-maltoside, and silybin β-lactoside. A soluble silybin prodrug has been finally synthesized with a high aqueous soluble polymeric carrier (polyethylene glycol)[23-30].

Some conjugations may in part affect the activity of silybin. For example, the radical-scavenging activity of the silybin 20-O-β-D-glucuronide is considerably lower than that of free silybin. In contrast, a considerable increase in radical-scavenging activity is observed in the other silybin, 7-O-β-D-glucuronide, in which position C-20 is free. The increase in the radical-scavenging activity of this latter conjugate cannot be simply ascribed to the addition of the glucuronyl moiety. The change in antiradical activity is, therefore, the result of the overall structural change. It is also quite interesting that one diastereoisomer of silybin (B) undergoes conjugation faster than the other (A); this difference indicates that the two silybin diastereoisomers are metabolized at different rates. These findings will aid the development of improved silybin formulations designed to inhibit the C-20 conjugation or, alternatively, to contribute to a modified dosage scheme that maintains free plasma silybin at sufficient levels[31-35]. Both free and conjugated silybin have a rapid plasma and tissue distribution that reaches maximum levels within one hour after 50 mg/kg silybin administration in mice[36]. The protein binding of silybin in rat plasma is 70.3% ± 4.6%[21].

In humans, the pharmacokinetics of silybin was evaluated after the administration of variable doses of silymarin, pure silybin, and silybin conjugated with phytosome in healthy volunteers. Hoh *et al*[37] for the first time identified the silybin plasma metabolites and measured the silybin tissue levels in humans who had ingested silybin. They demonstrated that silybin undergoes multiple conjugation reactions in humans and clearly identified the conjugated species: silybin monoglucuronide, silybin diglucuronide, silybin monosulfate and silybin glucuronide sulfate. The administration in humans of 240 mg of pure silybin induces a peak concentration of 240 ± 54 ng/mL in about 2 h that persists for 4 h. After administration of a single oral dose of 560 or 600 mg silymarin (about equivalent in total to 240 mg of silybin), the fractions of the free, sulfated, and glucuronidated silybin in human plasma are about 17%, 28%, and 55% of the total dose, with a higher plasma percentage of glucuronidated silybin B (71%) than of glucuronidated silybin A. The total percentage of dose recovered in urine, as free and conjugated, is very low, ranging from 1% to 7% of the dose (mean: 2.8 ± 0.6 ng/mL)[29,38-42]. The complex silybin phytosome (silybin + phosphatidylcholine) was recently formulated with the addition of vitamin E (Indena, IBI-Lorenzini spa Italy: Realsil®). This conjugation induces a greater solubility of silybin, as reported in Table 1. In 12 healthy volunteers aged 20-53 years, silybin was administered as Realsil® in two pharmaceutical forms (capsules and granules, both corresponding to 47 mg of silybin). Data were compared to those obtained by administering, in a cross-over manner after 1 wk of wash-out, a silymarin capsule containing 58 mg of silybin and silymarin granules containing 80 mg of silybin[43]. As summarized in Tables 1 and 2, the global results of the pharmacokinetic analysis indicated that the bioavailability of silybin phytosome is much higher than that of silymarin.

EFFECT OF LIVER DAMAGE ON PHARMACOKINETICS OF SILYBIN

In patients with well-compensated liver cirrhosis, silybin was administered as silybin phytosome at a dose of 120 mg

Table 1 Pharmacokinetics of silybin phytosome and silymarin in healthy participants[38-43]

	Silybin phytosome	Silymarin
Peak concentration (ng/mL)	298 ± 96	102 ± 22[a]
Time of peak (h)	1.6 ± 0.3	1.4 ± 0.3
Mean residence time (h)	3.6 ± 0.4	3.5 ± 0.4
AUC (ng/mL/h)	881 ± 207	257 ± 66[b]

[a]$P < 0.05$, [b]$P < 0.01$. NB: The analyses were performed using HPLC.

Table 2 Pharmacokinetics of silybin after administration to 12 healthy volunteers as silymarin or silybin-phosphatidylcholine-vitamine E complex (RA)[43]

Product	Cmax (ng/mL)	Tmax (h)	AUC (ng/mL per hour)
RA granules (47 mg silybin)	213 ± 166	0.5	246 ± 114
RA capsules (47 mg silybin)	117 ± 93	1.0	161 ± 85
Silymarin gran (58 mg silybin)	18 ± 17	0.5	25 ± 18
Silymarin caps (80 mg silybin)	5 ± 7	1.0	11 ± 5

× 3 /d, or as silymarin containing 84 mg of silybin 4 fold/d. The results showed that liver cirrhosis does not modify the kinetics of silybin and that in liver patients, the bioavailability of silybin is higher when it is administered as a phytosome adduct[44]. Similar data were obtained in rats with experimentally induced cirrhosis[45]. Table 3 summarizes the data obtained in humans.

More recently, the pharmacokinetics of silybin has been evaluated in patients with chronic hepatitis [hepatitis C virus (HCV) or non-alcoholic fatty liver disease (NAFLD)] and compensated liver cirrhosis[46,47]. A single, 600 mg p.o. dose of milk thistle extract was administered to healthy volunteers and to three patient cohorts, and blood samples were obtained over 24 h. Silybin A and B accounted for 43% of the exposure to the sum of total silymarin flavonolignans in healthy volunteers and only 31% to 38% in liver disease cohorts as a result of accumulation of silychristin (20%-36%). Area under the curve (AUC, 0-24 h) for the sum of total silymarin flavonolignans was 2.4, 3.3, and 4.7-fold higher for the HCV or NAFLD ($P \leqslant 0.03$) and HCV cirrhosis cohorts ($P \leqslant 0.03$), respectively, compared with healthy volunteers. Silymarin kinetics was correlated with plasma levels of caspases as an index of liver inflammation; caspase 3/7 activity correlated with the AUC (0-24 h) for the sum of all silymarin conjugates among all participants ($R^2 = 0.52$) and was 5-fold higher in the HCV cirrhosis cohort ($P \leqslant 0.005$ versus healthy participants). These findings suggest that the presence of liver damage, particularly as chronic inflammation, may affect the bioavailability of the different components of silymarin, possibly explaining the low beneficial effects of flavonoids in patients with liver damage.

Interactions and toxicity

There are substantial differences between silymarin and silybin in their interactions with metabolizing enzymes, and the reasons for these differences remain unknown. Silybin alone or as silybin β-galactoside, β-glucoside, β-lactoside, or β-maltoside at a final concentration of silybin 100 μmol/L has been tested *in vitro*. Under these conditions, silybin showed slow inhibitory effects (IC_{50} > 200 μmol/L) on marker substrates of CYP2E1, CYP2D6, CYP2C19, and CYP2A6. Silybin and silybin β-glycosides did not induce expression of CYP1A2 and CYP3A4 and did not affect the inducible expression of either of these enzymes[48-53].

Table 3 Comparison between silybin phytosome and silymarin pharmacokinetics in patients with liver cirrhosis[44]

	Silybin phytosome (360 mg)	Silymarin (336 mg)
C_{max} (ng/mL)	860 ± 166[b]	83 ± 15
Tmax (h)	2.7 ± 0.7[b]	2.6 ± 2.1
/2 (h)	3.3 ± 0.7[b]	2.6 ± 0.4
AUC	515 ± 665[b]	262 ± 39
Total bioavailability	252 ± 39[b]	19 ± 23

[b]$P < 0.001$.

In *in vivo* work, Sridar *et al*[54] investigated metabolic interactions involving silybin at doses ranging from 25 to 250 μmol/L and substrates metabolized by CYP3A4 or CYP2C9, showing that silybin may be a modulator/inactivator of P450s 3A4 and 2C9. What remains to be elucidated is whether this effect is brought about by competition at the site or inhibition for substrate binding and for metabolism at a particular binding pocket in the active site; another possibility is that it results from a conformational change in the active site. Despite these results, silybin has shown no effect on the metabolism of indinavir[55,56], which is mediated by CYP3A4, and others[57-60] have documented that silybin, at a concentration of 100 μmol/L, has no effect on either basal or inducible expression of CYP3A4 mRNA.

In any case, the silybin-drug interaction of these enzyme substrates is not clinically relevant, and the inhibitory effects of silybin occur only at concentrations that massively exceed the physiologically used doses[60-62]. *In vitro*, silybin inhibits the UGT glucuronyl transferases UGT1A6 and UGT1A9, but it is 14- to 20-fold more selective for UGT1A1[48,49,61-64] (see Table 4 as summary). The clinical relevance of this phenomenon is presently unknown because there are no published reports indicating the existence of a clinical interaction with bilirubin. UGT1A1 is the only enzyme responsible for bilirubin glucuronidation, and it contributes to the glucuronidation of several drugs. In animals, silybin affects the hepatobiliary elimination of various drugs[52,53,57,58,64]; oral feeding of pure silybin at doses of 100 and 200 mg/kg/bw/d showed a moderate to highly significant increase in both glutathione-S-transferase and quinine reductase activities in the liver, lung, stomach, skin, and small bowel in a dose- and time-dependent manner[58]. Recently, Flaig *et al*[27] provided the best evidence that silybin can be administered to humans at doses producing anticancer-relevant concentrations, with minimal or no side effects.

Table 4 Interference of silybin with cytochromes[48-64]

Interference	Possible interference	No interference
UGT1	CYP3A4	CYP2E1
	CYP2	CYP2D6
		CYP2C19
		CYP1A2
		CYP2A6

That study employed the largest doses ever used, ranging from 2.5 to 20 g of silybin-phosphatidylcholine (Indena's Siliphos® "silybin phytosome"), given daily in three divided doses for 4 wk to 13 men with a history of prostate carcinoma. At a dose escalation from 15 to 20 g/d, silybin was discontinued because of asymptomatic hyperbilirubinemia, most likely because of inhibition of the glucuronyl transferase UGT1A1. However, in all patients, this mild hyperbilirubinemia improved with treatment cessation.

The limitations of available clinical trials with regard to establishing safety are the same as those for establishing efficacy. Clinical trials testing safety are poor predictors of the fate of extracts in real-world settings, where patients ingest multiple drugs and herbs, take different formulations of the same product, and add alcohol and other compounds, often for extremely long periods. In the randomized trials that reported adverse effects, their frequency was approximately equal in the silybin and control groups. The majority of the observed adverse events were unrelated to the product or difficult to separate from the concomitant disease, and in available reports, causality is rarely addressed. There are no safety data in children or older adults, as there are no reported studies in children and very few studies that included patients older than 65 years. Adverse effects associated with oral ingestion of silybin include mainly gastrointestinal problems, but these are rare. Headache/dizziness and pruritus were reported in one clinical trial. Asymptomatic liver toxicity has been observed in recent clinical trials performed in cancer patients, in whom hyperbilirubinemia and increases in alanine aminotransferase (ALT) levels were observed; however, these effects were present only when very high dosages of silybin phytosome (between 10 and 20 g/d) were used. At high doses, a laxative effect of silybin phytosome is possible because of increased bile secretion and bile flow. Mild allergic reactions have also been noted, but they were not serious. Therefore, the available data indicate that milk thistle has few side effects when doses lower than 5 g/d are used, with possible adverse effects at doses greater than 10 g/d[6,7,62].

The documented effects of silybin in basic research

In liver cells, as well as in other types of cells, the common effects of silybin may be summarized as follows: (1) Antioxidant; (2) Direct and/or indirect (through the antioxidant capability) modulator of inflammation and fibrogenesis; and (3) indirect and/or direct modulator of some intrahepatic metabolic pathways.

Antioxidant action

The antioxidant effects of silybin have been demonstrated in all cells studied. Silybin acts as an antioxidant because it inhibits radical formation, binds some radical species (scavenger), interferes with lipid peroxidation of membranes (and therefore modulates membrane permeability), and increases the intracellular content of scavengers[65]. In fact, in the presence of oxidative and nitrosative stress, silybin inhibits the formation of superoxide anion radicals and of nitric oxide (NO), increases ATP content through the phosphorylation of ADP, decreases the content of malondialdeyde (MDA) and totally abolishes the decrease of glutathione, of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase[66-73]. These results, which are dose-dependent, have been documented in isolated rat Kupffer cells, hepatocytes, HEPG2 cells, isolated mitochondria from rat hepatocytes, and in models of ischemia-reperfusion of rat liver. In experimentally induced liver damage, one hour after intragastric administration to rats of conjugated silybin (0.6 g/kg bw), the content of silybin in the microsomes measured using a specific HPLC assay was approximately 2.5 μg/mg of protein, corresponding to a final concentration of 10 μmol/L of silybin. Under these conditions, lipid peroxidation, mitochondrial permeability and respiration, and membrane potential as well as cell death were reversed by silybin, particularly if it was conjugated as silybin + phosphatidylcholine. This last compound, at a dose of 20 μmol/L, directly scavenges hydroxyl, hydroxyethyl, lipodienyl, methyl, and trichloromethyl radicals[74,75].

Finally, silybin acts as an antioxidant because it also serves as an iron chelator[76-78]. More recently, it has been suggested that dehydrosilybin (DHS), an oxidized form of silybin, has greater antioxidant activity than silybin (about three times better), probably because of the presence of unsaturated bonds that contribute to hydrogen-donating capacity. The better scavenger activity of DHS may also be the result of its greater ability to react with cell membranes because it has higher lipophilicity than silybin[32,65].

Table 5 summarizes the main antioxidant effects of silybin.

Anti-inflammatory action

In general terms, silymarin and silybin interfere with the NF-κB-controlled transduction cascade. NF-κB is an inducible and ubiquitously expressed DNA-binding protein, acting as a transcription factor for genes involved in inflammation, cell survival, differentiation, and growth[79,80]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by interaction with inhibitory protein 1 κ B α (IκBα). Upon activation from oxidative stress, NF-κB dissociates from IκBα, and IκBα is degraded. NF-κB translocates to the nucleus and, through kinase phosphorylation, drives the activation of genes supporting inflammation. Consistent with its antioxidant activity, silybin has been demonstrated to inhibit NF-κB activation and translocation through suppression of IκBα phosphorylation and degradation[81-83]. In an acute model of liver damage

Table 5 Antioxidant effects of silybin[65-75]

Doses	Cells	Targets
From 10 to 100 μmol/L	Hepatocytes	Decreased formation of reactive oxygen species from mitochondria; iron chelator
	HepG2	Decreased formation of superoxide anion
Mean dose with documented effects: 20 μmol/L	Kupffer cells	Decrease of NO production
	Monocytes	Scavenger of lipodienyl, methyl, trichloromethyl radicals
	Endothelial cells	Decrease of hydrogen peroxide concentration
	Cancer cells	Block of membrane lipid peroxidation

Table 6 Anti-inflammatory/antifibrotic effects of silybin[79-99]

Doses	Cells	Targets
From 5 to 50 μmol/L	Hepatocytes	Inhibition of NF-κB-mediated signaling
Mean: 15 μmol/L	Endothelial cells	Suppression of IκBα phosphorylation
	Platelets	Inhibition of protein kinase kinase
	Cancer cells	Inhibition of c-jun N-terminal kinase
	Phagocytes	Inhibition of leukotriene formation
	Stellate cells	Inhibition of release of cytochrome c
	HepG2	Inhibition of ERK, MEK, and Raf phosphorylation; inhibition of release of caspase 9 and 3, IL-8, and of PDGF- and TGF-β-mediated signaling; decrease of MMP2; increase of TIMP2; inhibition of HCV replication

in which mice were treated with concanavalin A, silybin reduced plasma levels of transaminases and the plasma and liver content of pro-inflammatory cytokines, inhibited hepatic NF-κB activation, and increased plasma and tissue levels of IL-10[84]. In rats with dimethyl-nitrosamine-induced chronic liver damage, silybin conjugated with phosphatidylcholine and vitamin E (Realsil®, Ibi-Lorenzini, Italy) administered by gastric gavage could prevent loss of body and liver weight, as well as reducing the degree of liver injury, as determined by ALT values and necroinflammatory scores. This outcome was associated with reduced hepatic stellate cell activation and proliferation both after 1 and 5 wk of treatment[85]. The anti-inflammatory action of silybin is also related to its interference with multiple cytokine-induced signaling pathways to down regulate inducible nitric-oxide synthase (iNOS) expression[86-88]and to the inhibition of cyclooxygenase (COX)-2 expression and activity and leukotriene formation in human platelets, white blood cells, and endothelial cells. Finally, silybin inhibits activation of the protein kinases and of a c-jun N-terminal kinase[89-92].

In addition to its antioxidant and anti-inflammatory actions, silybin also shows an antiviral effect. In fact, at a concentration of 20 μmol/L, it inhibits the protein expression and the replication of HCV virus in infected polymorphonucleated cells derived from patients with chronic HCV infection[93-95].

Antifibrotic action

In an *in vitro* model of human hepatic fibrogenesis, silybin demonstrated both direct and indirect antifibrotic properties. In fact, in stellate cells from human liver, silybin reduced platelet-derived growth factor (PDGF)-induced DNA synthesis and cell proliferation at a dose of 25 μmol/L. Silybin also reduced PDGF-induced cell migration in a dose-dependent fashion. Finally, pre-treatment with 25-50 μmol/L of silybin significantly reduced the TGF-β-induced *de novo* synthesis of procollagen type I in cell supernatants[96].

To investigate the role of silybin in modulating the pro-inflammatory properties of hematopoietic stem cells, cells were stimulated with IL-1β (20 ng/ml), a potent pro-inflammatory cytokine; silybin inhibited, in a dose-dependent manner, IL-1-induced synthesis of human MCP-1 (monocyte chemoattractant protein 1) and human IL-8 as detected in cell supernatants. This effect was related to the effect of silybin on the inhibition of IκBα phosphorylation and to its capability to inhibit ERK, MEK, and Raf phosphorylation at any concentration used[96]. Antifibrotic effects were also documented in experimental animals and in humans[85,97-99].

Table 6 summarizes the main anti-inflammatory and antifibrotic effects of silybin.

Metabolic effects

Silybin interferes with some mechanisms of action of insulin. In fact, it modulates the uptake of glucose in adipocytes by blocking the insulin-dependent glucose transporter 4. In rat hepatocytes, silybin, in concentrations ranging from 25 to 100 μmol/L, lowers glucose formation from different gluconeogenic substrates through an inhibitory effect on pyruvate kinase activity[100]. As previously reported in cultured hepatocytes, low doses of silybin reduce reactive oxygen species formation from mitochondria; this reduction leads to a decrease in oxidation of carbons arising from glycolysis[101]. Moreover, silybin inhibits, in a dose-dependent manner, gluconeogenesis and glycogenolysis, both in basal conditions and after a glucagon-dependent stimulation, by blocking glucose-6-phosphate hydrolysis. This effect was demonstrated using different substrates, such as dihydroxyacetone, lactate/pyruvate, glycerol, and fructose[102] (Table 7).

Table 7 Effects of silybin on hepatic metabolism of glucose at doses of 25-100 μmol/L in hepatocytes[100-102]

Doses	Cell	Targets
25-100 μmol/L	Hepatocytes	Inhibition of pyruvate kinase Inhibition of glycolytic flux Inhibition of glucose-6-phosphate hydrolysis Inhibition of glucose-6-phosphatase

Table 8 Other effects of silybin on cellular signaling[106-122]

Induction of apoptosis through an inhibition of IGF-IR
Down regulation of survivin and an increase in p53 expression
Block of cycle-regulator cyclins and promoter activities
Decrease of MMP-1 production
Decrease of angiogenesis
Inhibition of VEGF expression
Inhibition of HIF-1α
Increase of IGFBP-3

In an animal model of type 1 diabetes mellitus[103], in a 6-mo, double-blind, randomized trial in patients with poorly controlled non-insulin-dependent diabetes mellitus and alcoholic liver disease[104], and in a randomized, double-blind, placebo-controlled trial in patients with type Ⅱ diabetes[105], silybin significantly affected plasma levels of glucose and triglycerides, with a trend toward lower hemoglobin A1c levels.

Other effects on cellular signaling

In cancer cells, silybin alters cell cycle regulators and induces apoptosis, both through antioxidant and anti-inflammatory properties and through the inhibition of growth factor receptor-mediated mitogenic and cell survival signaling, particularly related to the activation of tyrosine kinases[106-111]. PDGF receptor (PDGFR), epidermal growth factor receptor (EGFR), Bcr-Abl, and KIT are examples of tyrosine kinases overexpressed in most human cancers. Silymarin or silybin are particularly effective in inhibiting EGFR signaling with suppression of cyclin-dependent kinase expression (i.e. CDK4) and up regulation of CDK inhibitors (CDKIs). These effects lead to G1 and G2-M arrest in cancer cells[112-119], as recently confirmed in a model of human colon cancer[120,121]. In fact, at a dose ranging from 40-75 μmol/L, silybin significantly inhibited cell proliferation through cell cycle arrest *via* inhibition of cyclin promoter activity.

Because it inhibits constitutive NF-κB activation, silybin can induce apoptosis, consistent with a significant decrease in its nuclear level of p65 subunit. In addition, it activates caspase 3 and caspase 9 and decreases survivin levels[122].

Angiogenesis refers to the growth of capillary vessels from existing blood vessels and is considered obligatory for the growth and progression of solid tumors. Angiogenesis critically depends on several conditions; in fact, endothelial cells must: (1) proliferate to provide the necessary number of cells for the growing vessels; (2) secrete matrix metalloproteinases (MMPs), which are required to break down surrounding tissue matrix; and (3) be capable of movement and migration. In addition, the angiogenic stimuli like hypoxia and the production of angiogenic cytokines, such as vascular endothelial growth factor (VEGF), must be sustained. Silybin treatment decreases secreted VEGF levels and shows a strong, concentration-dependent inhibition of capillary tube formation on matrigel, retraction, and disintegration of preformed capillary network, inhibition of matrigel invasion and migration, and a decrease in MMP-2 secretion[92,106-122].

Table 8 summarizes the main cellular signaling affected by silybin.

The use of silybin in the clinical setting: efficacy and suggestions

The use of silymarin in the clinical setting has a long history; in its native Mediterranean region, it has been employed for liver damage since the Greco-Roman era. As mentioned in the introduction, "herbal therapy" use is consistently increasing worldwide, and some of the most common herbal supplement preparations are derived from the milk thistle plant (*Silybum marianum*). Despite a long history of its use and the large number of people who consume this substance, no conclusive data on its clinical efficacy can be identified. In fact, only a few well designed clinical trials have been performed. Most studies have been conducted using silymarin and with inclusion of patients with alcoholic or viral cirrhosis. Stickel *et al*[8] identified the 10 main controlled trials conducted in liver patients, with numbers of patients ranging from 20 to 200 and doses of silymarin ranging from 210 to 450 mg/d for 7 to 730 d. These clinical studies generally have suffered from the same shortcomings found in many other trials on herbal medicines, such as a small sample size, lack of appropriate randomization and of allocation concealment or blinding, very different periods of treatment, lack of information about type and dose of extract used as well as product characterization, ill-defined patient population, and a lack of etiology, severity of disease, and discussion of potential confounders.

We could argue that the history of studies on silybin reflects, in part, the epidemiological history of liver diseases. When liver cirrhosis was the only clearly identified manifestation of liver damage and alcohol was the only known pathogenetic factor, silymarin and other plant extracts represented the most frequently used drugs. Knowledge about the pathogenetic relationships between hepatitis viruses and liver damage induced researchers and industry to focus attention on antiviral drugs. In response to the discovery of metabolic liver diseases, as well as increased knowledge about the cellular and subcellular mechanisms of both induction and progression of liver damage, researchers in academia and industry have begun to reappraise natural products and to evaluate their therapeutic efficacy using appropriate methods.

The clinical experience with silybin is mainly related to its properties as a detoxifying agent and as a hepatoprotective compound in different acute and chronic liver diseases.

Silybin as a detoxifying and hepatoprotective substance

Several chemotherapeutic agents are metabolized by the liver and can exert hepatotoxicity, with the net result of drug reductions or withdrawal. Chemotherapeutic agents that are likely to produce hepatotoxicity include dactinomycin, daunorubicin, docetaxel, gemcitabine, imatinib, 6-mercaptopurine, methotrexate, and oxaliplatin. Cancer patients taking these therapies often self-medicate with milk thistle because of its reputation as a liver protectant. Clinicians also prescribe it to cancer patients for the same purpose. The rationale of milk thistle use is to provide support to the liver while it performs multiple functions, including responding to the increased metabolic demands caused by tumor growth, assisting in metabolizing products generated when a tumor is killed or reduced by chemotherapy and radiation, and aiding in the processing of drugs prescribed to cancer patients.

Silybin is also considered a potent inhibitor of human intestinal β-glucuronidase, blocking the release and reabsorption of free xenobiotics and their metabolites from their glucuronide conjugates. Because the liver is the primary organ that cleanses and detoxifies the blood, many detoxification programs include a component of liver support or what is often called liver cleansing. For these reasons, silybin is commonly included in detoxification regimens[6,7,62,108-111]. However, only one randomized, double-blind study has reported the effects of milk thistle in patients receiving cancer therapy; 50 children with acute lymphoblastic leukemia and grade 2 or higher hepatic toxicity were randomized to receive a milk thistle supplement (Siliphos, Thorne Research, Dover, Idaho) (5.1 mg/kg/day) or placebo for 28 days. The authors reported significant reductions in AST levels ($P < 0.05$) and a trend toward a significant reduction in ALT levels ($P < 0.07$). A significantly larger number of children in the milk thistle group developed a > 50% reduction in total bilirubin at day 28 compared to placebo ($P < 0.0069$)[123].

Silybin in acute liver damage

The administration of silybin within approximately 48 h after poisoning produced by the mushroom *Amanita phalloides* (death cap) seems to be an effective measure to prevent severe liver damage[124]. A retrospective analysis of 205 cases of clinical poisoning from 1971 to 1980 documented the efficacy of milk thistle silybin extract in increasing survival rates in adults and children exposed to this potentially lethal mushroom[125]. In January 2007, six family members in California suffering from aflatoxin poisoning caused by *A. phalloides* mushrooms were treated with intravenous milk thistle, provided by Madaus Pharma (Brussels, Belgium; division of Madaus AG, Cologne, Germany). The US Food and Drug Administration granted permission for the use of milk thistle after considering that all patients were at high risk of dying of liver failure. Ultimately, one of the six patients died, while the others had a full recovery after treatment. A case report mentions that silybin may offer protection from liver toxicity caused by the pharmaceutical drug phenytoin[126].

Silybin in chronic liver disease

The various flavonoid compounds that are included in the general term "milk thistle" have been recognized as a "safe and well-tolerated herb" with a limited adverse event profile[1-10]. As previously reported, the majority of studies have been conducted using silymarin and including patients with alcoholic or viral cirrhosis. The Cochrane Collaboration group[4,127] evaluated the studies related to the use of silymarin/silybin in patients with different types of acute and chronic liver diseases. Various items were analyzed to define the quality of trials, such as the number of participants, the criteria of randomization, the blinding, the modalities of follow-up, and the statistical methods. From 1,831 references, 67 publications addressed patients with alcoholic and/or hepatitis B or C liver diseases treated with milk thistle; of these, only 26 were selected according to the agreed criteria, and these were associated with 13 trials. The active treatment consisted of silymarin per os in 10 randomized clinical trials, silybin phytosome per os in two randomized clinical trials, and silybin + phosphatidylcholine in one randomized clinical trial. The Cochrane Collaboration studies concluded that milk thistle does not seem to significantly influence the course of the disease in treated patients. However, all causes of mortality were reduced by 50% in patients with alcoholic liver disease without HCV antibodies who took milk thistle extracts compared to placebo ($P < 0.05$); in trials that studied liver-related mortality, there was a significant effect of silybin (relative risk, 0.50; 95% CI 0.29 to 0.88; $P = 0.02$)[4,8,9,127, 128].

Seeff *et al*[5,129] examined the spontaneous use of herbal products in the US in patients affected by HCV chronic infection, including non-responders to a previous treatment with interferon and ribavirin. In 1145 patients, about 50% used herbal products; of these, silybin was used by 70%. Even if no changes in liver tests and/or in HCV-RNA serum levels were observed, the univariate analysis documented a lower incidence of symptoms and a better quality of life in patients who consumed silybin relative to those who did not. On multivariate analysis, adjusted for age, gender, educational status, alcohol use (n. drinks/d), physical activity, body mass index, and smoking, silybin positively affected more than one aspect of quality of life. An examination of the total number of patients treated with silymarin/silybin in both well-conducted trials and in pilot studies with a high quality identified about 2000 patients with liver cirrhosis or with chronic hepatitis of different etiology, with a mean duration of treatment of six months and a dose of silybin ranging from 160 to 360 mg/d (except in two cases, see below).

The main results regarding the efficacy of silybin in

Table 9 Main studies performed by using purified silybin as drug

Authors	Type of study, number of patients	Drug used, dose and duration of treatments	Outcomes	Results	Clinical relevance
Vailati *et al*[146]	A phase II randomised, open trial on 60 patients with chronic alcoholic or viral hepatitis	Three doses (160, 240, 360 mg) of silybin and phosphatidylcholine (IdB 1016, Indena, Italy) for two weeks. No placebo or no intervention group was used	Liver tests	Improvement of liver enzymes with all used doses	Scarce
Buzzelli *et al*[147]	Double blind with identical placebo. Twenty patients with HBV and/or HCV chronic active hepatitis	IdB1016 (complex with phosphatidylcholine and silybin) two capsules, twice a day (equivalent to 120 mg of silybin in each capsule) (480 mg/d). Duration of treatment and of follow-up: two months in total	Mortality. Liver biochemistry	Improvement of liver enzymes and bilirubin	Scarce
Buzzelli *et al*[148]	Unclear, described as double blind but the method to achieve this was not described. Trial characteristics: cross over design. Patients were assigned to the Silipide group for two months treatment, and one month washout. Ten patients with chronic hepatitis C. (non-responders) to a previous treatment with recombinant interferon α	Silipide (IdB1016) capsules 360 mg/d. Control group: placebo capsules. Duration of treatment and follow-up: two months of treatment and one month of washout	Mortality. Liver biochemistry	Results were not reported separately, only overall results. Improvement of liver tests	Data published only in abstract form
Lirussi *et al*[104]	Blinding: adequate, double blind with placebo of identical appearance. Sixty out-patients with chronic alcoholic liver disease and non-insulin dependent type 2 diabetes	Silybin-β-cyclodextrin (135 mg silybin) sachets t.i.d Duration of treatment: 6 mo	Mortality. Liver biochemistry	Decrease of fasting glucose and lipid peroxidation markers	Good
Bares *et al*[138]	Randomized study to 1 of 3 oral doses. Thirthy seven patients with chronic hepatitis C non responders to a previous IFN treatment	IdB1016 at 314, 628, 942 mg t.i.d.(120,240 and 360 mg t.i.d. silybin equivalents, respectively) for 12 wk	Effects on serum markers of iron status	There was a significant decrease in serum ferritin, that was independently associated with the stage Ⅲ-Ⅳ of liver fibrosi	Good
Falasca *et al*[134]	Observational study on forthy naïve HCV positive patients (30 treated and 10 observed without treatments)	Silybin-Vitamine E-Phospholipid Complex (Realsil®- Ibi-Lorenzini-Italy) in a dose of 4 pills per day (each pill: 47 mg of silybin) for 3 mo	Hepatoprotection and anti-inflammatory effect by determining cytokine pattern and markers of liver disease	Improvement of liver enzymes and of Il2 plasma levels. Improvement of insulin resistance markers in patients with contemporaneous liver steatosis	Medium
Federico *et al*[141]	Observational study on 85 outpatients: 59 with NAFLD and 26 with HCV related chronic hepatitis in combination with NAFLD, non-responders to previous antiviral treatment. 53 (39 NAFLD and 14 HCV) were treated, while the other 32 patients (20 NAFLD and 12 HCV) served as a control group (no treatment)	The complex silybin-vitamin E-phospholipids (Realsil®), 4 pieces/d for six months followed by another six months of follow up	Effects on insulin resistance and liver damage	US steatosis, liver enzymes, hyperinsulinaemia, and indices of liver fibrosis were improved in both treated groups	Suggestive
Ferenci *et al*[139]	Observational study on 36 patients with HCV chronic hepatitis non responders to IFN + ribavirin. Duration of the study: 7 d	Silybin i.v. (Madaus, Germany) at 5, 10, 15 and 20 mg/kg per day for 14 d	Effect on viral load. Safety	Good compliance, no side effects and potent antiviral effect against HCV	High

liver disease patients are summarized as follows.

In the past, most studies were focused on liver cirrhosis, particularly alcoholic cirrhosis, and the efficacy of silybin was evaluated in terms of improvement in liver test abnormalities and/or in mortality rate (see above for results)[126-128]. Silybin β-cyclodextrin has been studied as an antidiabetic drug in patients with alcoholic liver disease and concomitant non-insulin-dependent diabetes mellitus; in these patients, the drug (at a dose of 135 mg/d) did not influence liver function test results or insulin secretion but significantly reduced fasting glucose ($P < 0.03$) and serum triglyceride levels ($P < 0.01$) compared to placebo. The effects seem to be related to a reduction in insulin resistance[104].

More recently, studies have focused on chronic hepatitis, particularly that induced by HCV. Silybin phytosome, at a dose ranging from 240 mg/d to 942 mg t.i.d. (the highest dose used in patients with liver damage) was well tolerated without adverse effects and significantly improved liver damage as expressed by transaminase levels, oxidative stress (serum malondialdehyde levels) and both ferritin serum levels and iron global body storage[130-138]. Today, the attention of researchers is focused on a possible antiviral effect of silybin against HCV infection.

Polyak *et al*[95] tested the anti-inflammatory and antiviral action of different extracts from silymarin on polymorphonuclear cells from patients with chronic HCV infection, documenting an anti-inflammatory and antiviral effect of milk thistle extract, mainly for silybin, which presented the strongest anti NF-κB and anti-HCV replication effect. Ferenci *et al*[139] and Biermet *et al*[140] have demonstrated that silybin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. These studies also showed that very high doses of silybin i.v. (from 5 to 20 mg/kg/d for 14 d) were free of toxic effects.

Finally, non-alcoholic fatty liver disease (NAFLD) may occur as an expression of a metabolic syndrome or in association with HCV chronic infection. The simultaneous presence of NAFLD in this latter group of patients may negatively affect the progression of fibrosis and the response to antiviral treatment. Patients affected by primitive NAFLD and/or chronic HCV-related hepatitis in combination with NAFLD, all non-responders to previous antiviral treatment, were treated with four pieces/d of the complex silybin-vitamin E-phospholipids corresponding to about 200 mg of pure silybin/d for three or six months. The treatment induced a significant reduction of plasma markers of chronic inflammation (C-reactive protein and cytokines), metabolic parameters (triglycerides, cholesterol, insulin resistance), liver tests (transaminases and gamma-glutamyl transpeptidase), degree of ultrasonographic liver steatosis and, finally, of main indices of liver fibrosis TGF-β, hyaluronic acid and metalloproteinase 2)[99,141,142].

In a rodent model of non-alcoholic steatohepatitis, the same silybin phospholipid complex prevented mitochondrial dysfunction[143], and preliminary results in a large, multicenter Italian trial *vs* placebo indicate that complex silybin-vitamin E-phospholipids significantly improved liver damage in patients with NAFLD and markers of liver fibrosis in patients with HCV chronic hepatitis[144].

In Table 9 the main studies performed with silybin are reported and discussed.

CONCLUSION

The data reported in this review clearly indicate the increasing interest in silybin and its compounds as well as the continuous improvement in knowledge about the molecular actions of this substance. However, in the clinical setting, there is currently a lack of definitive data about its efficacy in patients with chronic liver disease. The only well-defined finding is the absence of adverse events at high doses. Generally, all clinical studies on herbal products suffer from similar limitations, in part related to the fact that well-designed trials require resources and natural products industries do not sponsor them with significant budgets. In addition, in the majority of cases, herbal products differ from pharmaceutical compounds because multiple ingredients could act through multiple pathways to therapeutically affect the host. In the case of silybin, clinical studies on the pure extract and/or its derivatives are few and with a limited number of patients. Silybin is the most active flavonolignan in silymarin, and most capsules are standardized to this compound, but many variations of complex mixtures and single extracts are available and may critically affect clinical outcome. A full phytochemical and biological profile is preferable before commencing any clinical study. Today, analytical techniques that examine a suite of compounds, including their respective ratios, provide a more rational approach to authentication and quality assessment of the products. There must be assurance that the administered dose increases plasma and/or target tissue concentrations consistent with those required to produce effects *in vitro*. One primary fault of many clinical studies of botanicals is that adequate pharmacokinetic analyses are not completed before initiating efficacy trials. Some botanicals may fail in efficacy trials, not because the botanical is itself without activity but because the dosing was not sufficient to achieve pharmacologically meaningful concentrations.

Several silybin clinical trials are ongoing at this time (see also online at www.nccam.nih.gov). In addition, manufacturers of milk thistle extracts are conducting clinical trials with their own products that will elucidate the effects of specific preparations.

There is a need to enhance the funding opportunities to evaluate these long-used products with evidence-based knowledge, but there is also a need to reiterate Kroll *et al*[60], "As biological studies progress, it remains important to make the distinction between silymarin and silybin and their respective and distinct compositions". This latter point should also be considered when clinical investigators turn to pooling existing studies for meta-analyses. The epidemiology of chronic liver disease is changing

worldwide: viral infections are declining, and patients with HCV/HBV chronic hepatitis are older; NAFLD and alcoholic liver diseases are increasing, and generally patients with these pathologies are younger. Finally, alcohol-related problems, metabolic disruptions, and viral infections frequently coexist in the same patient[145]. Therefore, in clinical practice, there is the need for drugs that can be used in the long term without serious adverse events. Researchers should definitively demonstrate if silybin has potential in this regard. Lastly, the absence of significant adverse effects of silybin even at high doses, the good compliance by the patient, the availability of a purified form of the compound, represent characteristics that allow to obtain commercially available products containing almost 600 mg of pure silybin to ensure a good concentration in tissues.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2302

World J Gastroenterol 2011 May 14; 17(18): 2302-2314
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Double-balloon-enteroscopy-based endoscopic retrograde cholangiopancreatography in post-surgical patients

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Received: June 20, 2010 Revised: September 26, 2010
Accepted: October 3, 2010
Published online: May 14, 2011

Abstract

AIM: To evaluate double balloon enteroscopy (DBE) in post-surgical patients to perform endoscopic retrograde cholangiopancreatography (ERCP) and interventions.

METHODS: In 37 post-surgical patients, a stepwise approach was performed to reach normal papilla or enteral anastomoses of the biliary tract/pancreas. When conventional endoscopy failed, DBE-based ERCP was performed and standard parameters for DBE, ERCP and interventions were recorded.

RESULTS: Push-enteroscopy (overall, 16 procedures) reached enteral anastomoses only in six out of 37 post-surgical patients (16.2%). DBE achieved a high rate of luminal access to the biliary tract in 23 of the remaining 31 patients (74.1%) and to the pancreatic duct (three patients). Among all DBE-based ERCPs (86 procedures), 21/23 patients (91.3%) were successfully treated. Interventions included ostium incision or papillotomy in 6/23 (26%) and 7/23 patients (30.4%), respectively. Biliary endoprosthesis insertion and regular exchange was achieved in 17/23 (73.9%) and 7/23 patients (30.4%), respectively. Furthermore, bile duct stone extraction as well as ostium and papillary dilation were performed in 5/23 (21.7%) and 3/23 patients (13.0%), respectively. Complications during DBE-based procedures were bleeding (1.1%), perforation (2.3%) and pancreatitis (2.3%), and minor complications occurred in up to 19.1%.

CONCLUSION: The appropriate use of DBE yields a high rate of luminal access to papilla or enteral anastomoses in more than two-thirds of post-surgical patients, allowing important successful endoscopic therapeutic interventions.

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Key words: Double balloon enteroscopy; Endoscopic retrograde cholangiopancreatography; Choledochojejunostomy; Hepaticojejunostomy; Pancreaticojejunostomy; Percutaneous cholangiodrainage

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Raithel M, Dormann H, Naegel A, Boxberger F, Hahn EG, Neurath MF, Maiss J. Double-balloon-enteroscopy-based endoscopic

retrograde cholangiopancreatography in post-surgical patients. *World J Gastroenterol* 2011; 17(18): 2302-2314 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2302.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2302

INTRODUCTION

With the technique of push-and-pull enteroscopy by a double balloon endoscope, it is possible to advance much deeper into the small intestine than using a conventional push-enteroscope[1-3]. Double balloon enteroscopy (DBE) has been successfully applied for diagnosis and treatment of various small intestinal diseases, such as mid-gastrointestinal bleeding, polyposis syndromes, Crohn's disease, lymphoma, foreign body impaction, or other inflammatory or neoplastic diseases in the jejunum or ileum[1-3]. Although the introduction of DBE by Yamamoto has brought a significant benefit for the management of various small intestinal diseases, its value in the diagnosis and treatment of biliary or pancreatic diseases in patients after complex abdominal or bilio-pancreatic surgery has recently been reported in some case studies of selected patients[4-10]. The emerging role of DBE in postoperative endoscopic procedures arises from the fact that conventional endoscopy using side viewing endoscopes, forward viewing push-enteroscopes, or (pediatric) colonoscopes has often been reported to be unsatisfactory in patients after partial or total gastrectomy (Billroth Ⅱ gastrojejunostomy, Roux-en-Y reconstruction), Whipple resection or bilio-pancreatic reconstructions (pancreaticojejunostomy, choledocho-choledochostomy, hepaticojejunostomy)[4,5,10-12]. For example, in the pre-DBE era, conventional endoscopic access to the afferent loop and/or choledocho-, hepatico- or pancreaticojejunostomy was extremely difficult because of various lengths of bowel to be traversed, unfortunate locations of low jejunal anastomoses, jejunal loops of differing lengths, fixed jejunal loops, angulation or postoperative strictures and changes[4,5,10-12].

Failure of endoscopic access and therapy in post-surgical patients with normal papilla, choledocho-, hepatico- or pancreaticojejunostomy often results in more invasive and cost-intensive procedures such as percutaneous transhepatic cholangiodrainage (PTCD), computed tomography (CT)-guided pancreatic drainage, or repeated surgery. A training model for balloon-assisted enteroscopy and hepatobiliary interventions has been established by our group to learn, facilitate and adequately perform modern enteroscopic interventions[13-17]. Therefore, this study describes our clinical results from the prospective use of DBE in performing cholangio- and pancreatography, including therapeutic interventions of the biliary and pancreatic tract in a group of 37 consecutive post-surgical patients.

MATERIALS AND METHODS

Patient population

Between August 2005 and December 2008, 45 consecutive patients after complex abdominal surgery were admitted to the Department of Medicine 1 of the University Erlangen-Nürnberg because of abdominal pain, cholestasis, inflammatory symptoms, cholangitis, choledocholithiasis, or for an enlarging pancreatic pseudocyst. During this study period, eight patients with partial gastrectomy (Billroth Ⅱ) and both afferent and efferent loops at the gastrojejunostomy were excluded from the study, because six could initially be successfully treated using the treatment gastroscope and two using the side-viewing duodenoscope.

Thirty-seven consecutive post-surgical patients were included in this study after having obtained informed consent and agreement to participate and for scientific documentation of the examination results. This clinical trial was carried out in accordance with the Helsinki declaration. The different indications for ERCP, previous surgery, localization of foot-point anastomosis, and depth of papilla or ostium localization are listed in Tables 1 and 2. In this prospective protocol, all patients underwent first usual, conventional endoscopy at least once using esophago-gastroduodenoscopy (GIF-Q160, GIF-1T140; Olympus, Hamburg, Germany), side-viewing duodenoscopy (TJF160; Olympus) and push-enteroscopy (PE; SIF Q140; Olympus) to exclude other diseases and to document postoperative anatomy, type of surgery, depth of anastomoses and, if possible, of papilla or biliary or pancreatic enteroanastomoses. Thirteen percent of all patients had two PEs in order to clarify the post-surgical situation and to reach the entero-anastomosis.

If this approach by conventional endoscopy failed to gain access to the papilla, the ostium of the bilio-digestive or pancreatico-digestive anastomosis, push-and-pull enteroscopy (DBE, EN-450T5; Fujinon Europe, Willich, Germany) was tried before admitting the patient for re-operation, CT-guided drainage or PTCD. Among these DBE examinations, the p-type enteroscope (EN-450P5/20; Fujinon Europe) was used in 13.7% and the t-type enteroscope (EN-450T5) in 86.2% of the patients.

All enteroscopic procedures were performed during conscious sedation (midazolam/pethidine or propofol/pethidine) by two experienced examiners (> 1500 ERCP) and two endoscopy assistants. Butylscopolamine was only used after reaching the end of the afferent loop for ERCP or at withdrawal of the enteroscope, respectively, in cases of vigorous peristalsis, to identify postoperative anatomy, hidden ostium or to facilitate cannulation of the ostium of the biliodigestive anastomosis.

PE

PE was started in the left lateral position using the Olympus SIF-Q140 forward-viewing enteroscope (working length 2.50 m, no elevator lever) without overtube[18]. If PE failed to come forward, the patient was turned to the prone position and X-rays were used to localize loops, to straighten the enteroscope, to direct manual compression to guide the enteroscope forward, or to minimize pain by adequate withdrawal of the enteroscope[18-21]. Post-surgical

anatomy, location of the foot-point anastomosis and the route to the afferent loop were each exactly documented, as well as time requirements for each diagnostic and therapeutic step. Foot-point anastomosis and the afferent loop were marked by India ink. Forward-viewing PE-based ERCP was performed using the typical ERCP technique as described previously[18-21].

DBE

DBE was performed using a standard technique, starting in the left lateral position, and thereafter changing to the prone position as described by Yamamoto and other authors[1-4]. At times, manual compression to guide the enteroscope in the abdomen and radiography were necessary. Provided that the anatomical situation and access to papilla or ostium of the enteroanastomoses were clarified, the afferent loop in proximity to the foot-point anastomosis was marked with clips and Indian ink on retraction of the enteroscope, so that this location would be found quicker in a future examination. Using a standardized protocol, the advance was exactly documented during DBE, and the respective anatomical depth of foot-point anastomosis, and papilla and ostium region were determined with the retracted and (as much as possible) straightened enteroscope. The time taken for this procedure and the whole procedure were also recorded. If during enteroscopy, advance failed, the enteroscope slid back, or if pain was experienced by the patient, radiography was applied to avoid kinking, to straighten loops and to retract the enteroscope carefully.

DBE-based ERCP

When papilla or pancreatico-, choledocho-, or hepaticojejunostomy were needed, ERCP was applied using the push-and-pull enteroscope, a forward-viewing endoscope of 2 m working length, without elevator lever[19-21]. This was assisted by X-rays for radiographic imaging of bile ducts and/or pancreatic ducts or a pancreatic cyst. Appropriate stabilization of the enteroscope with the overtube and/or enteroscope balloon was often required before performance of ERCP.

After administration of contrast medium and diagnosis, papillotomy or, an initial bougienage and/or incision of a stenotic ostium of the hepaticojejunostomy was performed. This was achieved by the use of a 5 and 6 Fr Huibregtse catheter and/or a 6 Fr papillotome (Olympus, intended for SIF Q140 enteroscope), or a snare. Further interventions aided by a 5-m guide wire (Metro guide wire; Cook, Limerick, Ireland) were implantation of endoprostheses (5-8 Fr) or of biliary 7 Fr nasobiliary probes, stone removal, or ostium and papilla dilation using either a CRE-dilation balloon (CRE 8-10mm balloon; Cook) or a basket.

With regard to prosthesis change, the old prosthesis was at first mobilized with a foreign-body forceps or a loop, and extracted and placed in the afferent loop. After DBE-ERCP implantation of the new prostheses was completed, the old prostheses were fixed again with the loop and extracted from the patient during the final retraction of the double balloon enteroscope.

RESULTS

Patient population

During the period between August 2005 and December 2008, 45 post-surgical patients were admitted to hospital for endoscopy. Eight of these patients with partial gastrectomy (Billroth Ⅱ, without Roux-en-Y reconstruction) could initially be successfully treated with gastroduodenoscopy or side-viewing duodenoscopy alone, and were therefore excluded from the prospective study. In the remaining 37 patients with complex abdominal surgery, neither a gastroscope nor duodenoscope gained initial access to the papilla or ostium, such that PE, and if it failed, then DBE were necessary.

Previous types of abdominal surgery

Previous abdominal surgery of the remaining 37 patients (Table 1) was partial gastrectomy in eight patients (Bilroth Ⅱ-resection, 21.6%, four patients had further resections after B-Ⅱ-resection, five patients with Roux-en-Y reconstruction); total gastrectomy with Roux-en-Y loop in seven patients (18.9%), and classical or modified Whipple operation with Roux-en-Y loop in seven patients (18.9%). Fifteen patients had normal stomach anatomy after biliary surgery with reconstruction of a choledocho- or hepaticojejunostomy *via* Roux-en-Y loop (40.5%).

Thus, 34 patients had previously undergone Roux-en-Y construction (91.8%), whereas only three had an end-to-side gastrojejunostomy that contained an afferent and efferent loop (8.1%).

Among all post-surgical patients, 24/37 patients (64.8%) had a final diagnosis of choledocho- or hepaticojejunostomy (23 Roux-en-Y, one dorsal gastrojejunostomy), while 13 patients (35.1%) still had a normal papilla. The pancreaticojejunostomy had to be searched additionally in only three of these patients (8.1%) (Table 2).

Indications for ERCP and interventional procedures

With regard to the indication, it was necessary to radiograph the bile ducts of 34 patients (91.8%), because these patients were admitted for cholestasis (59.3%), cholangitis (28.1%), or choledocholithiasis (13.3%), with a view to PTCD or re-operation. Radiography of the pancreatic duct was required in only three patients (8.1%), because of the presence of a pancreatic pseudocyst and suspected or advanced chronic pancreatitis, respectively (Table 1).

Due to the complex anatomical situation in seven patients (18.9%) with recurrent disease, 37 PTCDs had already been performed in these individuals before the introduction of DBE-ERCP (Table 2).

Access to papilla and entero-anastomoses by PE and DBE

The individual endoscopic accessibility and anatomical

Table 1 Characteristics of post-surgical patients receiving push-enteroscopy or double balloon enteroscopy-endoscopic retrograde cholangiopancreatography

Pts.	Age/sex	Indication	Previous surgery	Access by G/T/P
1	72 f	Recurrent cholangitis	LTX, Roux Y, hepaticojejunostomy	No
2[3]	76 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ)	No
3	60 m	Liver abscesses	Whipple resection, Roux Y, hepaticojejunostomy	P
4	66 m	Benign cholestasis	CHE, Roux Y, hepaticojejunostomy	P
5[3]	52 f	Benign cholestasis	Complicated CHE, Roux Y, hepaticojejunostom	No
6	79 f	Postsurgical bile duct leakage	Complicated CHE partial gastrectomy (B Ⅱ)	P
7	38 m	Recurrent cholangitis	Congenital bile duct atresia Roux Y, hepaticojejunostomy	No
8	66 m	Pancreatitis with pseudocyst	Pylorus preserving pancreatic head resection, Roux Y, hepatico-& pancreaticojejunostomy	No
9	58 f	Benign cholestasis abdominal pain	Total gastrectomy, Roux Y, hepaticojejunostomy	No
10	64 f	Benign cholestasis with cholangitis	CHE, right hemihepatectomy, Roux Y, hepaticojejunostomy	No
11	50 f	Benign cholestasis, bile ducht stones	Dorsal gastroenterostomy with hepaticojejunostomy	G[1]
12	51 f	Benign cholestasis	CHE, partial gastrectomy (B Ⅱ) with Roux Y	No
13	81 f	Malignant cholestasis	CHE, partial gastrectomy (B Ⅱ) with Roux Y	No
14[3]	52 f	Benign cholestasis	Compliated CHE, Roux Y, hepaticojejunostomy	No
15[3]	71 m	Malignant cholestasis	Complicated CHE, partial gastrectomy (B Ⅱ), Roux Y	No
16	69 f	Recurrent cholangitis	CHE, Roux Y, hepaticojejunostomy	No
17	47 f	Cholangitis, malignant cholestasis	Total gastrectomy, Roux Y, hepaticojejunostom	T[2]
18	67 m	Benign cholestasis	LTX, bile duct revision, Roux Y, hepaticojejunostomy	No
19	51 f	Benign cholestasis, bile ducht stones	LTX, bile duct revision, Roux Y, hepaticojejunostomy	No
20	68 f	Benign cholestasis, chronic pancreatitis	Total gastrectomy, Roux Y	No
21	71 m	Recurrent cholangitis	Modified Whipple resection, Roux Y, hepaticojejunostomy	No
22	68 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ) with Roux Y	No
23[3]	64 f	Malignant cholestasis	CHE, small bowel & colon resection, Roux Y, hepatico-jejunostomy	No
24	61 m	Suspected malignant cholestasis	Modified Whipple resection, Roux Y, hepaticojejunostomy	No
25	62 m	Malignant cholestasis	Total gastrectomy, Roux Y	P
26	73 m	Benign cholestasis	Pylorus preserving pancreatic head resection, Roux Y, hepatico& pancreaticojejunostomy	No
27	76 m	Benign cholestasis	Total gastrectomy, Roux Y	No
28[3]	76 f	Malignant cholestasis	Total gastrectomy, Roux Y	No
29	84 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ) with Roux Y	No
30	54 m	Choledocholithiasis, cholangitis	Complicated CHE, Roux Y, choledochojejunostomy	No
31	74 m	Choledocholithiasis	Total gastrectomy, Roux Y	No
32	61 m	Recurrent cholangitis	LTX, bile duct revision, Roux Y, choledochojejunostomy	P
33[3]	55 m	Suspected malignant cholestasis, chronic pancreatitis	Whipple resection, Roux Y, hepatico- & pancreatico-jejunostomy	No
34	34 f	Biliary colics, benign cholestasis hepatitis C	LTX, Roux Y, hepaticojejunostomy	P
35[3]	64 m	Suspected malignant cholestasis, chronic pancreatitis	Whipple resection, Roux Y, hepatico- & pancreatico-jejnostomy	No
36	51 f	Suspected choledocholithiasis,right abdominal pain	LTX, Roux Y, choledochojejunostomy	No
37	61 m	Recurrent cholangitis	Complicated CHE, Roux Y, hepaticojejunostomy	No

[1]Only after previous double balloon enteroscopy; [2]Only after previous double balloon enteroscopy and by use of a short-specialised, large caliber overtube (16.8 mm); [3]Patients indicate initial failure of DBE-based ERCP. G: Gastroscope; T: Side-viewing duodenoscope; P: Push-enteroscope; CHE: Cholecystectomy: B Ⅱ: Billroth Ⅱ resection; LTX: Liver transplantation; DBE: Double balloon enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography.

depth of the anastomoses, as well as of the papilla and the ostium of the choledocho- or hepaticojejunostomy and of the pancreaticojejunostomy using PE and DBE are described in Tables 1 and 2. The average depth of all anastomoses (three Billroth Ⅱ gastrojejunostomy, 34 footpoint anastomoses jejunojejunostomy) was 71 ± 21 cm, and the length of the afferent loop to the papilla or enteroanastomosis measured a further 53 ± 26 cm.

In total, a median of four (2-19, 25th-75th percentile) balloon-assisted enteroscopic cycles had to be performed after the passage of the anastomosis in the afferent loop, until the papilla or ostium were reached by DBE. Manual compression to guide the enteroscope was necessary in most patients.

The push-enteroscope could reach the papilla or the enteroanastomoses in only 6/37 cases (16.2%), while DBE had to be applied in 31 post-surgical patients (83.7%).

With DBE, access to papilla, choledocho-, hepatico- or pancreaticojejunostomy could be successfully and repeatedly achieved in 23 out of 31 patients (74.1%).

A total of 86 DBE-ERCPs were undertaken in those 31 patients, who failed to be successfully examined by PE. Seventy-five of the 86 DBE examinations (87.2%) were successfully carried out as a diagnostic or therapeutic

Table 2 Results of push-enteroscopy and double balloon enteroscopy-endoscopic retrograde cholangiopancreatography: postoperative anatomy and final diagnosis

Pts.	Foot-point anastomosis (cm)	Papilla/ ostium (cm)	ERCP diagnosis	PTCD before /after DBE
1	84	162	Stenotic hepaticojejunostomy (mucosal and intramural stricture 3 mm), putrid cholangitis	(2) Yes
2[1]	67	Not found	Swelling of anastomosis, afferent loop not found	No
3	65	90	Stenotic hepaticojejunostomy (mucosal, 11 mm stricture), cholangitis	No
4 P	75	110	Sludge, stenotic hepaticojejunostomy (mucosal, 3 mm stricture)	No
5[1]		Not found	PTCD stenotic hepaticojejunostomy (12 mm stricture)	(8) Yes(6)
6 P	52 (B Ⅱ)	78	Distal bile duct leakage and adhesion to abd. drainage	No
7	80	165	Stenotic hepaticojejunostomy (mucosal, 2 mm stricture), cholangitis	No
8	85 85	107 118	Normal choledochojejunostomy pancreaticojejunostomy with 10 mm diameter, 10 mm pancreatic Duct stricture, pancreatic pseudocyst	No
9	85	130	Normal hepaticojejunostomy, bile duct kinking	No
10	77	142	Stenotic hepaticojejunostomy (intramural, 4 mm) and stricture, common hepatic duct 4mm, bilioma	No
11	46	62	Obstructed hepaticojejunostomy by sludge/stones (hepaticolithiasis)	No
12	70	105	Papilla stenosis, bile duct kinking and stricture 3 mm	No
13	60	84	Bile duct stricture 18 mm due to papilla tumor	Yes (2)
14[1]	95	Not found	PTCD stenotic hepaticojejunostomy (12 mm stricture)	(12) Yes (6)
15[1]	57	110	PTCD edematous, tumorous papilla	Yes (2)
16	65	120	Stenotic hepaticojejunostomy (mucosal, 4 mm stricture)	(10) Yes
17	65	92	Malignant proximal bile duct stricture 22 mm	No
18	100	175	Hepaticolithiasis, normal hepaticojejunostomy	No
19	70	120	Stenotic hepaticojejunostomy (intramural, 12 mm stricture), cholestasis due to bile duct bleeding	(1) Yes
20	60	78	Papilla & bile duct stenosis due to chronic, pancreatitis, pancreatic duct stenosis	No
21	55	85	Stenotic hepaticojejunostomy,(mucosal, 2 mm stricture) & intrahepatic stricture	No
22	75	110	Distal bile duct stricture 45 mm due to ampullary tumor	No
23[1]		Not found	PTCD complete malignant stricture of hepaticojejunostomy due to progredient metastasis	Yes (1)
24	60	120	Hilar and hepatic duct strictures 9 and 26 mm, normal hepatico jejunostomy	No
25 P	65	110	Malignant obstruction biliary metal stent, sludge, cholangitis	(4) Yes
26	110	158	Stenotic hepaticojejunostomy, (intramural, 10 mm stricture)	No
27	76	112	22 mm bile duct stricture due to chronic pancreatitis	(2) Yes
28[1]	88	145	Polypoid papilla tumor	Yes (4)
29	100	140	Distal bile duct stricture 35mm due to suspected pancreatic tumor	No
30	105	151	Bile duct with sludge, normal choledochojejunostomy	No
31	51	165	Choledocholithiasis	(2) Yes (2)
32 P	78	147	Stenotic choledochojejunostomy, (intramural, 6 mm stricture) and bilioma segment Ⅳ	No
33[1]	66 66	Not found 126	PTCD: malignant stenotic hepatico-jejunostomy (filia), but normal pancreaticojejunostomy and chronic pancreatitis -	Yes (3)
34 P	80	132	Stenotic hepaticojejunostomy & hilar stenosis in ischemic cholangiopathy	No
35[1]	68 68	114 131	PTCD: recurrence of pancreatic tumor with malignant stenosis at hepaticojejunostomy, bile ducts and small intestine normal pancreaticojejnostomy and chronic pancreatitis	Yes (5)
36	70	131	Normal choledochojejunostomy	No
37	78	139	Stenotic hepaticojejunostomy (mucosal 2mm stricture)	No

[1]Patients indicate initial failure of double balloon enteroscopy (DBE)-based endoscopic retrograde cholangiopancreatography (ERCP). PTCD: Percutaneous transhepatic cholangiodrainage; PE: Push-enteroscopy.

DBE-ERCP (Tables 1-3), while 11 examinations (12.7%) in eight patients were unsuccessful.

After the initial, successful DBE-ERCP in two patients, the papilla and ostium of the hepaticojejunostomy, respectively, could be reached afterwards with the side-viewing endoscope or gastroscope. However, both treatments only worked after previous DBE, during which a large caliber overtube (17 mm, length 110 cm; Fujinon Europe) was inserted as a guide bar and the hepaticojejunostomy, located in an intestinal loop, was made visible through an inserted prosthesis.

Failure of PE and DBE to reach papilla or enteroanastomoses

In 8/31 patients (25.8%), despite DBE application, access to the bile ducts could not be achieved for a number of reasons (Tables 1 and 2): the anastomosis region was considerably swollen (one patient) or not visible because of metastasis (one patient); the afferent loop was technically not intubatable (one patient); the papillary or ostial region was infiltrated or covered by a tumor (four patients); or the ostium of the hepaticojejunostomy could not be found (one patient). Seven of these 8 patients (87.5%)

Table 3 Results of push-enteroscopy and double balloon enteroscopy-endoscopic retrograde cholangiopancreatography: therapeutic measures and (means ± SD) of sedation, X-rays and procedure time

Pts.	Push ERCP-/DBE-ERCP		Sedation		X-ray		Procedure Time (min)
	Procedures	Therapy	Dose (mg)	Drug	Time (min)	Dose (10^3 cGy/cm^2)	
1	7	Ostium incision (snare, papillotome) dilation, 2 stents inserted, regular change of 2 stents/1 yr	12.8 ± 3 132 ± 31	Midazolam Pethidine	19 ± 11	3.4 ± 2	122 ± 158
2	1	Not successful, re-operation	10.0 100 120	Midazolam Pethidine Butylscopolamine	3.3	1.0	82
3 P	3	Ostium incision (papillotome), dilation, stent insertion, regular change of stent/1 yr	15.0 ± 1 125 ± 35 40	Midazolam Pethidine Butylscopolamine	7.5 ± 7	1.8 ± 1.9	115 ± 79
4 P	4	Stent insertion, regular change of stent/1 yr	12 ± 2 137 ± 25 5	Midazolam Pethidine Diazepam	20 ± 29	3.1 ± 1.6	110 ± 171
5	2	Not successful, PTCD	12 ± 1 150 5 40	Midazolam Pethidine Diazepam Butylscopolamine	2.8 ± 1	4.0 ± 0.2	77 ± 11
6 P	2	Stent insertion, closure of bile duct leakage	7.8 ± 0.4 100	Midazolam Pethidine	4.0 ± 1	0.4 ± 0.1	135 ± 71
7	9	Ostium incision (papillotome), 2 stents inserted, regular change of stents/1 yr	1691 ± 867 135 ± 74 40	Propofol Pethidine Butylscopolamine	7.1 ± 6	1.8 ± 2.4	168 ± 131
8	4	Bougienage pancreaticojejunostomy, stent insertion into pancreatic duct and pseudocyst; normal hepatico-jejunostomy	13.3 ± 2 158 ± 38 40 ± 28	Midazolam Pethidine Butylscopolamine	11.8 ± 9	2.0 ± 2	161 ± 92
9	1	Normal hepaticojejunostomy	14 150	Midazolam Pethidine	10.1	0.5	91
10	4	3 stents inserted, one change of 2 stents	11.2 ± 5 133 ± 28	Midazolam Pethidine	12.6 ± 9	0.6 ± 0.4	61 ± 12
11	4	Insertion nasobiliary probe, dilation, stone extraction, insertion of stent	9.5 ± 1 125 ± 35 20	Midazolam Pethidine Butylscopolamine	8.1 ± 2	0.7 ± 0.4	61 ± 22
12	8	Bougienage, papillotomy, papilla dilation 8-10mm, stent insertion, regular change of stents/18 months	1082 ± 476 156 ± 77 47 ± 11	Propofol Pethidine Butylscopolamine	14 ± 8	3.1 ± 1.8	113 ± 97
13	3	Stent insertion, regular change of stent unsuccessful due to progredient papilla tumor, PTCD	10.8 ± 3 91 ± 52 40 ± 28	Midazolam Pethidine Butylscopolamine	13 ± 4	5.9 ± 2.9	177 ± 61
14	2	Not successful, PTCD	25 ± 7 175 ± 35	Midazolam Pethidine	5.4 ± 1	0.8 ± 0.1	155 ± 21
15	2	Not successful, PTCD	7.8 ± 3 100 ± 25 40	Midazolam Pethidine Butylscopolamine	5.9 ± 2	1.5 ± 0.3	122 ± 46
16	1	Ostium incision (papillotome), 2 stents inserted (perforation)	14 150 5	Midazolam Pethidine Diazepam	15.7	1.7	155
17	5	Papillotomy*, bougienage, nasobiliary probe; insertion of 2 stents, regular change of 2 stents/9 mo	16.8 ± 4 210 ± 74 16.7 ± 10 30 ± 11	Midazolam Pethidine Diazepam Butylscopolamine	11.6 ± 11	2.5 ± 2.6	198 ± 98
18	1	Stone extraction	19 200	Midazolam Pethidine	24.4	5.3	178
19	4	Extraction sludge & blood coagel, insertion nasobiliary probe, extraction of percutaneous drainage & insertion of 2 stents (rendezvous), regular change of 2 stents/ 9 mo	13 ± 1 116 ± 28 5 ± 5	Midazolam Pethidine Diazepam	9.7 ± 9	2.0 ± 1.8	82 ± 31
20	4	Papillotomy, stent insertion pancreatic duct, regular change of stent/6 mo, hemostasis with injection therapy	695 ± 275 75 ± 50 70 ± 14	Propofol PSSSethidine Butylscopolamine	8.7 ± 1	0.7 ± 0.4	61 ± 13
21	3	Insertion of 2 stents, regular change of 2 stents/6 mo	12 ± 1.8 158 ± 62	Midazolam Pethidine	15 ± 7	4.5 ± 1.9	185 ± 32
22	1	Papillotomy, insertion of 2 stents	19 200 40	Midazolam Pethidine Butylscopolamine	17.2	4.5	113

23	1	Not successful, PTCD	16 50 20	Midazolam Pethidine Butylscopolamine	0.6	0.2	63
24	2	Stent insertion	17.5 ± 2 100 ± 70	Midazolam Pethidine	18.9 ± 15	5.6 ± 2.8	150 ± 61
25 P	3	Stone/sludge extraction, dilation, biliary metal stent and malignant bile duct stricture, stent insertion, regular change of stent/9 mo	9 ± 4 200 ± 65	Midazolam Pethidine	12.9 ± 2	3.3 ± 1.1	54 ± 12
26	2	Ostium incision (papillotome), bougienage, stent insertion	7 ± 4 75 ± 35 40 ± 20	Midazolam Pethidine Butylscopolamine	4.5 ± 2	1.2 ± 0.6	61 ± 23
27	3	Papillotomy, extraction of percutaneous drainage and insertion of 2 stents (rendezvous)	5.7 ± 1 83 ± 28 40	Midazolam Pethidine Butylscopolamine	5.0 ± 1	1.0 ± 0.1	71 ± 12
28	1	Not successful, PTCD	5 50	Midazolam Pethidine	2.1	0.6	109
29	2	Papillotomy, bougienage, stent insertion	9 ± 2 150 ± 25	Midazolam Pethidine	9.2 ± 2	4.4 ± 0.3	113 ± 21
30	1	Sludge extraction, insertion nasobiliary	2.5 50	Midazolam Pethidine	16.4	7.8	123
31	2	Papillotomy, stone extraction, extraction of percutaneous drainage and insertion of stent (rendezvous)	7 ± 2 100 ± 25 80	Midazolam Pethidine Butylscopolamine	2.2 ± 0.5	1.9 ± 0.4	96 ± 31
32 P	1	Stent insertion	10 200 20	Midazolam Pethidine Butylscopolamine	27.1	7.9	161
33	2	Not successful, PTCD diagnostic pancreatography, extraction of percutaneous drainage with both ostium incision and insertion of 2 stents (rendezvous)	12 ± 5 150 ± 70 40 ± 28	Midazolam Pethidine Butylscopolamine	10.6 ± 9	3.3 ± 2.5	97 ± 80
34 P	3	Insertion of 2 stents, regular change of stents/12 mo	10 ± 7 183 ± 124 10 ± 5 20 ± 20	Midazolam Pethidine Diazepam Butylscopolamine	19.9 ± 10	3.6 ± 2.3	98 ± 33
35	1	Not successful, PTCD diagnostic pancreatography	11 150 40	Midazolam Pethidine Butylscopolamine	0.3	0.1	86
36	1	Normal choledochojejunostomy	7 50 20	Midazolam Pethidine Butylscopolamine	2.1	1.8	51
37	1	Ostium incision (papillotome), insertion of 2 stents	8.5 150	Midazolam Pethidine	4.4	2.0	72

Pts overall	Total number PE/DBE	Mean sedation dose per examination		Total x-ray time	Total x-ray dose	Total examination time
37	16 PE 86 DBE	11.7 ± 2.8 124 ± 45 20 ± 20 1156 ± 593	Midazolam Pethidine Butylscopolamine Propofol	9.0 ± 5.5	2.5 ± 1.3	111 ± 54

P: Push-enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography; DBE: Double balloon enteroscopy; PTCD: Percutaneous transhepatic cholangiodrainage; PE: Push-enteroscopy.

underwent subsequent PTCD or surgery (one patient, 12.5%).

Diagnosis, results and interventions at normal and malignant choledocho- and hepaticojejunostomy

In choledocho- or hepaticojejunostomies, 14 out of 24 (58.3%) were cicatricially changed, three were infiltrated by malignant tissue (12.5%), and seven (29.1%) appeared normal in width and were intact (Table 2).

DBE was able to achieve access to 15 of the 24 choledocho- or hepaticojejunostomies (62.5%), while PE reached only four out of 24 (16.6%), and the remaining five patients with failure of the enteroscopic approach (20.8%) had to undergo PTCD.

Among the seven normal appearing ostium of the choledocho- or hepaticojejunostomies (29.1%), sludge and

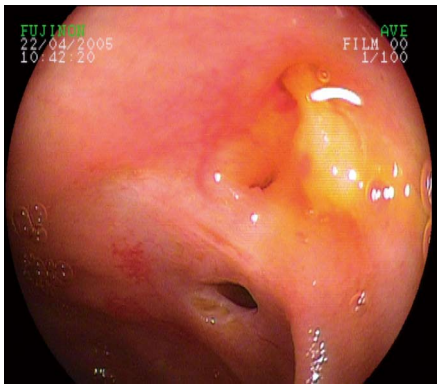

Figure 1 Endoscopic finding of stenotic hepaticojejunostomy in recurrent cholangitis with putrid secretion after careful ostium incision during double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique.

concrements had to be removed from one normal choledocho- and three normal hepaticojejunostomies in one patient suffering from cholangitis and choledocholithiasis, and three patients with hepaticolithiasis, respectively. In addition, endoprosthesis and/or nasobiliary probe insertion *via* the normal choledocho- or hepaticojejunostomy were necessary in two of these patients and in one with hilar and hepatic duct strictures, respectively.

Out of three tumor-induced malignant ostium stenoses (12.5%), the precise location of the enteroanastomosis could be identified twice, but in neither case could the stenosis be passed by a flexible hydrophilic guidewire and successfully treated. All three patients with tumorous hepaticojejunostomies required PTCD.

Diagnosis and results in post-surgical stenotic choledocho- and hepaticojejunostomy

Eight patients out of 14 (57.1%) with cicatricial ostial stenosis at the choledocho- or hepaticojejunostomy were treated successfully *via* DBE-ERCP, and a further four *via* PE (28.5%), while the remaining two patients (14.2%) required PTCD (Tables 2 and 3).

In one case with stenotic hepaticojejunostomy and previous PTCD (suspected hepaticolithiasis) at an outlying hospital, DBE-ERCP revealed blood in the afferent loop, bile duct bleeding from PTCD, and obstruction of the stenotic ostium including bile ducts due to blood clots. Thus, extraction of sludge and blood clots was performed, and insertion of a temporary nasobiliary drainage for irrigation of the bile duct. Then, after 3 d, a first DBE-based rendezvous technique was applied *via* the PTCD with successful extraction of the percutaneous drainage and endoscopic insertion of two internal stents.

Of note, a successful rendezvous technique was further achieved in three patients with non-malignant disease who were admitted to our hospital after construction of a PTCD, and in one patient with initial failure of DBE (Table 3). Thus, these four patients had most significant benefit from DBE-ERCP because they had endoscopically inserted endoprotheses and lost their percutaneous drainage within 1 wk.

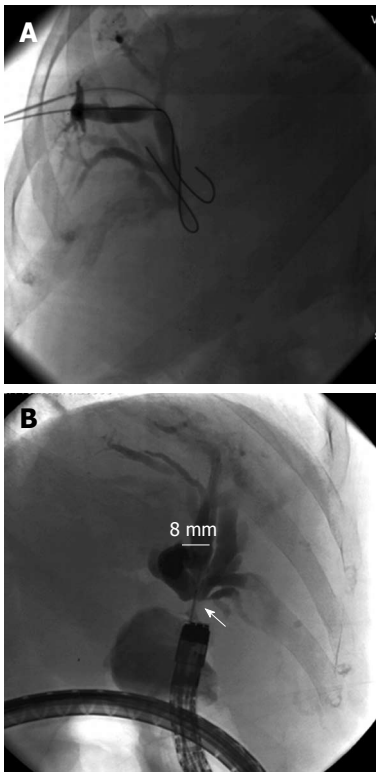

Figure 2 Radiological findings of stenotic hepaticojejunostomy in recurrent cholangitis with unsuccessful percutaneous drainage (A), but selective access to dilated bile ducts (width 8 mm) through a high-grade stricture (3 mm long, arrow) by double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique (B).

Ostium incision and dilation and endoprosthesis insertion at post-surgically strictured choledocho- and hepaticojejunostomy

Initial endoscopic interventions at the non-malignant post-surgical biliary anstomosis (choledocho- or hepaticojejunostomy), which could not be cannulated by a flexible guidewire, included a careful, 1-3-mm ostium incision (by snare and/or 6 Fr papillotome) of each narrowed ostium in 6 out of 12 cases (50.0%) during DBE-ERCP. Five ostial incisions were made during DBE-ERCP, and one during PE-based ERCP. All incisions resulted in significant widening of the ostium with subsequent successful cannulation and intervention in the biliary system. Perforation occurred in one of the 5 patients treated with ostial incision by DBE-ERCP (20.0%), which had to be treated surgically. None (0%) of the ostial incisions caused relevant bleeding, but in two cases (40.0%), pus was discharged from the opened ostium (Figures 1 and 2).

The other six patients (50.0%) with post-surgically strictured choledocho- or hepaticojejunostomy were initially cannulated using a guidewire and were treated either with a bougienage *via* a papillotome or nasobiliary probe, to widen the ostium ready to implant subsequently a prosthesis, or by dilation using a colonic CRE balloon.

Overall, in patients with cicatricial changed choledocho- or hepaticojejunostomies, on average 1.5 ± 0.7 endoprostheses were implanted per DBE-ERCP examination

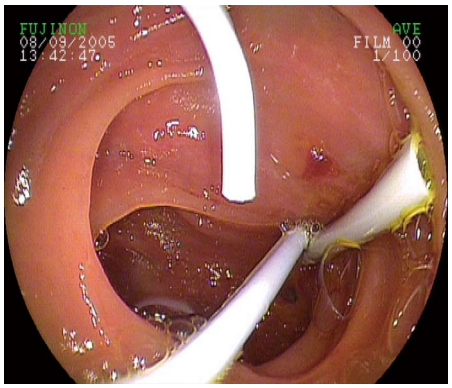

Figure 3 Endoscopic finding of stenotic hepaticojejunostomy in recurrent cholangitis after ostial incision and insertion of two endoprotheses during double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique.

(one double pigtail 5 Fr, 18 double pigtail 7 Fr and three double pigtail 8 Fr, as well as four straight 7 Fr endoprostheses and two 7 Fr nasobiliary probes; Figure 3).

At present, four patients with cicatricially changed ostium of the choledocho- and hepaticojejunostomy were treated several times by DBE-ERCP over a period of 1 year, with a regular exchange of prostheses every 3 mo (Table 3). After prosthesis implantation, all four patients had no further problems with cholangitis and cholestasis. In three out of four patients (75%), a sufficient widening of the ostium was achieved after the 1-year prosthesis therapy. Consequently, prosthesis therapy was no longer required and the cholestasis parameters stayed within the normal range over a prolonged period of time. However, the prosthesis exchange proved to be more difficult than the initial prosthesis implantation, because this procedure carries varying degrees of difficulty. In addition, an average treatment time of 12 ± 41 min had to be calculated for prostheses extraction and their temporary placing in the intestines.

DBE-ERCP with interventions at the pancreatic anastomosis

Among the 31 post-surgical patients, pancreaticojejunostomy was also found *via* DBE in three patients (9.6%) because of recurrent abdominal pain, inflammatory symptoms and an expanding cystic lesion in the pancreatic region. This could only be achieved successfully by DBE (Tables 1 and 2). The pancreaticojejunostomies (mean insertion depth: 128 ± 7 cm) were located mostly at 3-8 cm aborally of the biliodigestive anastomosis, and hence, required 1 ± 1.7 balloon-assisted cycles more to identify the pancreaticojejunostomy and to stabilize the DBE in front of it.

During the DBE-based pancreatography, two duct systems in patients with recurrent pancreatic tumor presented a similar appearance to those with chronic pancreatitis (clotted side branches, duct irregularities, but no acute strictures). In addition, one significantly dilated residual pancreatic duct was detected merging into a cystic lesion (pseudocyst). In the latter case, for the first time a 7 Fr double pigtail prosthesis had to be inserted for drainage of the pseudocyst *via* DBE-ERCP, because the patient suffered evidently from pain, weight loss, and inflammatory symptoms. After 2 d, the patient was free of symptoms. However, a mild lipase increase occurred post-interventionally, but there was no manifestation of post-ERCP pancreatitis. Within a week, the pseudocyst regressed noticeably, which was sonographically controlled and later documented with endoscopic ultrasound and CT. The prosthesis was removed 2 mo after insertion.

DBE-ERCP with interventions *via* the afferent loop at the papilla

Thirteen (41.9%) of the 31 patients still had a normal papilla. In 11 out of 13 patients (84.6%), the papilla was accessible *via* a Roux-en-Y loop, and only in two patients (15.3%) was it directly accessible from the Billroth Ⅱ stomach anastomosis *via* the afferent loop (Table 1).

The papilla could be reached with conventional PE in two of these 13 (15.3%) cases, and ERCP could be successfully performed with this forward-viewing enteroscope.

In the remaining 11 patients (84.6%) with normal papilla and prior abdominal surgery, the papilla had to be searched by push-and-pull-enteroscopy. DBE-ERCP could only be performed after appropriate stabilization of the enteroscope in front of the papilla, partly by use of the balloons. The DBE-ERCP and treatment was successful in eight of the 11 cases (72.7%; Tables 2 and 3), while in three cases (27.2%), DBE-based endoscopic retrograde cholangiography (ERC) failed because of tangential position to the papilla, or because of a papillary tumor (re-operation in one patient, and PTCD in two).

In the eight successful DBE-ERCs, seven patients (87.5%) had papillotomies of 3-7 mm in length using a 6 Fr papillotome, whereby moderate pancreatitis and bleeding (14.2% for each) occurred as side effects. In total, 1.2 ± 0.4 endoprostheses were successfully placed *via* the forward-viewing enteroscope (four double pigtail 7 Fr prostheses, one double pigtail 8 Fr prosthesis, seven straight 7 Fr endoprosthesis, and one 7 Fr nasobiliary probe).

In addition, apart from bougienage with the 6 Fr papillotome, dilatations using a CRE dilation balloon (8-10 mm, Cook) and removal of 5 ± 11 concrements and sludge using baskets were carried out in cases of papillary or distal bile duct stenoses. For treatment of purulent cholangitis with concrements, a nasobiliary drainage for irrigation was also placed *via* the enteroscope and left for 3 d to perform endoscopic shockwave lithotripsy and clean the bile system.

Laboratory results before and after DBE-ERCP with interventions

Before intervention, laboratory testing determined that the patients presented with distinct cholestasis and bilirubin elevation (2.8 ± 3.1 mg/dL) and/or inflammatory symptoms (leukocytes 12800 ± 10200/μL, C-reactive protein 51 ± 37 mg/L). By performing DBE-ERCP with ostial incisions, papillotomies and/or implantation of biliary endoprostheses, a clear reduction of cholestasis and chol-

angitis parameters was obtained. Values for bilirubin (1.6 ± 2.0 mg/dL), leukocytes (6800 ± 4000/μL) and C-reactive protein (18 ± 21 mg/L) decreased significantly ($P < 0.05$).

Complications of DBE-ERCP with interventions

Among 86 DBE-ERCPs, post-interventional cholangitis was not observed in any of the 31 patients treated by DBE-ERCP. However, after six of 86 examinations (6.9%) in 31 patients (19.3%), a lipase increase of more than twice the norm was seen on the day after DBE, whereas clinically significant post-ERCP pancreatitis (one mild and one moderate) was only seen after two examinations (2.3%) in two patients.

Post-interventional bleeding occurred in one of 86 examinations (1.1%) in 31 patients (3.2%) after papillotomy, which required emergency endoscopy, intensive care treatment, and blood transfusion.

Post-interventional stomach pain was experienced after six of 86 examinations (6.9%) in 31 patients (19.3%), whereas perforation occurred in two DBE-ERCPs (2.3%). One perforation developed immediately after ostial incision, while the second became evident 8 h later, with ileal perforation. Both perforations could be treated surgically, and no patient died due to complications of DBE-ERCP. No other fatalities following DBE-ERCP were recorded.

After two of 86 examinations (2.3%), two patients complained of abdominal pain that lasted > 24 h, and raised temperature developed on the day after the examination. Of note, one patient developed tonsillitis after DBE-ERCP (1.1%). No other serious side effects occurred.

Examination and radiography times and premedication during DBE-ERCP

The average duration of all DBE-ERCPs was 111 ± 54 min, and radiography took 9.0 ± 5.5 min with a dose of 2465 ± 1295 cGy/m^2. The individually required examinations for each patient are listed in Table 3, which included the exact therapeutic procedures, time measurements, and premedication.

With regard to premedication, an average of 11.7 ± 2.8 mg midazolam and 124.9 ± 45 mg pethidine or 1156 ± 593 mg propofol was needed per patient undergoing DBE-ERCP. In addition, butylscopolamine was administered at an average dose of 44.8 ± 20 mg. During conscious sedation for DBE-ERCP, one patient each developed hypoxia induced by midazolam/pethidine or propofol, which led in each case to abortion of the examination.

DISCUSSION

The difficulties involved with endoscopic access to the bile ducts and the pancreas in patients with prior abdominal surgery before the introduction of DBE have been described previously[4-6,10-12,19-21]. The success rate of ERCP with a side-viewing endoscope, push-enteroscope or pediatric colonoscope in patients with previous surgery depends on a number of factors, e.g. type of previous surgery, length of afferent loop, post-surgical changes, or experience of the endoscopist. Usually, results tend to be very variable (e.g. success rate of Billroth Ⅱ gastrojejunostomy up to 92%, Roux-en-Y reconstruction, 33%, and pancreaticojejunostomy, 8%) accompanied by high complication rates[4,6,19-21].

Access through conventional endoscopy was particularly difficult in our patients after several rounds of complex abdominal surgery (91.8% Roux-en-Y reconstruction, 8.1% gastrojejunostomy), and initially, access or treatment by gastroscope or duodenoscope was not possible. As recently outlined by several other investigators in small patients series[5-10,22-24], our stepwise approach with PE and DBE in 37 non-selected, consecutive post-surgical patients found that DBE-ERCP was clearly more efficient than PE. By the appropriate use of DBE in over two-thirds of cases, enteroanastomoses or papilla could be repeatedly reached, identified and satisfactorily visualized. The enteroscope could be stabilized also for bilio-pancreatic intervention. DBE-ERCP could be successfully conducted in 74.1% of the cases *via* the enteroscope, while PE reached biliary anastomoses or papilla in only 16.2% of the patients, which resulted in successful ERCP in only a minority of patients. Both results are in good agreement with recently published data for the approach by double- or single-balloon enteroscopy[5-10,22-26], as well as for earlier published data on postoperative or PE-based ERCP[4,11,19-21].

However, until a successful DBE-ERCP was achieved, several balloon-assisted enteroscopic cycles over an average length of 124 ± 47 cm of the small intestine, application of X-rays, and manual guidance of the enteroscope were necessary. In addition, a substantial effort in time, staffing and sedation had to be afforded. Compared with PE, the push-and-pull method by DBE proved to be markedly more effective, because pushing and stretching of small intestinal loops is reduced by regular retractions of the DBE cycle. The threading of the small intestine onto the DBE and the option to block the balloons at the enteroscope provides the enteroscope tip with a greater possibility of movement for identifying the biliary or pancreatic anastomoses or the papilla. In addition, sliding back of the enteroscope may be prevented by inflated balloons, which, compared with PE, explains the significantly higher effectiveness of interventions during DBE-ERCP.

Out of the 37 post-surgical patients with significant cholestasis and cholangitis, PE achieved a successful bile duct drainage in six (16.2%), whereas, before DBE was introduced, a far more invasive procedure, either PTCD or surgery, would have been carried out in the remaining 31 patients. PTCD carries a significantly higher morbidity and mortality risk compared to the endoscopic procedure[12,14-17,27,28], therefore, all consecutive patients with previous abdominal surgery were included in this prospective treatment protocol after DBE had been introduced in August 2005 at the University of Erlangen–Nuremberg. Of note, DBE facilitated successful ERCP with biliary

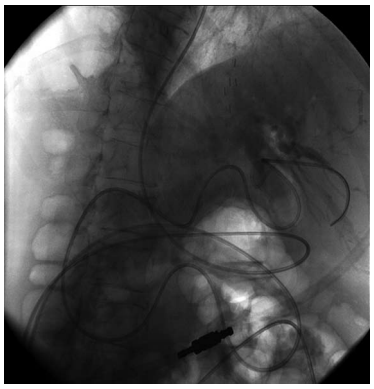

Figure 4 Radiological finding of insertion of a nasobiliary probe for irrigation in recurrent cholangitis with sludge after liver transplantation and hepaticojejunostomy by double balloon enteroscopy-endoscopic retrograde cholangiopancreatography through 120 cm of small bowel.

interventional procedures leading to significant reduction of cholestasis or cholangitis in 23 of 31 patients (74.1%). Thus, PTCD could be avoided in those 23 post-surgical patients, because endoscopic biliary drainage was achieved.

In comparison to reported PTCD-induced complication and infection rates of up to 55%, and even mortality[12,14-17,27,28] only one case of post-papillotomy bleeding (3.2%), two of post-ERCP pancreatitis (6.4%) and two perforations (6.4%) occurred following DBE-ERCP, but no cholangitis or mortality has been recorded to date. Thus, this first prospective investigation from a university tertiary referral center confirms that DBE-ERCP has considerable potential to treat successfully benign (postoperative) or malignant biliary and papillary stenoses, bile duct concrements, and cholangitis, even in non-selected post-surgical patients[4-10], and it helps to reduce the number of percutaneous approaches. Only in eight of 31 patients (25.8%), in whom the biliary or pancreatic anastomoses or papilla could not be found *via* DBE, was PTCD finally necessary. Even when the biliodigestive anastomoses could not be found and/or DBE-ERCP failed because of tumor-changed papilla or choledocho- and hepaticojejunostomy, a change in treatment procedure could be attempted after construction of PTCD by using DBE. After introduction of the percutaneous tube into the small intestine, percutaneous drainage was successfully changed in four patients to internal drainage inserted *via* DBE (Table 3). This was achieved by application of a DBE-PTCD rendezvous procedure, which was performed for the very first time in Erlangen in 2006. Before the DBE era, a longer-lasting bougienage and Yamakawa prosthesis therapy or biliary metal stent implantation were often indicated after the initial PTCD puncture[12,14-17]. By the use of DBE-ERCP, however, the external drainage could be extracted from all four patients after 1 wk. Practically, methylene blue injected externally through the PTCD helps to identify the afferent loop and/or biliary anastomoses or papilla, so that these are more easily and quickly detected by the subsequent DBE.

The key benefits of DBE-ERCP in the care of post-surgical patients with cholestasis/cholangitis and patients with installed percutaneous drainage are somewhat limited by the small caliber of bile duct prostheses that are applied *via* the enteroscope. According to the present state of technology, only an implantation of 5-8 Fr prostheses through an operating channel of 2.8 mm is possible. Consequently, several prostheses (1.5 ± 0.7) were implanted in our patients. In the case of strongly soiled bile ducts and concurrent cholangitis or sump syndrome, it is recommended first to apply a nasobiliary probe for irrigation of the bile ducts (Figure 4) to prevent rapid clogging of the small caliber bile duct prostheses.

The sequential coupling of two examinations (DBE and ERCP) explains the lengthy examination times, high doses of sedation, and applied fluoroscopy dosage. Considering the enormous benefit of DBE-ERCP with an approximately 74% successful biliary drainage and a significantly smaller complication rate than PTCD[11,12,14-17,27-29], the effort involved in such an examination seems justified.

In comparison to the more frequent cholestatic patients, only three of 37 patients also required radiography and interventions of the pancreatic duct after pancreatic resection. Overall, only a limited view could be gained as to which role DBE-ERCP might play in this area. In all three patients, the position of the pancreaticojejunostomy was only reached by DBE and was located deeper in the small intestine or considerably closer to the blind end of the afferent loop than was the choledocho- or hepaticojejunostomy. The technical conduction of the endoscopic retrograde pancreatography *via* DBE was undertaken in the same manner as described for ERCP. The ostium, however, was smaller, but in none of the cases stenotic. The main pathological changes of chronic pancreatitis were limited to the remaining pancreatic duct in the corpus area. During DBE-based pancreatography, a cystic lesion (pseudocyst) could be successfully drained *via* insertion of a 7 Fr double pigtail prosthesis for the first time, which led to a noticeable improvement of the patient, and regression of the pseudocyst within a week. Therefore, DBE offers also a novel option for pseudocyst drainage in postsurgical patients.

In conclusion, this prospective study from a single university tertiary referral center confirms the results from other investigators and shows that DBE-ERCP achieves a high rate of successful cholangiography and drainage in post-surgical patients[5-10,22-26,29], allows further treatment of pancreatic cystic lesions *via* pancreaticojejunostomy, and offers new possibilities in patients with PTCD as DBE-based rendezvous techniques are applicable.

ACKNOWLEDGMENTS

We thank our endoscopy assistants Hiwot Diebel, Sandra Raithel and Franz Kraus who assisted with the examinations and worked out the procedural standards for preparation, assistance and post-processing of the DBE-ERCP procedure. They were an invaluable help for this study.

COMMENTS

Background

Abdominal surgery involving the stomach, small bowel, pancreas, liver or biliary tract may change significantly the anatomy of these organs, with construction of small bowel anastomoses and small bowel limbs of differing length, angles or fixation. Thus, postoperative endoscopy with conventional endoscopes to reach the biliary tract or pancreas through small bowel limbs has often been described as unsatisfactory in postoperative disease.

Research frontiers

Balloon-assisted endoscopy has been developed since 2004, with the introduction of a double balloon enteroscopy (DBE) system, followed later by single balloon endoscopy or balloon-guided enteroscopy techniques. All balloon-assisted endoscopy techniques have the potential to access more deeply into the small bowel than conventional endoscopes, and they allow one to examine the whole small bowel (4-7 m long). Thus, this study investigated the value of the DBE for examination of postoperative patients with diseases of the biliary tract or pancreas.

Innovations and breakthroughs

Before the era of balloon-assisted endoscopy, only 20%-30% of patients with diseases of the biliary tract or pancreas (e.g. tumor, stones, inflammation, stenosis) could be effectively managed by conventional endoscopy, whereas the other 70%-80% had to be treated by more invasive percutaneous puncture techniques, external tube insertion, drainage procedures, and more cost-intensive computed tomography (CT)-based therapies, or even re-operation. This paper describes, in a large number of consecutive patients, successful use of DBE to perform effective endoscopic treatment in a majority (74%) of post-surgical patients with bilio-pancreatic diseases.

Applications

DBE-based examination of the biliary tract or pancreas represents a further important endoscopic treatment modality for postoperative patients after complex abdominal resections. It allows successful application and interventions in post-surgical patients with bile duct stenosis, obstruction, stones or pancreatic diseases (chronic inflammation, tumor) in terms of performing incision of the bile duct ostium, or papillotomy, endoprosthesis insertion, or stone extraction.

Terminology

DBE-based examination of the biliary tract and pancreas is achieved by forward-viewing optics in post-surgical patients, and requires examination of the small bowel by DBE, and includes endoscopic–radiological examination of the bile duct and/or pancreatic duct, with the aim of performing interventions in the case of bile duct, liver or pancreatic disease. This whole procedure is called DBE-based retrograde cholangiopancreaticiography and is indicated only when conventional endoscopy fails to reach the biliary tract or pancreas.

Peer review

This study describes the utility of modern enteroscopy, especially DBE, in symptomatic patients with cholestasis and cholangitis after complex abdominal surgery. A high rate of enteroscopic access and successful biliary interventional procedures, with a new intervention, ostial incision at biliary anastomoses is presented, which resulted in a substantial reduction in more invasive procedures such as transhepatic percutaneous biliary interventions or CT-guided punctures.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2315

World J Gastroenterol 2011 May 14; 17(18): 2315-2325
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

(-)-Epigallocatechin-3-gallate inhibits VEGF expression induced by IL-6 *via* Stat3 in gastric cancer

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Supported by National Natural Science Foundation of China, Grant, No. 30571833; Natural Science Foundation of Guangdong Province, 05001785; China Postdoctoral Science Foundation 20100470963
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Received: September 16, 2010 Revised: January 6, 2011
Accepted: January 13, 2011
Published online: May 14, 2011

Abstract

AIM: To demonstrate that (-)-Epigallocatechin-3-gallate (EGCG) inhibits vascular endothelial growth factor (VEGF) expression and angiogenesis induced by interleukin-6 (IL-6) *via* suppressing signal transducer and activator of transcription 3 (Stat3) activity in gastric cancer.

METHODS: Human gastric cancer (AGS) cells were treated with IL-6 (50 ng/mL) and EGCG at different concentrations. VEGF, total Stat3 and activated Stat3 protein levels in the cell lyses were examined by Western blotting, VEGF protein level in the conditioned medium was measured by enzyme-linked immunosorbent assay, and the level of VEGF mRNA was evaluated by reverse transcription polymerase chain reaction (RT-PCR). Stat3 nuclear translocation was determined by Western blotting with nuclear extract, and Stat3-DNA binding activity was examined with Chromatin immunoprecipitation (ChIP) assay. IL-6 induced endothelial cell proliferation was measured with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide assay, *in vitro* angiogenesis was determined with endothelial cell tube formation assay in Matrigel, and IL-6-induced angiogenesis *in vitro* was measured with Matrigel plug assay.

RESULTS: There was a basal expression and secretion of VEGF in AGS cells. After stimulation with IL-6, VEGF expression was apparently up-regulated and a 2.4-fold increase was observed. VEGF secretion in the conditioned medium was also increased by 2.8 folds. When treated with EGCG, VEGF expression and secretion were dose-dependently decreased. IL-6 also increased VEGF mRNA expression by 3.1 folds. EGCG treatment suppressed VEGF mRNA expression in a dose-dependent manner. EGCG dose-dependently inhibited Stat3 activation induced by IL-6, but did not change the total Stat3 expression. When treated with EGCG or AG490, VEGF expressions were reduced to the level or an even lower level in the tumor cells not stimulated with IL-6. However, PD98059 and LY294002 did not change VEGF expression induced by IL-6. EGCG inhibited Stat3 nucleus translocation, and Stat3-DNA binding activity was also markedly decreased by EGCG. Furthermore, EGCG inhibited IL-6 induced vascular endothelial cell proliferation and tube formation *in vitro* and angiogenesis *in vitro*.

CONCLUSION: EGCG inhibits IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer, which has provided a novel mechanistic insight into the anti-angiogenic activity of EGCG.

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Key words: Epigallocatechin-3-gallate; Vascular endothelial growth factor; Signal transducer and activator of transcription 3; Angiogenesis; Gastric cancer

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Zhu BH, Chen HY, Zhan WH, Wang CY, Cai SR, Wang Z, Zhang CH, He YL. (-)-Epigallocatechin-3-gallate inhibits VEGF expression induced by IL-6 *via* Stat3 in gastric cancer. *World J Gastroenterol* 2011; 17(18): 2315-2325 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2315.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2315

INTRODUCTION

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and active component of green tea, has shown to have chemopreventive and chemotherapeutic properties for a variety of cancers[1,2]. Previous studies demonstrated that EGCG inhibited tumor growth by anti-angiogenesis, as well as by inhibiting proliferation and inducing apoptosis[3,4]. Angiogenesis is necessary for the growth and metastasis of solid tumors, and vascular endothelial growth factor (VEGF) is the most potent angiogenic factor. EGCG inhibited angiogenesis mainly by targeting VEGF signaling pathway[5-7]. Recent studies have shown that EGCG directly inhibits VEGF expression in multiple tumors[8-10]. We also demonstrated that EGCG reduced VEGF production in gastric cancer, and the inhibitory effect was at transcriptional level, suggesting that EGCG inhibited VEGF expression by reducing VEGF gene transcription[11]. However, the detailed molecular mechanism underlying the inhibitory effect of EGCG on VEGF expression is not fully understood.

VEGF expression associates with a variety of cytokines, growth factors, transcription factors, and oncoproteins, such as interleukin-6 (IL-6)[12,13]. Significantly, many of these molecules transmit signals through signal transducer and activator of transcription 3 (Stat3), a member of Janus-activated kinase (JAK)/STAT signaling pathway[12-15]. Activation by phosphorylation of tyrosine residue is required for the activity of Stat3, which is normally a transient and tightly regulated process. Once activated, Stat3 translocates into nucleus, binds to specific DNA promoter sequence and induces downstream gene expression[15]. Aberrant activation of Stat3 is found in a variety of tumors and contributes to oncogenesis by enhancing proliferation and preventing apoptosis[16,17]. Recent studies showed that abnormal Stat3 activation directly promoted VEGF expression and angiogenesis, and blockage of Stat3 activation inhibited these effects[18-20]. Abnormal Stat3 activation is also found in various gastric cancer cell lines and specimens, and associated with tumor status[21-23]. Phosphorylated Stat3 expression is significantly correlated with VEGF expression and microvessel density in gastric cancer, and is an independently prognostic factor of poor survival[22,23]. Blockade of Stat3 activation induced cell apoptosis and growth inhibition in gastric cancer[21,22]. Furthermore, gastric cancer cells transfected with dominant-negative Stat3 exhibits a decreased VEGF expression and less angiogenic phenotype[23], suggesting that blockade of Stat3 activation could inhibit VEGF expression and angiogenesis in gastric cancer. Our recent study showed that EGCG inhibited Stat3 activation and VEGF expression in gastric cancer[11]. Previous studies also demonstrated that EGCG inhibited activation of Stat3 in multiple tumor cells[9,24,25]. However, to our knowledge, whether EGCG inhibits VEGF expression and angiogenesis *via* Stat3 remains to elucidate.

An etiologic relation between high risk of gastric cancer and chronic gastritis with *Helicobacter pylori* has been firmly established[26]. Consequently, various cytokines have been implicated in the pathogenesis of gastric cancer. As a multifunctional cytokine, IL-6 has received particular attention. IL-6 promotes tumor growth and metastasis by up-regulating VEGF expression and VEGF-mediated angiogenesis, and is closely associated with disease status and outcome of gastric cancer[27,28]. Recent studies demonstrated that IL-6 induced VEGF expression and angiogenesis *via* Stat3 in multiple tumors[29-31] and gastric cancer[32]. Blocking Stat3 signaling pathway down-regulated VEGF promoter activity, and effectively abolished IL-6-induced VEGF expression and angiogenesis[33,34]. Therefore, this study was designed to demonstrate that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer in an attempt to further understand the molecular mechanism underlying the anti-angiogenic activity of EGCG.

MATERIALS AND METHODS

Cell culture

Human gastric cancer (AGS) cells (Cell Bank of Sun Yet-San University, Guangzhou, China) were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), (Gibco BRL, Gaithersburg, MD) and incubated at 37℃ in a humidified incubator at 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were prepared from fresh human umbilical cord obtained from the Department of Obstetrics and Gynecology, First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China as described previously[11], and grown in human endothelial-serum free medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100U penicillin, streptomycin and fungizone, and incubated at 37℃ in a humidified incubator at 5% CO_2. To maintain a uniform condition, all experiments were carried out between cell passages 4-6.

Western blotting

After serum starvation for 24 h, AGS cells (5×10^5 cells/well)

seeded in 90 mm plates were stimulated with IL-6 (50 ng/mL, R&D systems, Minneapolis, Minn., USA) in the presence of EGCG (Sigma-Aldrich Chemical Co., St Louis, MO, USA) at concentrations indicated for another 24 h to determine the VEGF protein level or for 1 h to determine the Stat3 protein level. Total protein was extracted from the cell lysates with mammalian cell lysis kit (Bio Basic Inc., Ontario, Canada). Protein level was quantified with Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Total protein (100 μg) was separated in 12% sodium dodecyl sulfate (SDS)-PAGE, and transferred onto PVDF membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk and incubated at 4℃ overnight with a rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit polyclonal anti-Stat3 antibody (Santa Cruz), or a goat polyclonal anti-p-Stat3 antibody [Phospho-Stat3 (tyr-705); Santa Cruz]. After being washed with 0.1% Tween 20 in Tris-saline three times, the membrane was incubated with biotin-labeled anti-rabbit or anti-goat IgG for 1 h at room temperature with agitation. The probe proteins were detected using enhanced chemiluminescence system (Amersham International, Piscataway, NJ, USA). The same membrane was stripped and re-blotted with an antibody specific to β-actin (Santa Cruz). Protein expression levels were normalized by β-actin.

Enzyme-linked immunosorbent assay

AGS cells were seeded in 90 mm plates at 5×10^5 cells per well and stimulated with IL-6 (50 ng/mL) for another 24 h in the presence of EGCG at concentrations indicated after serum starvation for 24 h. The conditioned media were harvested and centrifuged. VEGF concentrations in the supernatant were measured using VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D systems).

Reverse transcription polymerase chain reaction

AGS cells (5×10^5 cells/well) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 12 h in the presence of EGCG at concentrations indicated. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The levels of human VEGF mRNA were evaluated by reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription was performed with 1 μg total RNA and 100 pmol random hexamers in a total volume of 20 μL to produce first-strand cDNA. PCR experiments were performed with 1 μL of the first-strand cDNA in a 50 μL reaction mixture. Human VEGF cDNA was amplified with specific primers (sense primer, 5'-TGCATTCACATTTGTTGTGC-3'; antisense primer, 5'-AGACCCTGGTGGACATCTTC-3'; a 200 bp product) and β-actin specific primers (sense primer, 5'-TCATCACCATTGGCAATGAG-3'; antisense primer, 5'-CACTGTGTTGGCGTACAGGT-3'; a 150 bp product). Amplification protocol was as follows: denaturation at 94℃ for 1 min, annealing at 60℃ (for β-actin, 55℃) for 1 min, and extension at 72℃ for 1 min. All PCRs were linear up to 30 cycles. The PCR products were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and quantified by densitometry using the Image Master VDS system and associated software (Pfizer, NY, USA).

Signaling inhibitors blocking the VEGF induction by IL-6

AGS cells were seeded into a 90 mm plate at 5×10^5 cells per well. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for another 24 h in the presence or absence of 50 μmol EGCG or signaling inhibitors: 20 μmol AG490 (Calbiochem, La Jolla, Calif., USA), 25 μmol PD98059 (Sigma) or 25 μmol LY294002 (Sigma). AG490 is a JAK2 inhibitor, PD98059 is a MAPK/ERK kinase (MEK) inhibitor, and LY294002 is a phosphatidylinositol-3-kinase (PI3K) inhibitor. Total proteins were extracted from the cell lysates and subjected to Western blotting analysis for VEGF expression.

Stat3 nuclear translocation

AGS cells (5×10^5 cells/well) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 1 h in the presence or absence of 50 μmol EGCG. The cells were washed with cold phosphate buffered saline and collected with a policeman cell scraper. The cells were suspended in a hypotonic buffer (10 mmol HEPES, 2 mmol $MgCl_2$, 10 mmol KCl, 0.1 mmol EDTA, 1 mmol DTT, 0.5 mmol phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40) and incubated on ice for 10 min. The cell lysates were then centrifuged at 15000 × *g* for 5 min and the pellets were resuspended in a high salt buffer (50 mmol HEPES, 300 mmol NaCl, 50 mmol KCl, 0.1 mmol EDTA, 1 mmol DTT, 0.5 mmol phenylmethylsulfonyl fluoride and 10% glycerol), and then incubated with rotation for 30 min at 4℃. Lysates were centrifuged at 15000 × *g* at 4℃ for 30 min. The supernatant was collected as a nuclear fraction and used for Stat3 nuclear translocation assay with an anti-p-Stat3 antibody, Phospho-Stat3 (tyr-705; Santa Cruz) by Western blotting.

Chromatin immunoprecipitation assay

AGS cells (1×10^7 cells) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 6 h in the presence or absence of 50 μmol EGCG. Chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described[35]. Briefly, cells were cross-linked using 1% formaldehyde at room temperature for 10 min. After sonication, the soluble chromatin was diluted 10-fold with ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mmol EDTA; 16.7 mmol Tris-HCl, pH 8.1; 167 mmol NaCl), and precleared with Protein A beads blocked with 1% salmon sperm DNA and 1% BSA. The precleared chromatin solution was immunoprecipitated by anti-p-Stat3 antibody (Santa Cruz) overnight at 4℃ with rotation. The immunoprecipitates were then pelleted, washed, and the antibody/protein/DNA complex was eluted off the beads by resuspending the pellets in 50 mmol $NaHCO_3$ and 1% SDS for 30 min. Cross-linking was reversed, and protein and RNA were removed by

adding 10 μg Proteinase K and 10 μg RNase A, followed by incubation at 42℃ for 3 h. Purified DNA was subjected to PCR with primers for VEGF promoter as follows: forward: 5'-AGACTCCACAGTGCATACGTG-3' and reverse: 5'-AGTGTGTCCCTCTGACAATG-3', which amplify 235 bp fragments flanking the Stat3 binding element. The final products were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and quantified by densitometry.

HUVECs proliferation and tube formation assay for in vitro angiogenesis

AGS cells seeded in 90 mm plates at 5×10^5 cells per well were stimulated with 50 ng/mL IL-6 for another 24 h in the presence or absence of 50 μmol EGCG or 20 μmol AG490 after serum starvation for 24 h. The conditioned media were generated from the supernatants centrifuged using Amicron® Ultra-15 Centrifugal Filter Devices (Millipore Filter, Bedford, Mass., USA) at 4000 × *g* for 15 min.

For proliferation assay, HUVECs were seeded in 96-well plates pre-coated with 1% gelatin at 5×10^3 cells per well and cultured with 100 μL conditioned media supplemented with 2% FBS in the presence or absence of 10 ng/mL VEGF neutralizing antibody. After cultured for 48 h, the viable cells were quantified by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide assay.

For tube formation assay, Matrigel was thawed at 4℃ in an ice-water bath. Matrigel (300 μL per well) was carefully added to a pre-chilled 24-well plate using a cold pipette and allowed to polymerize for 1h at 37℃. After polymerization, HUVECs (5×10^4 cells/well) in the conditioned media supplemented with 2% serum in the presence or absence of 10 μg/mL VEGF neutralizing antibody were layered on the top of the polymerized gel. Cells were incubated for 48 h at 37℃ in a humidified incubator at 5% CO_2, and the formed tubes were fixed with 10% buffered neutral formalin, stained with Diff-Quick Solution Ⅱ (Baster, McGraw Park, IL), and photographed (100 ×). For quantification of tube formation, the total length of tubes formed in a unit area was measured using national institute of health image program.

Matrigel plug assay for in vivo angiogenesis

Matrigel plug assay was performed as described previously with some modifications[36]. Briefly, 6-8-wk female BALB/c nude mice, weighing 18-22 g (Experimental Animal Center of Sun Yet-san University, Guangzhou, China) were subcutaneously injected with 0.5 mL of a liquid mixture composed of Matrigel (350 μL, 10mg/mL) and the conditioned media (150 μL) prepared as described above with or without 10 μg/mL of VEGF neutralizing antibody near the abdominal midline. The Matrigel was quickly polymerized *in vivo* to form a single and solid gel plug, which allowed the angiogenic factors to release and stimulate angiogenesis. One week later, the Matrigel plugs were harvested, weighed, and then minced and digested in 5 mL Drabkin reagent (Drabkin reagent kit 525; Sigma) for hemoglobin content measurement. Final hemoglobin concentration was calculated from a standard calibration curve.

Statistical analysis

All data were presented as means ± SE. Statistical significance was calculated using unpaired Student's *t* test. A *P* value less than 0.05 was considered statistically significant. All analyses were performed using SPSS version 13.0 (SPSS Inc, USA).

RESULTS

EGCG inhibits VEGF induction by IL-6 in AGS cells

To assess the effect of EGCG on VEGF expression induced by IL-6 in human gastric cancer, we first examined VEGF expression induced by IL-6 in AGS cells treated with EGCG. AGS cells were treated with EGCG at different concentrations and stimulated with IL-6 (50 ng/mL) for 24 h. VEGF protein levels in tumor cell lysates were analyzed by Western blotting. As shown in Figure 1A, there was a basal expression of VEGF in AGS cells. After stimulation with IL-6, VEGF expression was apparently up-regulated and a 2.4-fold increase was observed. When treated with EGCG, VEGF expressions were dose-dependently decreased. This inhibitory effect was not due to the toxic effect of IL-6, because IL-6 at the concentration less than 100 ng/mL did not cause growth inhibition of AGS cells within 24 h (data not shown).

VEGF secretion is a crucial step for tumor-induced angiogenesis, so we further evaluated the effect of EGCG on VEGF secretion induced by IL-6. VEGF protein levels in the conditioned medium were measured by ELISA. IL-6 also induced VEGF secretion in AGS cells and a 2.8-fold increase was observed. Consistent with the result of Western blotting analysis, the secreted proteins of VEGF induced by IL-6 in the conditioned media were reduced by EGCG in a dose-dependent manner (Figure 1B). These data provided direct evidence that EGCG inhibited VEGF production induced by IL-6 in gastric cancer cells.

EGCG inhibits IL-6-induced VEGF expression at transcription level

To determine whether the inhibitory effect of EGCG on IL-6-induced VEGF expression was at transcriptional level in gastric cancer, we examined VEGF mRNA expression in AGS cells by RT-PCR. We found that IL-6 induced VEGF mRNA expression in AGS cells. When stimulated with IL-6 (50 ng/mL) for 12 h, a 3.1-fold increase in VEGF mRNA expression was observed. Treated with EGCG, VEGF mRNA expressions were dose-dependently reduced (Figure 1C). These findings suggested that EGCG inhibited VEGF expression induced by IL-6 in gastric cancer cells at transcriptional level.

EGCG inhibits IL-6-induced VEGF expression *via* Stat3 pathway

IL-6 is known to signal through Stat3, MAPK and PI3K in gastric cancer[32]. To elucidate the signaling pathway that

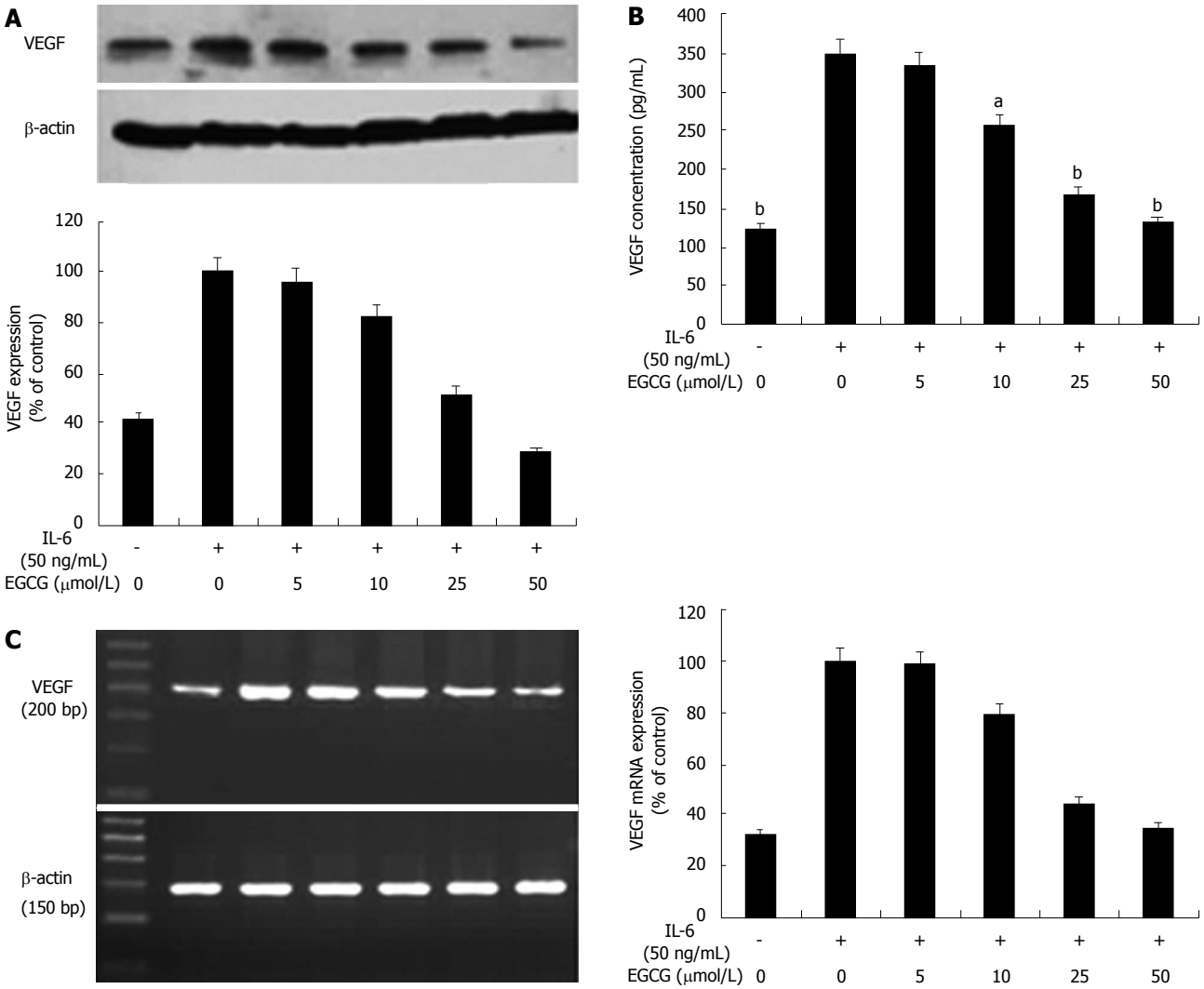

Figure 1 (-)-Epigallocatechin-3-gallate inhibits vascular endothelial growth factor production induced by interleukin-6 in human gastric cancer cells. A: When treated with (-)-Epigallocatechin-3-gallate (EGCG), vascular endothelial growth factor (VEGF) expression was dose-dependently decreased; B: Enzyme-linked immunosorbent assay showed that interleukin-6 (IL-6) induced VEGF secretion in human gastric cancer (AGS) cells and a 2.8-fold increase was observed. EGCG treatment dose-dependently reduced VEGF protein level in the conditioned medium. C: IL-6 also induced VEGF mRNA expression and a 3.1-fold increase in VEGF mRNA expression was observed. When treated with EGCG, VEGF mRNA expression was dose-dependently decreased. Values are expressed as percent of control (means ± SE, $n = 3$, $^{a}P < 0.05$, $^{b}P < 0.01$).

EGCG inhibited VEGF expression in gastric cancer, we tested the effect of several signaling pathway inhibitors, including JAK/STAT, MAPK and PI3K signaling pathway. IL-6 markedly increased VEGF expression in AGS cells. When treated with 50 μmol EGCG or 20 μmol AG490, VEGF expressions were reduced to near the basal level or even lower. However, the other two groups treated with PD98059 or LY294002 still exhibited enhanced VEGF expression, approximating the level of that stimulated with IL-6 (Figure 2). Because PI3K, MEK/ERK, and Stat3 activities could all be suppressed by EGCG, we further analyzed the combined effect of EGCG and the specific inhibitors on IL-6-induced VEGF expression. We found that treatment with 50 μmol EGCG or 20 μmol AG490 alone apparently inhibited IL-6-induced VEGF expression. However, EGCG treatment combined with AG490 did not suppress VEGF expression, and EGCG or AG490 treatment combined with PD98059 or LY294002 did not suppress VEGF expression either (data not shown). These data suggested that EGCG reduced VEGF expression induced by IL-6 *via* Stat3 signaling pathway in gastric cancer.

EGCG reduces IL-6-induced Stat3 activation in AGS cells

We previously demonstrated that EGCG inhibited Stat3 activation without changing Stat3 expression in gastric cancer[11]. In this study, we further assessed the effect of EGCG on Stat3 expression and activation induced by IL-6 in gastric cancer. AGS cells were treated with or without EGCG at different concentrations and stimulated with 50 ng/mL IL-6 for 1 h. Total Stat3 and phospho-Stat3 protein levels were examined using Western blotting with anti-Stat3 antibody to detect total Stat3 protein expression, and with anti-p-Stat3 antibody (specific for tyr-705) to detect phospho-Stat3, respectively. As shown in Figure 3, Stat3 was constitutively activated in AGS cells. When stimulated

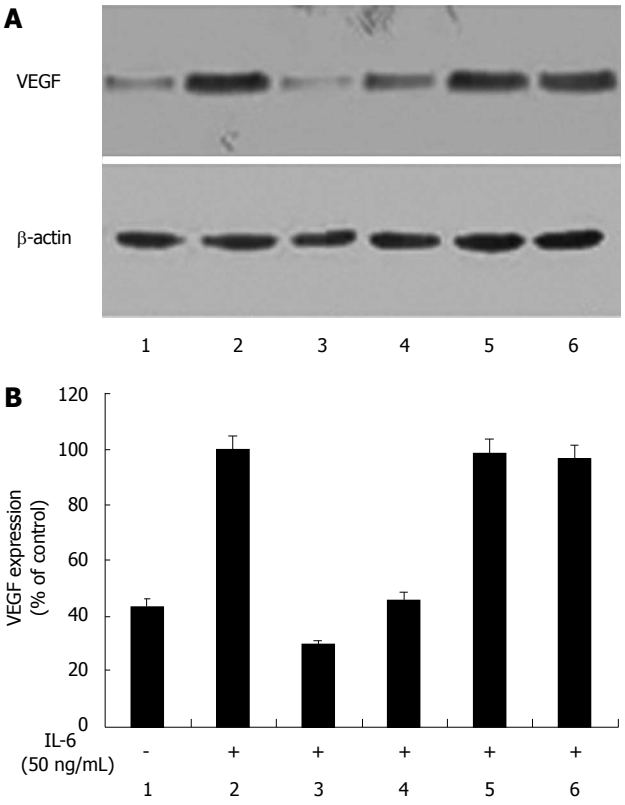

Figure 2 (-)-Epigallocatechin-3-gallate inhibits interleukin-6-induced vascular endothelial growth factor expression in human gastric cancer cells *via* JAK/STAT pathway. Human gastric cancer (AGS) cells were stimulated with interleukin-6 (IL-6) (50 ng/mL) for 24 h in the presence of 50 μmol (-)-epigallocatechin-3-gallate (EGCG) or signaling inhibitors. Vascular endothelial growth factor (VEGF) protein levels in tumor cell lysates were analyzed by Western blotting. IL-6 markedly increased VEGF expression in AGS cells. When treated with EGCG or AG490, VEGF expression was significantly reduced. PD98059 and LY294002 did not change IL-6-induced VEGF expression. 1: Without IL-6 stimulation; 2: Stimulated with IL-6; 3-6: Treated with 50 μmol EGCG or signaling inhibitors of 20 μmol AG490, 25 μmol PD98059 or 25 μmol LY294002.

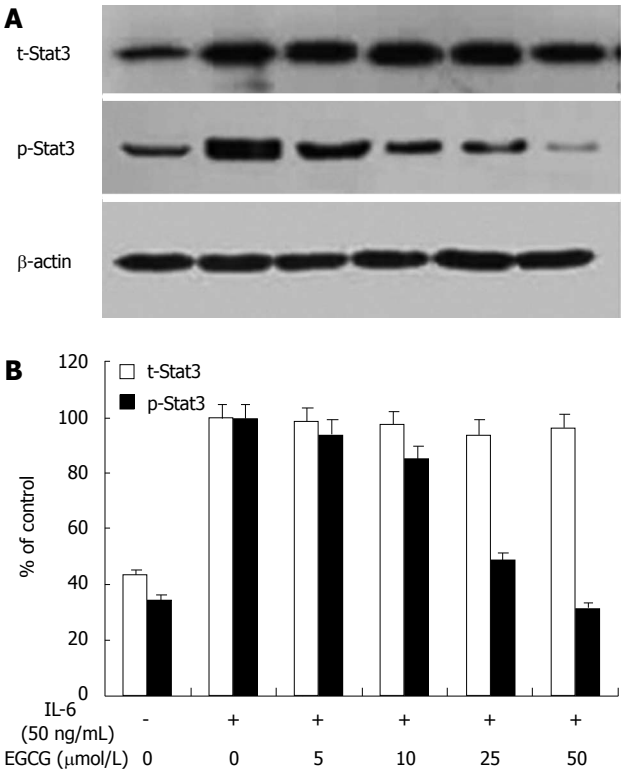

Figure 3 (-)-Epigallocatechin-3-gallate inhibits signal transducer and activator of transcription 3 activation induced by interleukin-6 in human gastric cancer cells. Human gastric cancer (AGS) cells were stimulated with interleukin-6 (IL-6) (50 ng/mL) in the presence of (-)-epigallocatechin-3-gallate (EGCG) at concentrations indicated for 1 h. Total signal transducer and activator of transcription 3 (Stat3) and activated Stat3 were examined by Western blotting. Stat3 was constitutively activated in AGS cells. After stimulated with IL-6, total Stat3 and activated Stat3 expression were both markedly increased by 2.3 folds and 2.9 folds, respectively. When treated with EGCG, activated Stat3 expression decreased in a dose-dependant manner, but total Stat3 expression remained unchanged.

with IL-6 (50 ng/mL) for 1 h, phosphorylation of Stat3 at tyrosine 705 increased by 2.9 folds and the total Stat3 expression increased by 2.3 folds. EGCG treatment inhibited IL-6-induced activation of Stat3 in a dose-dependant manner, without affecting total Stat3 expression. These findings suggested that EGCG reduced IL-6-induced VEGF expression in gastric cancer by inhibiting Stat3 activation instead of Stat3 expression.

EGCG inhibits Stat3 nuclear translocation and DNA binding activity

Our results showed that EGCG inhibited Stat3 activation and reduced VEGF expression at transcriptional level in gastric cancer. Activated Stat3 acts as a transcription activator, and is capable of translocating into nucleus, binding to the Stat3 consensus sequence in VEGF promoter region, thereby up-regulating VEGF expression[15]. To clarify whether EGCG affected Stat3 nuclear translocation and DNA binding activity, we first performed Western blotting with extraction of nuclear protein to visualize the nuclear translocation of phospho-Stat3 after IL-6 stimulation. As shown in Figure 4A, before IL-6 stimulation, less phospho-Stat3 was localized in the nucleus. Once stimulated with IL-6 for 1 h, phospho-Stat3 was apparently increased and translocated into the nucleus. After EGCG treatment, phospho-Stat3 in the nucleus was markedly decreased.

To further evaluate the Stat3-DNA binding activity, ChIP assay was performed. Immunoprecipitation was conducted with an anti-p-Stat3 antibody followed by PCR using oligonucleotide primers that amplified a 235 bp region spanning Stat3 binding site in VEGF promoter. IL-6 apparently increased this band in AGS cells, suggesting that IL-6 up-regulated VEGF expression by promoting Stat3 binding to VEGF promoter and activating VEGF transcription. When treated with EGCG, Stat3-DNA binding activity was markedly decreased (Figure 4B). Taken together, these data provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 through inhibiting Stat3 translocating into nucleus and binding to VEGF promoter in gastric cancer.

EGCG inhibits IL-6-induced angiogenesis in vitro

We further evaluated the effect of EGCG on IL-6-induced angiogenesis *in vitro* by assessing proliferation and

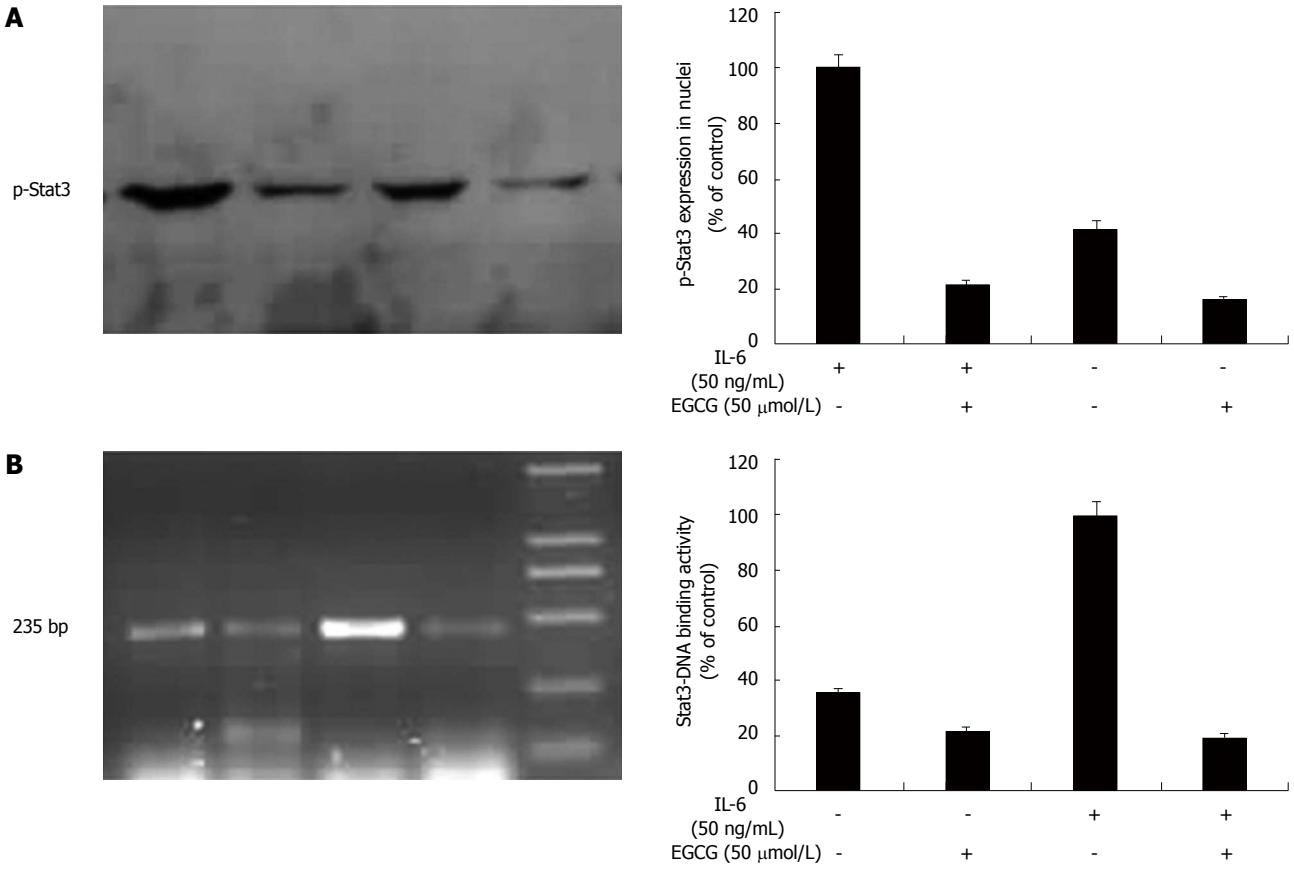

Figure 4 (-)-Epigallocatechin-3-gallate inhibits signal transducer and activator of transcription 3 nuclear translocation and DNA binding activity in human gastric cancer cells. Signal transducer and activator of transcription 3 (Stat3) nuclear translocation was determined by Western blotting with extraction of nuclear proteins. A: After treated with 50 μmol (-)-epigallocatechin-3-gallate (EGCG) and interleukin-6 (IL-6) (50 ng/mL) for 1 h, phospho-Stat3 in the nucleus was visualized with an anti-p-Stat3 antibody; B: IL-6 apparently increased phospho-Stat3 expression in the nucleus, but EGCG treatment markedly decreased this effect. Stat3-DNA binding activity was determined by chromatin immunoprecipitation (ChIP) assay. Immunoprecipitation was conducted with Stat3 antibody followed by polymerase chain reaction (PCR) using oligonucleotide primers that yielded a 235 bp band spanning Stat3 binding site in vascular endothelial growth factor promoter. IL-6 apparently increased Stat3-DNA binding activity. When treated with EGCG, Stat3-DNA binding activity was also markedly decreased.

tube formation of HUVECs cultured in the conditioned media. As shown in Figure 5A and B, the conditional media stimulated with IL-6 promoted HUVECs proliferation and tube formation when compared with the non-stimulated cell culture media. VEGF neutralizing antibody effectively blocked the enhancement of proliferation and tube formation of HUVECs cultured in the IL-6-stimulated conditional media, suggesting that IL-6 increased the angiogenic ability of AGS cells through up-regulating VEGF induction. Treatment with EGCG or AG490 also abrogated the effect of IL-6 on HUVECs cell proliferation and tube formation. These data further confirmed that EGCG inhibited angiogenesis induced by IL-6 through targeting Stat3/VEGF signaling pathway.

EGCG inhibits IL-6-induced angiogenesis in vivo

The effect of EGCG on angiogenesis induced by IL-6 *in vivo* was assessed using Matrigel plug assay. Matrigel plugs were harvested on the 7th d and examined by measuring the density of hemoglobin, as an indicator of vascularization. Hemoglobin concentrations were determined and normalized to represent the vascular densities in the plug. Grossly, the Matrigel plugs embedded with the conditioned media from IL-6-stimulated AGS cells developed substantial vasculature, as compared with the non-stimulated media. However, plugs that contained the conditioned media treated with VEGF neutralizing antibody, EGCG and AG490 exhibited considerably less vascularization (Figure 5C). Collectively, Matrigel plug assay demonstrated that IL-6 markedly potentiated AGS cells to enhance angiogenesis *in vivo* by inducing VEGF production, and EGCG abolished the pro-angiogenesis ability of AGS cells induced by IL-6 through inhibiting VEGF induction *via* Stat3.

DISCUSSION

Both VEGF over-expression and Stat3 over-activation occur at high frequency in human tumors[12-23], and IL-6 has shown to induce VEGF expression and angiogenesis by promoting Stat3 activity[29-31]. In gastric cancer, IL-6 induces VEGF expression *via* Stat3 signaling pathway[32], and is associated with tumor angiogenesis and disease status[27,28]. Previous studies have indicated that EGCG inhibits VEGF expression and Stat3 activation in multiple cancers[9,11,24,25]. However, whether EGCG inhibits

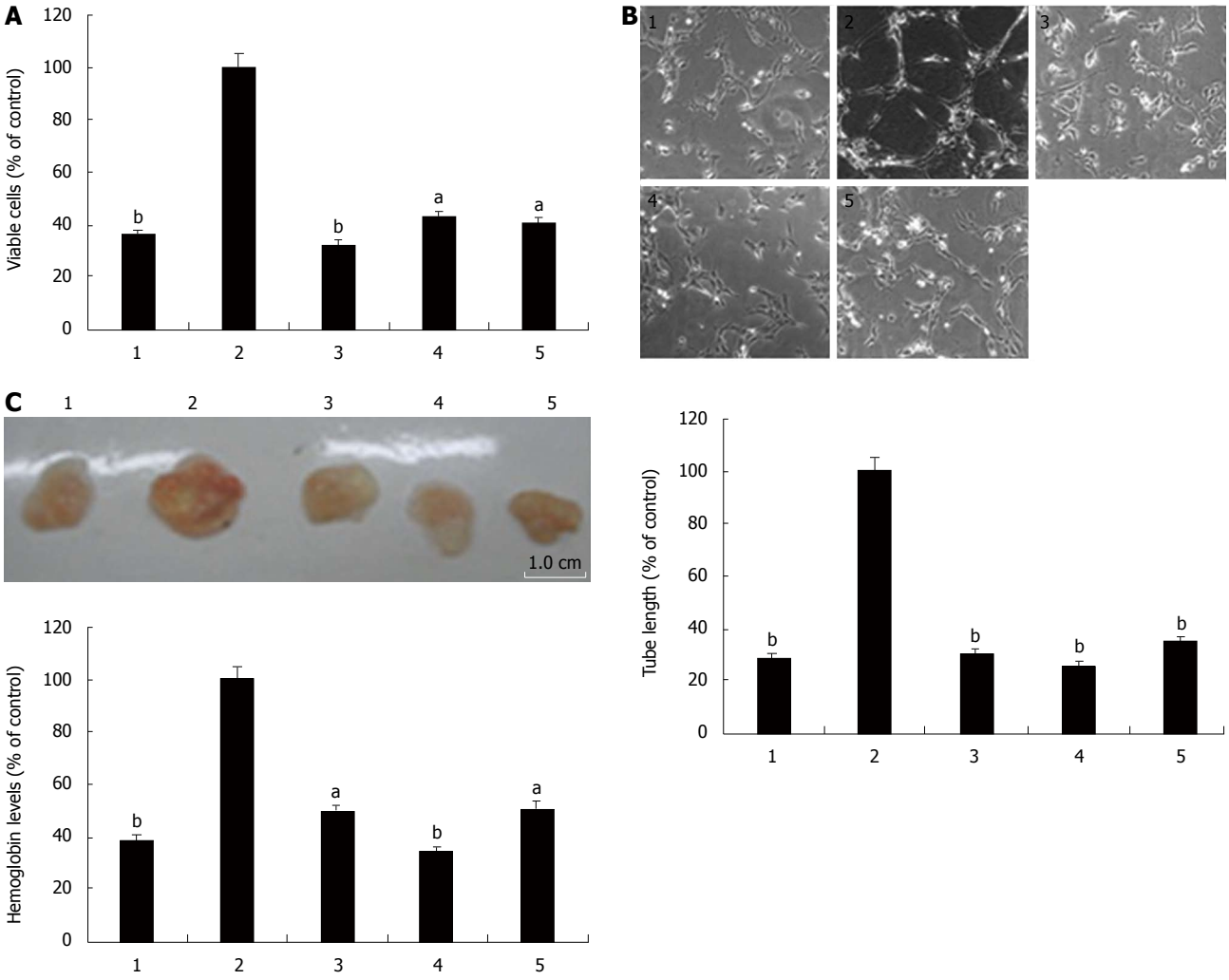

Figure 5 (-)-Epigallocatechin-3-gallate inhibits interleukin-6 induced angiogenesis *in vitro* and in *vivo*. The *in vitro* angiogenesis was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) and tube formation assay. Human umbilical vein endothelial cells (HUVECs) were cultured with the conditioned media in the presence of vascular endothelial growth factor (VEGF) neutralizing antibody, (-)-epigallocatechin-3-gallate (EGCG) or AG490. After 48 h incubation, the viable cells were quantified by MTT assay (A), and the formed tubes were fixed, stained, photographed and analyzed (B). The *in vivo* angiogenesis was determined by Matrigel plug assay. Matrigel plugs containing the conditioned media with VEGF neutralizing antibody, EGCG or AG490 were subcutaneously injected into nude mice. One week later, the Matrigel plugs were harvested and examined by measuring the density of hemoglobin (C). interleukin-6 (IL-6) apparently promoted vascular endothelial cell proliferation and tube formation *in vitro* and vascularization of Matrigel plugs *in vivo*. VEGF neutralizing antibody, EGCG and AG490 all markedly decreased these effects. 1: Without IL-6 stimulation as a negative control; 2: With IL-6 stimulation as a control; 3-5: With IL-6 stimulation and VEGF neutralizing antibody, EGCG or AG490. Values are expressed as percent of control (means ± SE, $n = 3$, $^aP < 0.05$, $^bP < 0.01$).

VEGF expression and angiogenesis *via* Stat3 remains to be elucidated. In this study, we demonstrated that EGCG inhibited IL-6-induced VEGF expression and angiogenesis in gastric cancer *via* suppressing Stat3 activity.

EGCG has shown to inhibit VEGF expression and angiogenesis in a variety of tumors[8-11], and is most effective in inhibiting VEGF expression and angiogenesis among the four main catechins of green tea[7]. However, the exact mechanism for the inhibitory effect of EGCG on VEGF expression and angiogenesis is not well understood. IL-6 is reported to induce VEGF expression and tumor vasculature in gastric cancer[27,28]. To evaluate the effect of EGCG on VEGF expression induced by IL-6 in gastric cancer, we examined VEGF expression in AGS cells treated with EGCG and IL-6. As shown in Figure 1A and B, after stimulated with 50 ng/mL of IL-6, a 2.4-fold increase of VEGF protein in tumor cells and a 2.8-fold increase in the conditioned media were observed. When treated with EGCG, VEGF expression and secretion were decreased in a dose-dependent manner. EGCG also dose-dependently inhibited VEGF mRNA expression in AGS cells (Figure 1C). Here we demonstrated that EGCG inhibited IL-6-induced VEGF expression in gastric cancer cells, and this inhibitory effect was at transcriptional level.

The important role of VEGF in angiogenesis has been well established. Therefore, we further evaluated the effect of EGCG on IL-6-induced angiogenesis both *in vitro* and *in vivo*. As shown in Figure 5, the conditioned media promoted vascular endothelial cell proliferation and tube formation *in vitro* and vascularization of Matrigel plugs *in vivo*. VEGF neutralizing antibody effectively

blocked these effects, confirming that IL-6-induced angiogenesis is VEGF-dependent. EGCG treatment also abrogated IL-6-induced angiogenesis *in vitro* and *in vivo*. These findings suggested that EGCG inhibited IL-6-induced angiogenesis *via* down-regulation of VEGF production in gastric cancer.

Several mechanisms have been proposed for the inhibitory effect of EGCG on VEGF expression[3-11]. In the present study, we found that EGCG inhibited IL-6-induced VEGF expression in AGS cells. IL-6 might act through several classic protein kinase cascades, such as MAPK, PI3K and Stat3[31,32]. To elucidate the signaling pathway that EGCG inhibited VEGF expression induced by IL-6 in AGS cells, we tested the effect of several signaling pathway inhibitors, and found that PD98059 and LY294002 did not affect IL-6-induced VEGF expression, suggesting that IL-6 did not induce VEGF expression in AGS cells through MAPK or PI3K signaling pathway, which was consistent with a previous study[32]. In contrast, EGCG and AG490 effectively inhibited VEGF expression induced by IL-6. In addition, EGCG and AG490 also effectively inhibited IL-6-induced vascular endothelial cell growth and tube formation *in vitro* and vascularization of Matrigel plug *in vivo*, indicating that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* Stat3 signaling pathway. A previous study also demonstrated that Stat3 pathway was predominantly involved in the signaling of IL-6 stimulation in VEGF expression in gastric cancer[32]. These data suggested that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* Stat3 signaling pathway in gastric cancer, and provided a novel mechanistic insight into the effect of EGCG on VEGF expression.

Activation by tyrosine phosphorylation is an indispensable prerequisite for the activity of Stat3. Compared with normal cells and tissues, abnormally activated Stat3 has been detected in a wide variety of human cancer cells and tissues, and associate with VEGF expression and tumor angiogenesis[14-23]. Previous studies have shown that EGCG inhibits activation of Stat3 in various cancer cells[9,24,25]. Our study also demonstrated that EGCG inhibited activation of Stat3 and VEGF expression in gastric cancer[11]. In this study, we found that Stat3 was constitutively activated in AGC cells. IL-6 induced a remarkable increase in Stat3 expression and activation. When treated with EGCG, Stat3 activation was inhibited in a dose-dependent manner, but the total Stat3 expression remained unchanged when compared with the control. EGCG treatment did not affect Stat3 mRNA expression, either (data not shown). These findings suggested that EGCG reduced VEGF expression in gastric cancer by suppressing Stat3 activation.

Once activated, Stat3 translocates into the nucleus, binds to specific DNA promoter sequence and induces downstream gene expression[15]. Stat3-binding site in VEGF promoter has been identified, providing evidence that VEGF is a direct target gene of Stat3. The activated Stat3 acts as a transcriptional activator and is capable of binding directly to the Stat3 consensus sequence in VEGF promoter region, thereby promoting the VEGF expression[18-20]. Previous studies showed that IL-6 up-regulated VEGF expression by promoting Stat3 binding to VEGF promoter[29-31]. Furthermore, blockage of Stat3 activation was associated with a decline in Stat3-DNA binding activity and VEGF mRNA expression in gastric cancer[32]. In this study, we found that IL-6 stimulation apparently increased Stat3 translocation into nucleus and Stat3-DNA binding activity. When treated with EGCG, Stat3 nuclear translocation and Stat3-DNA binding activity was markedly decreased. Masuda *et al*[9] also found that inhibition of Stat3 by EGCG significantly decreased VEGF promoter activity. Taken together, these data provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity.

Increasing evidences have suggested that cytokine/Stat3 signaling pathway plays an important role in tumor development and progression. Stat3 represents a point of convergence for these cytokine signaling pathways and has become a novel promising molecular target for intervention in cancer treatment. Great efforts have been made to disrupt Stat3 signaling pathway for inhibiting angiogenesis and tumor growth[37]. However, the toxicity with these approaches might be increased because multiple down-stream targets are affected. Recently, pharmacological approaches to Stat3 inhibition aim to identify natural products as inhibitors of Stat3 signaling pathway[38,39]. In this study, we demonstrated that EGCG inhibited IL-6-induced VEGF expression and angiogenesis in gastric cancer by the suppression of Stat3 activation, nuclear translocation and Stat3-DNA binding activity. As a natural, low cost and non-toxic product, EGCG has drawn special attention, and may become a promising inhibitor of Stat3 and an angiogenic inhibitor.

COMMENTS

Background

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and active component of green tea, has shown to have chemopreventive and chemotherapeutic properties for a variety of cancers, especially gastrointestinal cancers. Anti-angiogenic activity is one of its main effects against cancer. However, the detailed molecular mechanism is not fully understood.

Research frontiers

Angiogenesis is necessary for solid tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor, and EGCG has shown to inhibit angiogenesis and tumor growth *via* suppressing VEGF expression. However, the molecular mechanism remains unclear.

Innovations and breakthroughs

Increasing evidences suggest that cytokine/Stat3 signaling pathway plays an important role in tumor development and progression. Signal transducer and activator of transcription 3 (Stat3) represents a point of convergence for cytokine signaling pathways and has become a novel promising molecular target for intervention in cancer therapy. In this study, the authors demonstrate that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer, and provided further evidence of the molecular mechanism underlying the anti-angiogenic activity of EGCG.

Applications

By understanding how EGCG inhibits IL-6-induced VEGF expression and

angiogenesis *via* suppressing Stat3 activity, this study may represent a future strategy for therapeutic intervention in the treatment of gastric cancer.

Terminology

Stat3 represents a point of convergence for cytokine signaling pathways. IL-6 induces VEGF expression and angiogenesis *via* Stat3 in multiple tumors. Non-surprisingly, EGCG down-regulates VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity.

Peer review

The authors for the first time examined how EGCG inhibits IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity. It provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity. The results may represent a new molecular mechanism of anti-angiogenic activity of EGCG, and identified a new natural product as an inhibitor of Stat3 signaling pathway.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2326

World J Gastroenterol 2011 May 14; 17(18): 2326-2331
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

LBP and CD14 polymorphisms correlate with increased colorectal carcinoma risk in Han Chinese

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Supported by The National Natural Science Foundation of China, No.30571924
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Received: February 21, 2011 Revised: April 11, 2011
Accepted: April 18, 2011
Published online: May 14, 2011

Abstract

AIM: To explore the associations of polymorphisms of lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), toll-like receptor 4 (TLR-4), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) with the colorectal carcinoma (CRC) risk in Han Chinese.

METHODS: Polymorphisms of LBP (rs1739654, rs2232596, rs2232618), CD14 (rs77083413, rs4914), TLR-4 (rs5030719), IL-6 (rs13306435) and TNF-α (rs35131721) were genotyped in 479 cases of sporadic colorectal carcinoma and 486 healthy controls of Han Chinese in a case-control study. Single-nucleotide polymorphisms (SNPs) between cases and controls were analyzed by unconditional logistic regression.

RESULTS: GA and GG genotypes of LBP rs2232596 were associated with a significantly increased risk of CRC [odds ratio (OR) = 1.51, 95% confidence interval (CI) 1.15-1.99, *P* = 0.003; OR = 2.49, 95% CI 1.16-5.38, *P* = 0.016, respectively]. A similar association was also observed for the CG genotype of CD14 rs4914 (OR= 1.69, 95% CI 1.20-2.36, *P* = 0.002). In addition, a combination of polymorphisms in LBP rs2232596 and CD14 rs4914 led to a 3.4-fold increased risk of CRC (OR = 3.44, 95% CI 1.94-6.10, *P* = 0.000).

CONCLUSION: This study highlights the LBP rs2232596 and CD14 rs4914 polymorphisms as biomarkers for elevated CRC susceptibility in the Chinese Han population.

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Key words: Colorectal carcinoma; Cluster of differentiation 14; Lipopolysaccharide binding protein; Single-nucleotide polymorphisms

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Chen R, Luo FK, Wang YL, Tang JL, Liu YS. LBP and CD14 polymorphisms correlate with increased colorectal carcinoma risk in Han Chinese. *World J Gastroenterol* 2011; 17(18): 2326-2331 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2326.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2326

INTRODUCTION

Colorectal carcinoma (CRC) is one of the major causes of cancer death throughout the world. In China, the incidence rate of newly diagnosed CRC cases is increasing rapidly[1]. Both environmental and genetic factors contribute to the tumorigenesis of CRC. The classical adenoma-carcinoma sequence model proposes that genetic mutations of K-ras, adenomatous polyposis coli (APC), the deleted in colorectal cancer (DCC), and p53 play important roles in the malignant transformation and cancer progression

of CRC[2]. Recent studies have demonstrated that chronic inflammation is also an important factor in the carcinogenesis of CRC[3,4]. In the tumor microenvironment, inflammatory cells, especially the so-called tumor-associated macrophages (TAMs), induce suppression of host antitumor activities, stimulate tumor cell growth, and promote malignant transformation, angiogenesis and metastasis[4-8].

TAMs are key regulatory components of cancer-related inflammation. TAMs mainly derive from monocytic precursors in blood circulation, and are recruited to the tumor sites by tumor-derived chemokines such as C-C motif ligand 2 (CCL2) as well as cytokines in the tumor microenvironment, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) and macrophage colony stimulating factor (M-CSF). These chemokines and cytokines also regulate the survival and differentiation of TAMs[7,9]. Further studies have demonstrated that TAMs are defective in IFN-γ/lipopolysaccharide (LPS) responsiveness to bacterial invasion[10,11]. In solid tumors, TAMs are reprogrammed to have pro-tumor properties and therefore fail to respond to LPS stimulation that should have killed the cancer cells[12]. Whether LPS-induced signaling pathways play important roles in tumor progression warrants further investigation.

Lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), and toll-like receptor 4 (TLR-4) are pattern-recognition receptors (PRRs) that mediate innate immune response to LPS challenge[13,14]. LBP is a secretory class I acute phase protein, which can drastically increase LPS-induced activation of immune cells by binding with LPS and transferring it to CD14. CD14 is a glycosylphosphatidylinositol (GPI)-linked LPS receptor which exists as either membrane-bound forms on the surface of immune cells or soluble forms in the serum. TLR4 belongs to a family of innate immune receptors expressed on the surface of monocytes and macrophages, recognizing pathogen-associated molecular patterns (PAMPs) such as LPS. In the LPS signaling pathway, TLR4 can specifically recognize LPS with the aid of LBP, CD14 and MD-2 molecular complex and activate macrophages in response to LPS-induced inflammation.

Genetic variations of inflammatory factor genes are correlated with increased risk in several malignant tumors. Previous studies have demonstrated a strong association between IL-1beta, IL-8 polymorphisms and gastric carcinoma[15,16], IL-6 polymorphism and cervical carcinoma[17], IL-8, IL-10, TLR4 polymorphisms and prostate carcinoma[18,19], TNF-α polymorphism and non-small cell lung cancer[20]. However, the association between polymorphisms of LPS-signaling-related genes and CRC susceptibility in the Chinese Han population remains elusive. In this study, we directly addressed this issue and investigated the association between polymorphisms of LBP, CD14, TLR4, IL-6 and TNF-α genes and the CRC risk in a case-control study.

MATERIALS AND METHODS

Study population

All subjects were genetically unrelated Chinese Han people living in the southwest region of China. The characteristics of CRC cancer patients and controls included in this study are summarized in Table 1. Patients were chosen from Chongqing Xinqiao Hospital, the second affiliated hospital of Third Military Medical University who were treated from 2008 to 2010. The diagnosis of CRC was confirmed histologically. Patients with histories of previous cancers other than CRC and radiotherapy or chemotherapy were excluded. The controls were healthy people matched to cases by age, sex and dietary habits. Informed consent was obtained from all subjects and the study was approved by the Ethical Committee of Third Military Medical University. Individuals who had smoked over 100 cigarettes were classified as smokers, including current smokers and former smokers who had stopped smoking for at least one year. Individuals who had been drinking alcohol at least once a week for more than 6 months were labeled as drinkers, including current drinkers and former drinkers. Former drinkers were those who had abstained from drinking for more than one year.

Table 1 Characteristics of colorectal carcinoma cases and controls *n* (%)

Parameters	Cases	Controls	*P* value
Age (mean ± SD, yr)	57.85 ± 10.05	58.10 ± 13.47	0.751
Sex			
Male	259 (54.1)	254 (52.3)	
Female	220 (45.9)	232 (47.7)	
Total	479	486	0.574
Smoking status			
Never	334 (69.7)	357 (73.5)	
Former	15 (3.1)	39 (8.0)	
Present	130 (27.1)	90 (18.5)	
Total	479	486	0.199
Drinking status			
Never	10 (2.1)	16 (3.3)	
Former	280 (58.5)	207 (42.6)	
Present	189 (39.5)	263 (54.1)	
Total	479	486	0.248
Histological grade			
High	11 (2.3)		
Intermediate	296 (61.8)		
Low	172 (35.9)		
Location			
Colon	233 (48.6)		
Rectum	242 (50.5)		
Colon and rectum	4 (0.8)		
Stage			
Ⅰ	142 (29.7)		
Ⅱ	110 (23.0)		
Ⅲ	142 (29.6)		
Ⅳ	85 (17.7)		

DNA extraction, polymorphism selection and genotyping

Genomic DNA was extracted from whole blood samples of subjects by the TIANamp Blood DNA Kit (Tiangen, China) according to the manufacturer's instructions. Eight polymorphisms in 5 genes (rs1739654, rs2232596 and rs2232618 in LBP; rs4914 and rs77083413 in CD14; rs5030719 in TLR4; rs13306435 in IL-6; and rs35131721 in TNF-α) examined in our study have been reported with

the minor allele frequency over 1%[21]. SNP genotyping was carried out by the two-step SNaPshot assay. The first step was amplification of gene fragments containing these polymorphic sites. The polymerase chain reaction (PCR) was performed in 25 μL reaction mixture containing 1 × master mix (Tiangen, China), 30 ng genomic DNA templates and 0.4 μmol/L primer sets. Primer sequences (5' to 3') were presented in the order of forward, reverse and SNaPshot sequences, including LBP rs1739654: ACAGAAATGCAGGGACACCTCT, CCTGAGGCTCTCTCTTCCTCAC, GCGGCCCAGGAGGGGCTATT; LBP rs2232596: TCCAACTGGACCCTAGTGGAGT, CGCCTGGCCCTTAATTTTTACT, CCATGTTTTTCAGATTTGCGAAATGATCCAGAAATC; LBP rs2232618: TATGTTGGCACACACAGAACCA, CACTTCCATGTGTCCCTCTGTC, GCTCCTCAACTATTACATCCTTAACACC; CD14 rs77083413: TGAATTGGTCGAAAAGTCCTCA, CCCTGAACTCCCTCAATCTGTC, TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACCACGCCGGAGTTCATTGAGCCCTCGTG; CD14 rs4914: ACACGGACCCGTTGTTTAAGAT, TGGAACAGGTGCCTAAAGGACT, TTTTTTTTTTTTTTCTTGGATCTTAGGCAAAGCCCCGGGCCCCTTGGAG; TLR4 rs5030719: CAGAGTTGCTTTCAATGGCATC, TGCAGGAAACTCTGGTGTTCA, TTTTTTTTTTTTTTTTTTTTTTTTCCTGGACCTCTCTCAGTGTCAACTGGAGCA; IL-6 rs13306435: ATGGAAGGGTCCTACTCAGAGC, CATAAGTTCTGTGCCCAGTGGA, CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCACTTTCATTTTCCTTCAGGCAAAGAATCTAGA; TNF-α rs35131721: GCGGGAAATATGACAGCTAAGG, CTCCCCCAAGACCAAAACTTTA, TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAACCATTCTCCTTCTCCCCAACAGTTCC. A similar amplification condition was used for all genes: one cycle of denaturation at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 30 s, annealing at 68℃ for 30 s, elongation at 72℃ for 40 s; and an eventual elongation at 72℃ for 5 min. The size of PCR products were confirmed by 1.5% agarose gel electrophoresis. The second step was genotyping using the SNaPshot Multiplex Kit (Applied Biosystems, USA). Three μL PCR amplification products were mixed with 5 μL SNaPshot Multiplex Kit and 1 μL 10 μmo/L SNaPshot primer. The SNaPshot PCR condition was 25 cycles of 10 s at 96℃, 5s at 50℃ and 30 s at 60℃. Subsequently, samples were mixed with Liz120 (Applied Biosystems, USA) and were electrophoresed using Genetic Analyzer 3130 instrument (Applied Biosystems, USA). The data were analyzed using the 4.0 Genemapper software (Applied Biosystems, USA).

Statistical analysis

Differences in demographic variables, smoking status, drinking status, grouped genotypic frequencies between cases and controls were evaluated by Student's *t* test and χ^2 test. Two-sided *P* values were considered significant at levels less than 0.05. The associations between polymorphisms of LPS-signaling-related genes and CRC risk were estimated from unconditional regression analysis using the SPSS 13.0 software (PASW, USA). All the eight SNPs were tested for the Hardy-Weinberg equilibrium.

RESULTS

The characteristics of 479 CRC cases and 486 healthy controls are summarized in Table 1. In this case-control study, eight polymorphisms of five genes involved in the LPS-signaling pathway were assayed, in which TLR4 rs5030719 and TNF-α rs35131721 SNPs were excluded due to data bias. All the other six polymorphisms satisfied the Hardy-Weinberg equilibrium ($P > 0.05$).

The effects of the polymorphisms of LPS-signaling-related genes on the risk of colorectal cancer are shown in Table 2. In the genetic model, the G allele of LBP rs2232596 SNP was significantly associated with CRC (GA genotype: odds ratio (OR) = 1.51, 95% confidence interval (CI) 1.15-1.99, $P = 0.003$; GG genotype: OR = 2.49, 95% CI 1.16-5.38, $P = 0.016$). Similarly, the G allele of CD14 rs4914 SNP showed a strong association with the risk of CRC (CG genotype: OR = 1.69, 95 %CI 1.20-2.36, $P = 0.002$). To examine the interaction between epidemiological factors and genetic variances, stratified analysis using logistic regression was performed and no significant difference was found in the genotype distribution of LBP rs2232596 and CD14 rs4914 with respect to age, sex, tumor location and stages (data not shown).

Gene-tobacco exposure interactions and gene-alcohol exposure interactions were also evaluated by stratification analysis using logistic regression (Tables 3 and 4). Smokers with GA genotype of LBP rs2232596 SNP had a significant association with the CRC risk (OR = 1.68, 95% CI 1.17-2.40, $P = 0.005$), whereas in non-smokers, an increased CRC risk with CG genotype of CD14 rs4914 SNP (OR = 2.82, 95% CI 1.64-4.85, $P = 0.000$) was observed. In alcohol drinkers, the presence of GA and GG genotypes of LBP rs2232596 SNP (OR = 1.61, 95% CI 1.23-2.11, $P = 0.001$) and CG genotype of CD14 rs4914 SNP (OR = 1.80, 95% CI 1.28-2.55, $P = 0.001$) was associated with increased risk of CRC.

Further evaluations of the combinatory effects of LBP rs2232596 and CD14 rs4914 SNPs were conducted using logistic regression analysis (Table 5). Subjects carrying risk genotypes in both LBP and CD14 (GA and GG in LBP rs2232596, CG in CD14 rs4914) showed synergistic effects on the associations with the CRC risk (OR = 1.46, 95% CI 1.11-1.92, $P = 0.007$ and OR = 3.44, 95% CI 1.93-6.10, $P = 0.000$).

DISCUSSION

Our study aimed to investigate the association of SNPs in LPS-signaling-related genes and the risk of CRC in the Chinese Han population. We found that the G alleles of both LBP rs2232596 and CD14 rs4914 were significantly associated with CRC. In addition, a combination of these two polymorphisms dramatically increased the CRC risk. To our knowledge, this is the first report on the relationship of these two polymorphisms with gastrointestinal malignancies.

Table 2 Genes, polymorphism and frequencies in colorectal carcinoma cases and controls

SNP	Genotype	Cases (*n* = 479)	Controls (*n* = 486)	Odds ratio (95% CI)	[1]*P* value
LBP					
rs1739654	GG	377 (78.7%)	360 (74.1%)		
	GA	93 (19.4%)	118 (24.3%)	0.75 (0.55-1.02)	0.07
	AA	9 (1.9%)	8 (1.6%)	1.07 (0.41-2.82)	0.88
	GA+AA	102 (21.3%)	126 (25.9%)	0.77 (0.57-1.04)	0.09
rs2232596	AA	289 (60.3%)	343 (70.6%)		
	GA	169 (35.3%)	133 (27.4%)	1.51 (1.15-1.99)	0.003
	GG	21 (4.4%)	10 (2%)	2.49 (1.16-5.38)	0.016
	GA+GG	190 (39.7%)	143 (29.4%)	1.58 (1.21-2.06)	0.001
rs2232618	TT	385 (80.4%)	396 (81.5%)		
	CT	93 (19.4%)	88 (18.1%)	1.09 (0.79-1.50)	0.613
	CC	1 (0.2%)	2 (0.4%)	0.51 (0.05-5.70)	0.581
	CT+CC	94 (19.6%)	90 (18.5%)	1.07 (0.78-1.48)	0.662
CD14					
rs77083413	GG	403 (84.1%)	400 (82.3%)		
	GC	70 (14.6%)	81 (16.7%)	0.858 (0.605-1.215)	0.388
	CC	6 (1.3%)	5 (1%)	1.19 (0.36-3.93)	0.774
	GC+CC	76 (15.9%)	86 (17.7%)	0.88 (0.63-1.23)	0.447
rs4914	CC	369 (77%)	415 (85.4%)		
	CG	102 (21.3%)	68 (14%)	1.69 (1.20-2.36)	0.002
	GG	8 (1.7%)	3 (0.6%)	3.00 (0.79-11.39)	0.091
	CG+GG	110 (23%)	71 (14.6%)	1.74 (1.25-2.42)	0.001
IL-6					
rs13306435	TT	415 (86.6%)	420 (86.4%)		
	AT	60 (12.5%)	65 (13.4%)	0.93 (0.64-1.36)	0.723
	AA	4 (0.9%)	1 (0.2%)	4.05 (0.45-36.37)	0.177
	AT+AA	64 (13.4%)	66 (13.6%)	0.98 (0.68-1.42)	0.921

[1]Adjusted for age, sex, smoking and drinking status.

Table 3 Stratification analyses for rs2232596 by smoking or drinking status

Genotype frequencies (%)	Status	Cases (*n* = 479)	Controls (*n* = 486)	[1]*P* value	Odds ratio (95% CI)
	Smoking				
AA	No	131 (27.3%)	155 (31.9%)		
GA	No	59 (12.3%)	55 (11.3%)	0.303	1.26 (0.81-1.95)
GG	No	10 (2.1%)	5 (1.0%)	0.292	1.86 (0.59-5.87)
GA/GG	No	69 (14.4%)	60 (12.3%)	0.190	1.32 (0.87-2.02)
AA	Yes	158 (33.0%)	188 (38.7%)		
GA	Yes	100 (20.9%)	78 (16.0%)	0.005	1.68 (1.17-2.40)
GG	Yes	11 (2.3%)	5 (1.0%)	0.084	2.59 (0.88-7.63)
GA/GG	Yes	111 (23.2%)	85 (17.0%)	0.002	1.73 (1.22-2.46)
	Drinking				
AA	No	6 (1.3%)	9 (1.9%)		
GA	No	4 (0.8%)	6 (1.2%)	0.892	0.89 (0.16-5.07)
GG	No	0 (0%)	1 (0.2%)	0.998	
GA/GG	No	4 (0.8%)	7 (1.4%)	0.739	0.75 (0.14-4.03)
AA	Yes	283 (59.1%)	334 (69.7%)		
GA	Yes	165 (34.4%)	127 (26.1%)	0.003	1.53 (1.16-2.03)
GG	Yes	21 (4.4%)	9 (1.9%)	0.015	2.68 (1.21-5.97)
GA/GG	Yes	186 (38.8%)	136 (28.0%)	0.001	1.61 (1.23-2.11)

[1]Adjusted for age, sex, and drinking or smoking status.

The LPS-signaling pathway is a crucial player in the innate immunity regulatory system, which includes LBP, CD14, TLR4/MD2 and other molecules involved in LPS-induced NF-κB activation such as MyD88, TIR, IRAK and TRAF6[22]. In intestinal mucosa, continuous exposure to LPS activates M1 type macrophages to perform tumoricidal tasks. TAMs in the cancer micro-environment belong to M2 macrophages and, on the contrary, promote tumor growth. In the presence of M2 macrophages, the LPS signaling pathway is down-regulated but the mechanisms remain unclear[10-12].

Previous studies in this field mainly focused on the effects of genetic variations of aforementioned genes in non-tumoral diseases such as bacterial infections[23,24], sepsis[25] and myocardial infarction[26]. Recently, how these genetic variations contribute to the risk of developing var-

Table 4 Stratification analyses for rs4914 by smoking or drinking status

Genotype frequencies (%)	Status	Cases (n = 479)	Controls (n = 486)	[1]P value	Odds ratio (95% CI)
	Smoking				
CC	No	146 (30.5%)	191 (39.3%)		
CG	No	50 (10.4%)	23 (4.7%)	0.000	2.820 (1.64-4.85)
GG	No	4 (0.8%)	1 (0.2%)	0.144	5.210 (0.57-47.55)
CG/GG	No	54 (11.2%)	24 (4.9%)	0.000	2.920 (1.72-4.96)
CC	Yes	223 (46.6%)	224 (46.1%)		
CG	Yes	52 (10.9%)	45 (9.3%)	0.524	1.155 (0.74-1.80)
GG	Yes	4 (0.8%)	2 (0.4%)	0.430	1.993 (0.36-11.05)
CG/GG	Yes	56 (11.7%)	47 (9.7%)	0.429	1.190 (0.77-1.83)
	Drinking				
CC	No	9 (1.9%)	10 (2.1%)		
CG	No	1 (0.2%)	5 (1.0%)	0.215	0.227 (0.02-2.37)
GG	No	0 (0%)	1 (0.2%)	0.998	
CG/GG	No	1 (0.2%)	6 (1.2%)	0.154	0.190 (0.02-1.88)
CC	Yes	360 (75.2%)	405 (83.3%)		
CG	Yes	101 (21.1%)	63 (13.0%)	0.001	1.800 (1.28-2.55)
GG	Yes	8 (1.7%)	2 (0.4%)	0.060	4.470 (0.94-21.18)
CG/GG	Yes	109 (22.8%)	65 (13.4%)	0.000	1.890 (1.34-2.64)

[1]Adjusted for age, sex and drinking or smoking status.

Table 5 Colorectal carcinoma risk with combined lipopolysaccharide binding protein rs2232596 and CD14 rs4914 SNPs

No. of risk genotype	Cases (n = 479)	Controls (n = 486)	[1]P value	Odds ratio (95% CI)
"0"	235 (49.1%)	296 (60.9%)		
"1"	194 (40.5%)	172 (35.4%)	0.007	1.46 (1.11-1.92)
"2"	50 (10.4%)	18 (3.7%)	0.000	3.44 (1.94-6.10)
"1+2"	244 (50.9%)	190 (39.1%)	0.000	1.66 (1.28-2.15)

[1]Adjusted for age, sex, smoking and drinking status.

ious cancers has drawn much attention. Polymorphisms in the CD14 promoter can affect the susceptibility to CRC[27] and *Helicobacter pylori* infection–related gastric carcinoma[28] in Chinese patients, and prostate cancer in African American men[29]. Effects of polymorphisms of TLR4 and other PRRs on cancer risk have also been reported[30,31].

Our study of the genetic variances in LBP rs2232596 and CD14 rs4914 provided strong evidence of interactions between LPS-signaling-related genes and the risk of CRC, indicating that the genetic modulation of LPS-induced inflammation may contribute to CRC development and progression. TAMs with defective LPS responsiveness are common components of the micro-environment of different cancers. In addition, the current study and several previous studies revealed that functional polymorphisms in LPS-signaling-related genes are associated with various cancer risks. More studies are needed to shed light on the underlying genetic mechanisms.

Tobacco and alcohol exposure have been identified as high-risk factors for CRC[32, 33]. However, our data failed to show any significant associations of tobacco and/or alcohol exposure with CRC susceptibility. We found that smokers and drinkers carrying LBP rs2232596 polymorphisms had a higher risk of CRC. But only drinkers carrying CD14 rs4914 polymorphism showed modest risk of CRC. One possible explanation is that different mechanisms regulate tobacco-gene and alcohol-gene interactions. This study lacked detailed information on the smoking and drinking status of the subjects. Further stratification analysis is needed to evaluate the risk of lifestyle factors.

What mediates the observed association between gene polymorphisms and CRC susceptibility still remains unknown. It would be interesting to compare the serum levels of LBP and CD14 from different genotypes to examine the relationship between gene polymorphisms and their expression levels.

In conclusion, the functional G alleles in both LBP rs2232596 and CD14 rs4914 SNPs showed significant associations with a high CRC risk. Further studies are needed to elucidate the effects of these genotypes on gene transcription, expression and functions in CRC and other types of malignancies.

COMMENTS

Background

Colorectal carcinoma (CRC) is a leading cause of cancer death in China and throughout the world. Chronic inflammation is considered to be important in the carcinogenesis of CRC. In this study, the authors examined the association between the gene polymorphisms of several lipopolysaccharide (LPS)-signaling factors and the risk of CRC to better elucidate the mechanism of inflammation in tumorigenesis.

Research frontiers

LPS-induced signaling is an important innate immune response that involves many different molecules, such as lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), and toll-like receptor 4 (TLR-4). Recently, it has become a hot area to employ polymorphism analysis to identify genetic mutations in the immune system that are significantly correlated with tumor development.

Innovations and breakthroughs

To date, there has been no study on the polymorphisms of LPS-signaling-related genes and CRC susceptibility in the Chinese Han population. In this study, the authors directly addressed this issue and performed genetic analysis to screen for polymorphisms that are associated with increased CRC risk. The authors also explored the gene-environment interactions by studying the effects of smoking or drinking exposure on CRC susceptibility.

Applications

By demonstrating the association of LBP rs2232596 and CD14 rs4914 polymorphisms with increased CRC risk, the authors identified two important biomarkers for predicting CRC and further improved the understanding of the inflammation-related mechanisms in CRC development.

Terminology

Single-nucleotide polymorphism (SNP): a DNA sequence variation occurring when a single nucleotide in the genome differs between members of the same biological species or paired chromosomes in an individual. SNP analysis can shed light on how genetic variations affect disease development in humans.

Peer review

This is an interesting well-conducted and well-written study.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2332

World J Gastroenterol 2011 May 14; 17(18): 2332-2337
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Utility of pancreatography for diagnosing autoimmune pancreatitis

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Received: October 21, 2010 Revised: December 14, 2010
Accepted: December 21, 2010
Published online: May 14, 2011

Abstract

AIM: To identify pancreatographic findings that facilitate differentiating between autoimmune pancreatitis (AIP) and pancreatic cancer (PC) on endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP).

METHODS: ERCP findings of 48 AIP and 143 PC patients were compared. Diagnostic accuracies for AIP by ERCP and MRCP were compared in 30 AIP patients.

RESULTS: The following ERCP findings suggested a diagnosis of AIP rather than PC. Obstruction of the main pancreatic duct (MPD) was more frequently detected in PC ($P < 0.001$). Skipped MPD lesions were detected only in AIP ($P < 0.001$). Side branch derivation from the narrowed MPD was more frequent in AIP ($P < 0.001$). The narrowed MPD was longer in AIP ($P < 0.001$), and a narrowed MPD longer than 3 cm was more frequent in AIP ($P < 0.001$). Maximal diameter of the upstream MPD was smaller in AIP ($P < 0.001$), and upstream dilatation of the MPD less than 5 mm was more frequent in AIP ($P < 0.001$). Stenosis of the lower bile duct was smooth in 87% of AIP and irregular in 65% of PC patients ($P < 0.001$). Stenosis of the intrahepatic or hilar bile duct was detected only in AIP ($P = 0.001$). On MRCP, diffuse narrowing of the MPD on ERCP was shown as a skipped non-visualized lesion in 50% and faint visualization in 19%, but segmental narrowing of the MPD was visualized faintly in only 14%.

CONCLUSION: Several ERCP findings are useful for differentiating AIP from PC. Although MRCP cannot replace ERCP for the diagnostic evaluation of AIP, some MRCP findings support the diagnosis of AIP.

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Key words: Autoimmune pancreatitis; Pancreatic cancer; Endoscopic retrograde cholangiopancreatography, Magnetic resonance cholangiopancreatography

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Takuma K, Kamisawa T, Tabata T, Inaba Y, Egawa N, Igarashi Y. Utility of pancreatography for diagnosing autoimmune pancreatitis. *World J Gastroenterol* 2011; 17(18): 2332-2337 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2332.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2332

INTRODUCTION

Autoimmune pancreatitis (AIP) is a newly described entity of pancreatitis, the pathogenesis of which appears to involve autoimmune mechanisms[1-4]. Clinically, AIP patients and patients with pancreatic cancer (PC) share many features, such as preponderance of elderly males,

frequent initial symptom of painless jaundice, development of new-onset diabetes mellitus, and elevated levels of serum tumor markers. Radiologically, focal swelling of the pancreas, the "double-duct sign", representing strictures in both biliary and pancreatic ducts, and encasement of peripancreatic arteries and portal veins are sometimes detected in both AIP and PC[4-6]. AIP often mimics PC, and 2.4% of 1808 pancreatic resections in the USA were reported to have AIP on histological examination[7]. AIP responds dramatically to steroid therapy; therefore, accurate diagnosis of AIP can avoid unnecessary laparotomy or pancreatic resection.

Serum IgG4 levels were elevated in 77%[8]-81%[9] of AIP patients, but they were also elevated in 4%[8]-10%[10] of PC patients. There is no definite serological marker for AIP; therefore, AIP is currently diagnosed using a combination of characteristic radiological, serological, and pathological findings. Irregular narrowing of the main pancreatic duct (MPD) on endoscopic retrograde cholangiopancreatography (ERCP) is a characteristic radiological feature of AIP, and this pancreatographic finding on ERCP is mandatory in the Japanese diagnostic criteria for AIP[11]. We previously compared the pancreatograms of 17 AIP patients having a mass-forming lesion in the pancreatic head and 40 patients with pancreatic head cancer[6]. In the present study, we further compared pancreatograms of 48 AIP patients and 143 PC patients, and evaluated more accurately by further date.

Although pancreatographic findings are useful to differentiate AIP and PC, ERCP can cause adverse effects, such as pancreatitis. Since magnetic resonance cholangiopancreatography (MRCP) has become popular as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree, MRCP is replacing diagnostic ERCP in many pancreatobiliary diseases. In the new Korean diagnostic criteria, AIP can be diagnosed by MRCP without the need for ERCP[12]. In our previously study of the utility of MRCP for diagnosing AIP in 20 AIP patients who were examined before 2008, MRCP could not replace ERCP, because narrowing of the MPD in AIP was not visualized on MRCP[13]. However, with development of MRCP models, spatial resolution of the pancreatic duct has improved on MRCP. We again studied the usefulness of MRCP for diagnosing AIP in 30 AIP patients and assessed whether MRCP could replace ERCP for diagnosing AIP.

MATERIALS AND METHODS

From 1992 to August 2010, pancreatograms on ERCP were obtained in 48 AIP patients [34 males, 14 females; age, 64.1 ± 12.1 (mean ± SD) years; age range, 27-83 years]. They were diagnosed as having AIP according to the Asian Diagnostic Criteria for AIP[12]: pancreatic enlargement on computed tomography (CT) and ultrasonography (US) (n = 48), irregular narrowing of the MPD on ERCP (n = 48), elevated serum IgG4 (n = 42), presence of autoantibodies (n = 26), histological findings of lymphoplasmacytic sclerosing pancreatitis (LPSP, n = 18), and steroid responsiveness (n = 38). Overall, 23 patients had diffuse enlargement of the pancreas, and 25 patients had segmental enlargement of the pancreas (head, n = 17; body and/or tail, n = 8). An initial dose of oral prednisolone of 30-40 mg/d was administered for 2-3 wk. It was then tapered by 5 mg every 1-3 wk until it reached 5 mg/d. After about three months of induction, maintenance therapy of 2.5-5 mg/d was administered for six months to 143 mo. Malignant diseases, such as pancreatic or biliary cancers, were excluded by long follow up in the 30 patients without histological examination. During the same period as the AIP cases, ERCP pancreatograms were examined in 143 PC patients (81 males, 62 females; age, 63.5 ± 10.9 years; range, 42-79 years). These PCs were resected or histologically confirmed after adequate imaging studies, such as US, CT, and magnetic resonance imaging (MRI). The locations of PC were confirmed to include the head (n = 88), body (n = 37), and tail (n = 18) on these imaging examinations.

The pancreatographic findings were evaluated in the 44 AIP patients and the 143 PC patients to measure the length of the narrowed MPD and the diameter of the upstream MPD (dilated MPD above the narrowed part). It was also determined whether obstruction and skipped lesions in the MPD (discrete narrowed lesions scattered in the almost normal MPD) or side branch derivation from the narrowed portion of the MPD were present. The length of the narrowed MPD and the maximal diameter of the dilated upstream MPD were measured using measuring function on computerized images or a manual goniometer. The cholangiographic findings were evaluated to identify morphological modifications in the 38 AIP patients and 77 PC patients with bile duct involvement.

Furthermore, 30 of the 48 AIP patients also underwent MRCP within three weeks, and MRCP and ERCP findings were compared. MRCP was performed using a 1.5-T magnetic resonance imaging machine (INTERA, Philips Co. Ltd., The Netherlands) by two-dimensional (up to 2005) and three-dimensional (2006-2010), coronal, heavily T2-weighted, signal-shot, rapid acquisition with relaxation enhancement. Since 2006, a 1.5-T magnetic resonance imaging machine (MAGNETOM Avanto, Siemens Co. Ltd., Germany) was also used. Diagnostic accuracies for AIP on ERCP and MRCP were compared separately in diffuse-type AIP patients (n = 16) and segmental-type AIP patients (n = 14). Two endoscopists and radiologists, who were blind to clinical information, reviewed the radiological findings.

Statistical analyses were performed with the Mann-Whitney's U test and Fisher's exact probability test. P values of less than 0.05 were considered significant.

RESULTS

Pancreatographic differences between AIP and PC

Whereas obstruction of the MPD was more frequently detected in PC (AIP: 4% *vs* PC: 69%, $P < 0.001$), skipped lesions of the MPD were detected only in AIP (27% *vs* 0%,

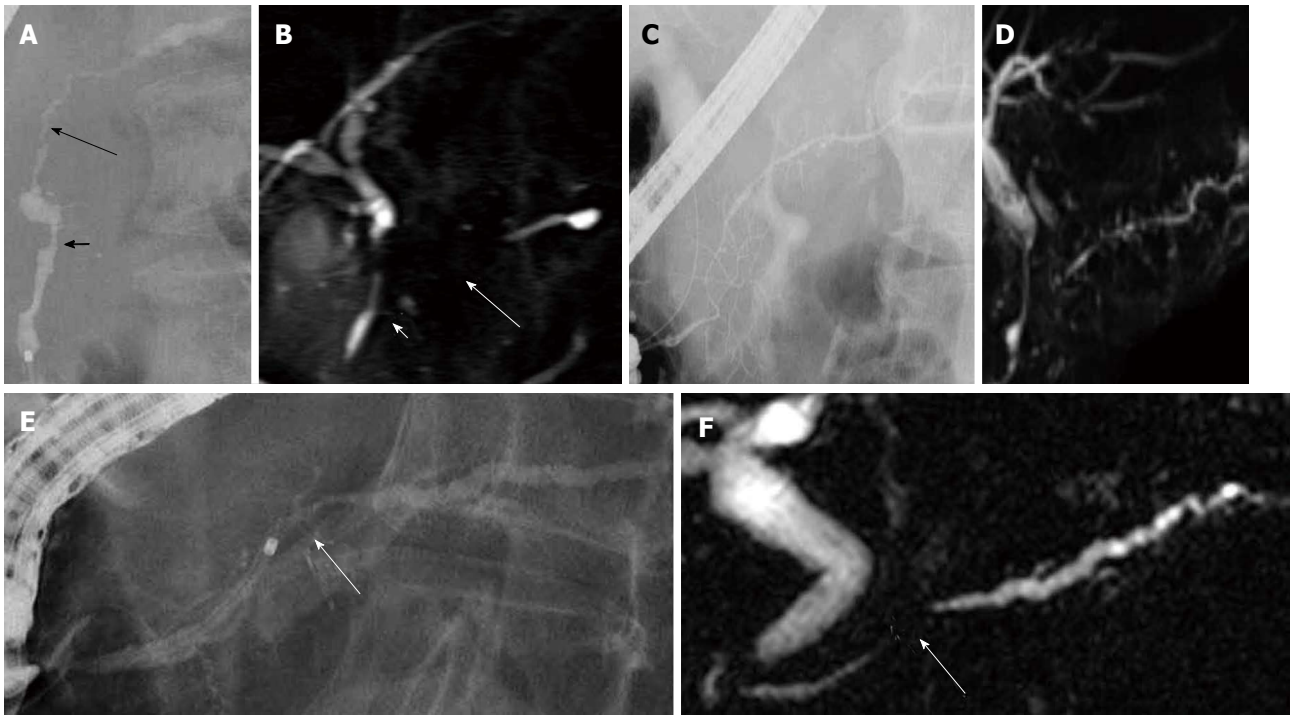

Figure 1 Pancreatography of autoimmune pancreatitis. A: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing skipped lesions of the main pancreatic duct (short and long arrows); B: On magnetic resonance cholangiopancreatography, skipped lesions (short and long arrows) on endoscopic retrograde cholangiopancreatography were not visualized; C: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing side branch derivation from the narrowed portion of the main pancreatic duct; D: Recent magnetic resonance cholangiopancreatography could show diffuse narrowing of the main pancreatic duct fairly well; E: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing a short narrowed main pancreatic duct (arrow) with upstream dilatation less than 5 mm; F: On magnetic resonance cholangiopancreatography, the narrowed portion (arrow) was not visualized.

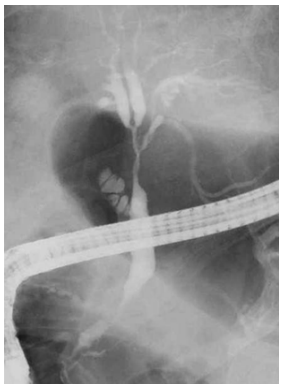

Figure 2 Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing stenosis of the hilar and intrahepatic bile duct.

$P < 0.001$) (Figure 1A), and side branch derivation from the narrowed portion of the MPD was more frequent in AIP (81% *vs* 22%, $P < 0.001$) (Figure 1C). The narrowed MPD was longer in AIP (7.6 ± 4.3 cm *vs* 2.5 ± 0.9 cm, $P < 0.001$), and a narrowed MPD longer than 3 cm was more frequent in AIP (90% *vs* 27%, $P < 0.001$). The maximal diameter of the upstream MPD was smaller in AIP (2.9 ± 0.8 mm *vs* 6.8 ± 2.1 mm, $P < 0.001$), and upstream dilatation of the MPD less than 5 mm was more frequent in AIP (95% *vs* 27%, $P < 0.001$) (Figure 1E) (Table 1). A short narrowed MPD was difficult to differentiate from stenosis of the MPD in PC (Figure 1E).

Cholangiographic differences between AIP and PC

Stenosis of the lower bile duct was smooth in 33 (87%) AIP patients, but irregular in 50 (65%) PC patients ($P < 0.001$). Left-side deviation of the lower bile duct was detected in both groups. Stenosis of the intrahepatic or hilar bile duct was detected in only six AIP patients ($P = 0.001$) (Figure 2) (Table 2).

Diagnostic accuracy for AIP by ERCP and MRCP

In diffuse-type AIP, diffuse narrowing of the MPD on ERCP was shown as skipped non-visualized lesions in 50% (Figure 1B), faint visualization in 19%, and non-visualization in 31% on MRCP. Diffuse narrowing of the MPD could be fairly well visualized in two recent patients (Figure 1D). Side branches from the narrowed portion of the MPD shown on ERCP were visualized faintly only in 21% on MRCP. Stenosis of the bile duct was also detected on MRCP. After steroid therapy, the MPD was visualized in 100%, and the branches were visualized in 50% on MRCP. Resolution of bile duct lesions was seen completely in 53% and incompletely in 47% on MRCP (Table 3).

In segmental-type AIP, segmental narrowing of the MPD on ERCP was shown as faint visualization in 14% and non-visualization in 86% on MRCP (Figure 1F). Side branches from the narrowed MPD shown on ERCP were visualized faintly only in 18% on MRCP. After steroid therapy, the MPD was visualized in 100%, and the branches were visualized in 60% on MRCP. Resolution of bile duct lesions was seen completely in 50% and incompletely in 50% on MRCP (Figure 3A and B) (Table 4).

Table 1 Pancreatographic differences between autoimmune pancreatitis and pancreatic cancer *n* (%)

	Autoimmune pancreatitis (*n* = 48)	Pancreatic cancer (*n* = 143)	*P*-value
Obstruction of the MPD +/-	2/46 (4)	98/45 (69)	< 0.001
Skipped lesions of the MPD +/-	13/35 (27)	0/143 (0)	< 0.001
Side branch derivation from the narrowed MPD +/-	39/9 (81)	10/35 (22)	< 0.001
Length of the narrowed MPD (cm)	7.6 ± 4.3	2.5 ± 0.9	< 0.001
Length of the narrowed MPD > 3 cm +/-	43/5 (90)	12/33 (27)	< 0.001
Diameter of upstream MPD (mm)	2.9 ± 0.8	6.8 ± 2.1	< 0.001
Diameter of upstream MPD < 5 mm +/-	19/1 (95)	12/33 (27)	< 0.001

MPD: Main pancreatic duct.

Table 2 Cholangiographic differences between autoimmune pancreatitis and pancreatic cancer *n* (%)

	Autoimmune pancreatitis	Pancreatic carcinoma	*P*-value
Stenosis of the lower bile duct	*n* = 38	*n* = 77	
Smooth stenosis	33 (87)	27 (35)	< 0.001
Irregular stenosis	5 (13)	50 (65)	
Left-side deviation of the lower bile duct +/-	20 (53)	49 (64)	NS
Stenosis of the intra/hilar bile duct	6 (16)	0 (0)	0.001

NS: Not significant.

Table 3 Diagnostic accuracy for diffuse-type autoimmune pancreatitis by endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography

ERCP before steroid	MRCP	
	Before steroid	After steroid
MPD narrowing (*n* = 16)	Skipped non-visualization 8 (50%) Faint visualization 3 (19%) Non-visualization 5 (31%)	Visualization 16/16 (100%)
Branches from the narrowed MPD (*n* = 14)	Faint visualization 3 (21%) Non-visualization 11 (79%)	Visualization 7/14 (50%)
Bile duct stenosis (*n* = 15)	Stenosis 15 (100%)	Resolution Complete 8/15 (53%) Incomplete 7/15 (47%)

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct.

DISCUSSION

AIP responds dramatically to steroid therapy; therefore, it is of the utmost importance that AIP be differentiated from PC to avoid unnecessary laparotomy or pancreatic resection. Histopathological findings of AIP in Japan are characterized by dense infiltration of T lymphocytes and IgG4-positive plasma cells, storiform fibrosis, and obliterative phlebitis in the pancreas; this is called LPSP. Abundant lymphoplasmacytic cells infiltrate around interlobular ducts with fibrosis, especially the medium and large ducts, including the MPD. The periductal inflammation is usually extensive and distributed throughout the entire pancreas. However, the degree and extent of periductal inflammation differ from duct to duct according to the location of the involved pancreas. The infiltrate is primarily subepithelial, with the epithelium only rarely being infiltrated by lymphocytes. It encompasses the ducts and narrows their lumen by infolding of the epithelium. If the inflammatory process affects the pancreatic head, it usually also involves the lower common bile duct, where it leads to a marked thickening of the bile duct wall due to fibrosis with lymphoplasmacytic infiltration. The epithelium of the bile duct is also well preserved. The thickening of the bile duct wall sometimes spreads extensively to the bile duct, where stenosis is not apparent on cholangiography[4,14-16]. On the other hand, PC cells infiltrate scirrhously, destroy the epithelium of the pancreatic and bile ducts, and frequently obstruct the main and branch pancreatic ducts. These histopathological differences around the ducts represent the different cholangiopancreatographic findings between AIP and PC. Pancreatographic findings, such as no obstruction of the MPD, skipped lesions of the MPD, side branch derivation from the narrowed portion of the MPD, narrowed portion of the MPD > 3-cm-long, a maximal diameter < 5 mm of the upstream MPD, and smooth stenosis of the lower bile duct are highly suggestive of AIP rather than PC. Stenosis of the intrahepatic or hilar bile duct is recognized as other

Table 4 Diagnostic accuracy for segmental-type autoimmune pancreatitis by endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography

ERCP before steroid	MRCP	
	Before steroid	After steroid
MPD narrowing (n = 14)	Faint visualization 2 (14%) Non-visualization 12 (86%)	Visualization 10/10 (100%)
Branches from the narrowed MPD (n = 11)	Faint visualization 2 (18%) Non-visualization 9 (82%)	Visualization 6/10 (60%)
Bile duct stenosis (n = 8)	Stenosis 8 (100%)	Resolution Complete 4/8 (50%) Incomplete 4/8 (50%)

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct.

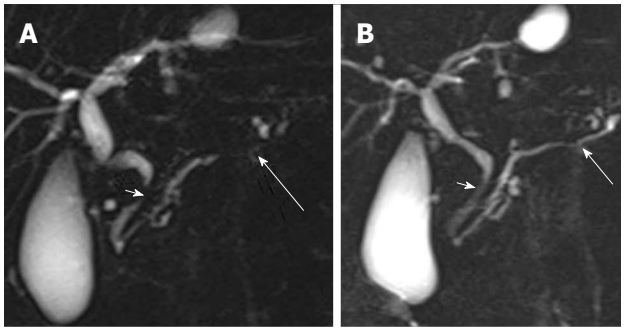

Figure 3 Magnetic resonance cholangiopancreatography finding of autoimmune pancreatitis. Stenosis of the lower bile duct (short arrow) and the narrowing of the main pancreatic duct (long arrow) (A) improved after steroid therapy (B).

organ involvement of AIP and supports the diagnosis of AIP rather than PC, although it should be distinguished from cholangiocarcinoma and primary sclerosing cholangitis[17,18]. Wakabayashi *et al*[19] compared pancreatograms of nine segmental-type AIP patients and 80 PC patients. They reported that obstruction of the MPD was frequent in PC [11% (1/9) *vs* 60% (48/80)]. A narrowed portion of the MPD ≥ 3-cm-long [100% (8/8) *vs* 22% (6/27)] and a maximal diameter < 4 mm of the upstream MPD [(67% (4/6) *vs* 4% (1/23)] were frequently detected in AIP, and side branch derivation from the narrowed portion of the MPD was detected in 50% (4/8) of AIP patients[19]. In Nakazawa's cholangiopancreatographic study of 37 AIP patients, skipped lesions of the MPD were detected in six (16%) patients, and stenosis of the intrahepatic or hilar bile duct was detected in 16 (43%) patients[20].

The major problem with MRCP for diagnosing AIP is that the narrowed MPD seen on ERCP cannot be visualized on MRCP, because of the inferior resolution of MRCP compared with ERCP. Segmental narrowing of the MPD seen on ERCP was not visualized in 86% on MRCP, and distinguishing between AIP and PC was quite difficult on MRCP. However, in these cases, less upstream dilatation of the MPD on MRCP may suggest AIP rather than PC. In diffuse-type-, diffuse narrowing of the MPD on ERCP was shown as skipped, non-visualized lesions in 50% and faintly visualized in 19% on MRCP. With the development of MRCP models, diffuse narrowing of the MPD could be visualized fairly well on MRCP in two recent patients. Skipped, non-visualized lesions and a faintly visualized, narrowed MPD can suggest AIP with typical diffuse enlargement of the pancreas on CT or MRI. Side branch derivation from the narrowed portion of the MPD is a useful pancreatographic finding suggesting AIP, but the finding can rarely be seen on MRCP. However, stenosis of the bile duct was also detected on MRCP and resolution of the pancreatic and bile ducts after steroid therapy can be fully evaluated on MRCP. Park *et al*[21] also reported that skipped MPD narrowing and less upstream MPD dilatation on MRCP suggest AIP. Although secretin is not available in Japan currently, secretin-MRCP is reported to enable demonstration of the integrity of the MPD and lead to a correct diagnosis of AIP[22].

In conclusion, the following ERCP findings are fairly specific for AIP and are useful to differentiate AIP from PC: less obstruction of the MPD, skipped lesions of the MPD, side branch derivation from the narrowed portion of the MPD, length of the narrowed MPD > 3 cm, maximal diameter of the upstream < 5 mm, smooth stricture of lower the bile duct, and stenosis of intra or hilar bile duct. Although MRCP cannot fully replace ERCP for the diagnostic evaluation of AIP, MRCP may deserve to be used in some diffuse-type AIP cases and is useful for follow-up of AIP.

COMMENTS

Background

Autoimmune pancreatitis (AIP) is a peculiar type of pancreatitis of presumed autoimmune etiology. Since AIP responds dramatically to steroid therapy, it is most important that AIP be differentiated from pancreatic cancer (PC) to avoid unnecessary laparotomy or pancreatic resection. However, some cases were still difficult to distinguish between AIP and PC.

Research frontiers

Pancreatographic findings to facilitate differentiating between AIP and PC on endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP) were investigated.

Innovations and breakthroughs

Obstruction of the main pancreatic duct (MPD) was more frequently detected in PC. Skipped MPD lesions and side branch derivation from the narrowed MPD were detected in AIP. The narrowed MPD was longer in AIP. Maximal diameter of the upstream MPD was smaller in AIP. Stenosis of the lower bile duct was smooth in AIP and irregular in PC patients. Stenosis of the intrahepatic or hilar

bile duct was detected only in AIP. Diffuse narrowing of the MPD on ERCP was shown as a skipped non-visualized lesion in 50% and faint visualization in 19% on MRCP.

Applications

Several ERCP findings are useful to differentiate AIP from PC. MRCP cannot replace ERCP for the diagnostic evaluation of AIP, but some MRCP findings support the diagnosis of AIP.

Peer review

This is a well-written manuscript about pancreatography for diagnosing autoimmune pancreatitis and is of some interest.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2338

World J Gastroenterol 2011 May 14; 17(18): 2338-2342
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Prospective randomized controlled trial investigating the type of sutures used during hepatectomy

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Received: June 11, 2010 Revised: November 25, 2010
Accepted: December 2, 2010
Published online: May 14, 2011

Abstract

AIM: To determine whether absorbable sutures or non-absorbable sutures are better in preventing surgical site infection (SSI), in this paper we discuss the results of a randomized clinical trial which examined the type of sutures used during hepatectomy.

METHODS: All hepatic resections performed from January 2007 to November 2008 at the Department of Surgery at Iizuka Hospital in Japan were included in this study. There were 125 patients randomly assigned to an absorbable sutures (Vicryl) group or non-absorbable sutures (Silk) group.

RESULTS: SSI was observed in 13.6% (17/125) patients participating in this study, 11.3% in the Vicryl group and 15.8% in the Silk group. Incisional SSI including superficial and deep SSI, was observed in 8% of the Vicryl group and 9.5% of the Silk group. Organ/space SSI was observed in 3.2% of the Vicryl group and 6.0% of the Silk group. There were no significant differences, but among the patients with SSI, the period for recovery was significantly shorter for the Vicryl group compared to the Silk group.

CONCLUSION: The incidence of SSI in patients receiving absorbable sutures and silk sutures is not significantly different in this randomized controlled study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures.

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Key words: Hepatectomy; Absorbable suture; Surgical site infection

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Harimoto N, Shirabe K, Abe T, Yukaya T, Tsujita E, Gion T, Kajiyama K, Nagaie T. Prospective randomized controlled trial investigating the type of sutures used during hepatectomy. *World J Gastroenterol* 2011; 17(18): 2338-2342 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2338.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2338

INTRODUCTION

Despite recent developments in surgery and patient management during the perioperative period, critical complications still develop in a few patients who undergo hepatic resection[1,2]. Surgical site infection (SSI) is one of the most important morbidities of surgery and leads to prolonged hospital stays. Previous studies revealed absorbable suture material in gastrointestinal surgical procedures to reduce the risk of SSI[3,4] although most of those studies have been limited to skin closures. Absorbable sutures are used in most hospitals in the United States and Europe based on the available clinical studies. On the other hand, silk sutures are still used in most hospitals in Japan. A

study by Kobayashi *et al*[5] revealed patients with intraoperative bowel injury, blood loss > 2000 mL, and age > 65 years are at risk of developing SSI after hepatectomy for liver cancers and then all blood vessels and bile ducts were ligated with silk or vessel clips during parenchymal resection. Absorbable sutures are now widely used for abdominal closure in Japan according to the CDC guidelines[6]. During the resection of the liver, silk sutures are generally used for the vessels in Japan, because silk sutures are easier to handle and less expensive than absorbable sutures. However, foreign materials, especially silk, are known to accelerate infection[6,7] and thus lead to a prolonged hospital stay. Togo *et al*[8] reported the usefulness of absorbable sutures to prevent SSI with a historical control study using an animal model. There has been no known randomized clinical trial for suture material used in the ligation of the cut surface of the liver. In this paper the authors provide the outcome of a randomized clinical trial investigating the type of sutures used during hepatectomy to determine which is better in preventing SSI, absorbable sutures or non-absorbable sutures.

MATERIALS AND METHODS

Study design

All hepatic resections without biliary reconstruction performed from January 2007 to November 2008 at the Department of Surgery at Iizuka Hospital in Japan were included in this study. Only patients who met the following criteria were enrolled: (1) liver resection without biliary reconstruction; (2) without other malignancy; and (3) until compensated cirrhosis with Child-Pugh class A or B. At the author's department, the rate of SSI during hepatectomy was 25% according to previous data. The sample size required to detect a difference by chi-square test with 80% power at the 5% significance level was 124 patients. There were 130 patients enrolled in this study. One patient refused to participate in the study. The authors obtained approval from the local ethics committee and obtained informed consent from the other patients. Finally 125 patients were randomly assigned to an absorbable sutures (Vicryl, Johnson & Johnson Corp., Tokyo, Japan) or a non-absorbable sutures (Silk) group. Because the operative procedure used on 4 patients was either conversion to radiofrequency ablation or biliary reconstruction (Figure 1) they were not included in the study. Randomization was achieved by providing sealed envelopes to be opened by the operator before laparotomy. Data analysis was performed for 125 patients.

Liver function was evaluated preoperatively using Child classification and indocyanine green retention test at 15 min (ICGR15) in patients with underlying liver disease. The presence of ascites and ICGR15 test value of more than 40% were considered an absolute contraindication for resection. Hepatitis B surface antigen (HBs Ag) and hepatitis C antibody (HCV Ab) were routinely measured preoperatively.

Surgical technique and intra-operative care were standardized by the same team in this study. These included a J-shaped incision for routine abdominal access, a slow and gentle hepatic dissection using an ultrasonic dissector with coagulator (CUSA Excel, Integra Co., USA), with systematic ligation of all sizable vessels, and close ultrasonographic guidance along the transection line. Cholecystectomy was performed in all patients if the gallbladder was present. Intraoperative bile leakage test was routinely performed to identify bile leakage[9]. With this procedure, we recognized small bile leakage sites on the cut liver surface and could repair these sites by Z-suturing using 6-0PDSII (Johnson & Johnson Corp., Tokyo, Japan). Intraoperative vascular control was achieved using the Pringle maneuver[10]. In order to prevent backflow bleeding central venous pressure was decreased to as low as 5 mm Hg when possible with careful circulating volume and respiratory assistance. When central venous pressure control was insufficient, outflow control was achieved by selective vascular exclusion, or by clamping of the infrahepatic inferior vena cava[11]. One or two closed drains were inserted close to the cut surface of liver parenchyma. Before the subcutaneous tissue was closed, the wound was washed with 1 L saline. Vicryl (Johnson & Johnson Corp., Tokyo, Japan), an absorbable suture material, was used during abdominal wound closure. Drains were removed when no bleeding or bile leakage were observed within 3 d. Bile leakage was defined as the drainage of macroscopic bile from the surgical drains for more than 7 d after surgery[9]. If bile leakage is clinically suspected after the removal of drains, percutaneous drainage is performed. Intravenous ampicillin/sulbactam (ABPC/SBT) 1.5 g was administered 30 min before surgery and additional ABPC/SBT was administered every 3 h. Systemic antibiotics were used for only two days after surgery. During the operation gloves were changed every 3 h.

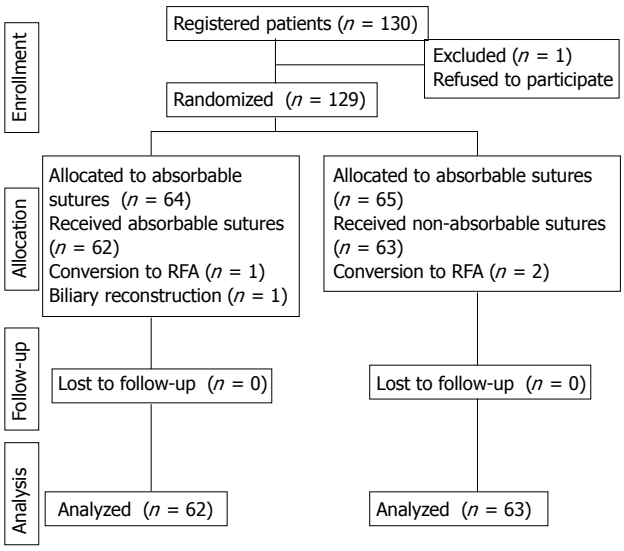

Figure 1 The trial profile of the patients in this study. RFA: Radiofrequency ablation.

Postoperative infection

SSI was defined as a condition in which purulent dis-

Table 1 Comparison of clinicopathological data of the groups

Variables	Vicryl group (n = 62)	Silk group (n = 63)	P-value
Age	68 ± 10	67 ± 12	0.684
Male/female	37/25	41/22	0.720
Body mass index	22.6 ± 3.0	23.7 ± 12	0.159
Diabetes mellitus (%)	19.4	33.0	0.104
HBV (%)	17.7	20.6	0.560
HCV (%)	50.0	39.7	0.666
Albumin (g/dL)	3.6 ± 0.5	3.6 ± 0.5	0.694
Total bilirubin (mg/dL)	0.7 ± 0.4	0.7 ± 0.4	0.813
AST (IU/L)	46 ± 31	43 ± 27	0.592
Platelet count (x 10^4/μL)	21.0 ± 11.0	19.7 ± 10.1	0.460
ICGR15 (%)	15.4 ± 9.0	16.4 ± 11.9	0.589
Child-Pugh A/B	58/4	59/4	0.999
HCC/non-HCC	46/16	47/16	0.826
Liver cirrhosis (ch/lf/lc)	15/20/27	15/22/26	0.948
Anatomical/nonanatomical	20/42	20/43	0.618
Operation time (min)	273 ± 88	276 ± 105	0.876
Blood loss (g)	698 ± 894	763 ± 1329	0.749
Transfusion (%)	12.9	17.4	0.620
Bile leakage (%)	3.2	6.3	0.680
Total SSI (%)	11.3	15.8	0.603
Incisional SSI (%)	8.0	9.5	0.999
Organ/space SSI (%)	3.2	6.3	0.680
Remote infection (%)	3.2	1.6	0.546
Hospital stay (d)	16 ± 15	16 ± 14	0.880

SSI: Surgical site infection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AST: Aspartate aminotransferase.

Table 2 Comparison of clinicopathological data of the groups

Variables	SSI(+) (n = 17)	SSI(-) (n = 108)	P-value
Age	68 ± 10	67 ± 11	0.667
Male/female	13 /4	65/43	0.287
Body mass index	24.3 ± 5.2	23.0 ± 3.9	0.208
Diabetes mellitus (%)	35.3	25.0	0.384
HBV (%)	23.5	18.5	0.703
HCV (%)	64.7	41.7	0.560
Albumin (g/dL)	3.6 ± 0.4	3.6 ± 0.5	0.977
Total bilirubin (mg/dL)	0.7 ± 0.3	0.7 ± 0.4	0.938
AST (IU/L)	56 ± 22	43 ± 29	0.070
Platelet count (x 10^4/μL)	20.7 ± 7.3	20.4 ± 11.0	0.904
ICGR15 (%)	19.9 ± 8.3	15.3 ± 10.7	0.101
Child-Pugh A/B	17/0	100/8	0.597
HCC/non-HCC	15/2	78/30	0.638
Liver cirrhosis (ch/lf/lc)	2/7/8	28/35/45	0.435
Anatomical/nonanatomical	8/9	32/76	0.228
Operation time > 265 min (%)	76.5	44.4	< 0.050
Blood loss > 1000 g (%)	41.2	18.5	< 0.050
Transfusion (%)	11.8	15.7	0.999
Bile leakage (%)	35.3	0	< 0.010
Vicryl/silk	7/10	55/53	0.603
Hospital stay (d)	29 ± 23	15 ± 12	< 0.010

SSI: Surgical site infection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AST: Aspartate aminotransferase.

charge was observed from any incision or space that was manipulated during an operation with or without microbiological evidence as in the guideline issued by CDC[6] and it was identified prospectively by direct observation of the surgical site**.** Patients were followed up 30 d after hospital discharge. SSI occurring after hospital discharge was included in this study. Remote infection was defined as a condition in which fever and leukocytosis were present with bacteria in sputum, urine, catheter-tip, blood, or other body fluid/space with or without microbiological evidence.

Statistical analysis

We analyzed associations between the continuous and categorical clinico-pathologic variables using the Student's t tests and χ^2 tests, respectively. Multivariate analysis was performed with a logistic regression test. A P value less than 0.05 was considered statistically significant.

RESULTS

Incidence of postoperative infection

The clinicopathological factors between the Vicryl and Silk groups were compared (Table 1). No significant differences were recognized during the evaluation of the host factors and the operative factors. SSI was observed in 13.6% (17/125) of patients in this study, 11.3% in the Vicryl group and 15.8% in the Silk group (P = 0.620). Incisional SSI including superficial and deep SSI, were observed in 8% of the Vicryl group and 9.5% of the Silk group (P = 0.999). Organ/space SSI was observed in 3.2% of the Vicryl group and 6.0% of the Silk group (P = 0.680). Organ/space SSI was namely abdominal abscess and percutaneous drainage was needed. Remote infection was observed with an incidence of 3.2% in the Vicryl group and 1.6% in the Silk group (P = 0.546). Pneumonia and urinary tract infection were observed in the Vicryl group and pneumonia in the Silk group. There were no significant differences. There was no hospital mortality in this study.

Risk factor for SSI

In the univariate analyses, operation time > 265 min, blood loss > 1000 g, and bile leakage differed significantly as predictive factors of SSI (Table 2). The type of sutures was not significant. Five factors including operation time > 265 min, blood loss > 1000 g, bile leakage and the type of sutures were analyzed with multivariate logistic regression. Results are given in Table 3. Blood loss > 1000 g was the only independent variable among the five factors.

Comparison of duration to recovery in the patients with SSI

Among the patients with SSI, the period for recovery in the Vicryl group was significantly shorter compared to the Silk group (Table 4).

DISCUSSION

Togo *et al*[8] reported the usefulness of absorbable sutures to prevent SSI with a historical control study using an animal model. This is the only report that the use of

Table 3 Multivariate logistic stepwise regression analysis for predictive factors of surgical site infection

	Adjusted odds ratios (95% CI)	*P* value
Blood loss > 1000 g	7.598 (1.396-41.364)	< 0.01

Table 4 Comparison of the period for recovery in patients with surgical site infection

Vicryl	28 ± 11 d
Silk	54 ± 30 d

$P < 0.05$.

absorbable sutures contributed significantly to the prevention of development of SSI. In our prospective study, the incidence of SSI in the Vicryl group was lower compared to the Silk group for both incisional SSI and organ/space SSI, however there were no other significant differences. Watanabe *et al*[12] reported the use of absorbable sutures in seromuscular suturing or intra-abdominal ligation was associated with a significantly lower incidence of SSI than non-absorbable sutures in the lower alimentary tract procedure, but not in the upper. The incidence of SSI is low so any significant difference is difficult to observe. The duration for recovery from SSI was significantly shorter in the Vicryl group than in the Silk group. Since SSI increases treatment costs and diminishes patient satisfaction markedly, the use of an absorbable suture is recommended in view of the medical economy and patient's quality of life.

The incidence of SSI as a form of postoperative infection was previously reported to be 20%-25%[8,13,14]. In our clinical experience, the incidence of SSI was observed to be 13.1% in this study and the rate of occurrence is decreasing every year because of SSI surveillance and development of surgical techniques such as closed suction drain and the prevention of bile leakage. Togo *et al*[8] reported the incidence of postoperative infection gradually decreased significantly with additional countermeasures. A larger sample size as well as a multi-institutional study is required for a randomized controlled study.

Intraoperative blood loss, long operation time and bile leakage were associated with SSI in our study. Both transfusion and operation time are reported to be risk factors of SSI in hepatectomy or other surgery[5,15]. Transfusion could induce immunosuppression in postoperative patients by reduction of the natural killer cell and cytotoxic T-cell populations[16,17]. Operation time could influence the concentration of systemic antibiotics, leading to the incidence of SSI. Bile leakage was observed at 4.8% (6/125) in this study. Generally the incidence of bile leakage was 4.0%-7.2% in recently reported large series[9,18-20]. The presence of bile, blood, and devitalized tissues in the dead space after hepatectomy may provide the ideal environment for bacterial growth and development of organ/space SSI, which frequently results in liver failure. To reduce organ /space SSI, it is most important to prevent bile leakage.

In conclusion, there were no significant differences between absorbable sutures and silk sutures on the incidence of SSI during the resection of the liver in this randomized control study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures. Further study is needed to provide evidence on the use of absorbable or non-absorbable suture materials in a multi-institutional randomized clinical trial.

COMMENTS

Background

Surgical site infection (SSI) is one of the most important morbidities of surgery and leads to prolonged hospital stays. In this paper the authors provide the outcome of a randomized clinical trial investigating the type of sutures used during hepatectomy to determine which is better in preventing SSI, absorbable sutures or non-absorbable sutures.

Research frontiers

During the resection of the liver, silk sutures are generally used for the vessels in Japan, because silk sutures are easier to handle and less expensive than absorbable sutures.

Innovations and breakthroughs

This is a randomized clinical trial investigating the type of sutures used during hepatectomy.

Applications

The incidence of SSI was not significantly different between absorbable sutures and silk sutures in this randomized controlled study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures.

Terminology

Vicryl is an absorbable suture material and silk is a non-absorbable suture material, which is widely used in Japan.

Peer review

Authors report prospective randomized study of surgical site infection comparing silk to vicryl ties in liver resection.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2343

World J Gastroenterol 2011 May 14; 17(18): 2343-2348
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

XPD Lys751Gln polymorphism and esophageal cancer risk: A meta-analysis involving 2288 cases and 4096 controls

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Received: September 16, 2010 Revised: December 21, 2010
Accepted: December 28, 2010
Published online: May 14, 2011

Abstract

AIM: To evaluate the association between xeroderma pigmentosum group D (XPD), genetic polymorphism Lys751Gln and esophageal cancer risk.

METHODS: We searched PubMed up to September 1, 2010 to identify eligible studies. A total of 10 case-control studies including 2288 cases and 4096 controls were included in the meta-analysis. Statistical analysis was performed with Review Manage version 4.2. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the strength of the association.

RESULTS: The results suggested that there is no significant association between XPD Lys751Gln polymorphism and esophageal cancer susceptibility in the overall population. However, in subgroup analysis by histology type, a significant association was found between XPD Lys751Gln polymorphism and esophageal adenocarcinoma (for CC *vs* AA: OR = 1.25, 95% CI = 1.01-1.55, P = 0.05 for heterogeneity).

CONCLUSION: Our meta-analysis suggested that XPD Lys751Gln polymorphism may be associated with increased risk of esophageal adenocarcinoma.

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Key words: Xeroderma pigmentosum group D; Polymorphism; Esophageal cancer; Mwta-analysis

Peer reviewer: Haruhiko Sugimura, First Department of Pathology,Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, 431-3192, Japan

Yuan L, Cui D, Zhao EJ, Jia CZ, Wang LD, Lu WQ. XPD Lys751Gln polymorphism and esophageal cancer risk: A meta-analysis involving 2288 cases and 4096 controls. *World J Gastroenterol* 2011; 17(18): 2343-2348 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2343.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2343

INTRODUCTION

Esophageal cancer, with a 5-year survival rate of < 20%, is considered as one of the most deadly malignancies[1,2]. It has already been identified that cigarette smoking, alcohol drinking, obesity, dietary factors, history of Barrett's esophagus, and esophageal reflux disease can contribute to the development of esophageal cancer[3-6]. However, only a fraction of exposed individuals develop esophageal carcinoma, which suggests that genetic variations in sensitivity to carcinogen exposure and DNA repair capacity

might be important inherited risk components in carcinogenesis[7,8]. DNA damage caused by exogenous, endogenous carcinogens or mutants is viewed as a crucial event in carcinogenesis. It can be repaired through activation of various pathways such as the nucleotide excision repair pathway (NER), base excision repair pathway (BER) and double-strand break pathway. The xeroderma pigmentosum group D (XPD) enzyme is involved in the NER pathway which plays an important role in the repair of bulky DNA adducts, such as pyrimidine dimmers, photoproducts and cross-links[9]. Several single-nucleotide polymorphisms (SNPs) have been identified in the XPD gene. Among them, a polymorphism in the XPD gene, codon 751 A to C, resulting in an amino acid alteration from lysine (Lys) to glycine (Gln) has been reported to be associated with an increased susceptibility to lung cancer, and head and neck carcinoma[10-12]. Other malignancies such as esophageal cancer have also been investigated.

To date, many molecular epidemiological studies have explored the association between XPD Lys751Gln polymorphism and esophageal cancer risk[12-23]. However, results of these studies are controversial, which may be caused by the limitation of individual studies. Therefore, we performed a meta-analysis of 10 published case-control studies covering 6384 subjects in order to get a more precise evaluation of the relationship between the XPD Lys751Gln polymorphism and esophageal cancer risk.

MATERIALS AND METHODS

Search strategy

We conducted a comprehensive search in the US National Library of Medicine's PubMed database (as of September 1, 2010) using search terms including "XPD", "xeroderma pigmentosum group D", "ERCC2", "excision repair cross-complementing rodent repair deficiency", "polymorphism", "esophageal", "esophagus" and the combined phrases for all genetic studies on the relationship between XPD polymorphism and esophageal cancer. Moreover, we reviewed the references from original articles to search for more studies. No language restrictions were imposed. Two investigators conducted all searches independently. Studies were absorbed in this meta-analysis if they met the following criteria: (1) a case-control study of the XPD Lys751Gln polymorphism and esophageal cancer risk; and (2) the authors must offer the size of the sample, odds ratios (ORs) with 95% confidence intervals (CIs) or the information that can help infer the results in the articles. If data were reported in more than one study, the most recent and complete study was chosen for this analysis.

Data extraction

Two investigators independently extracted data and reached a consensus on all of the items. Information was collected from each article, including the first author's name, year of publication, country of origin, racial descent of the subjects (categorized as Asian, European and mixed populations), sources of controls, genotyping method, histological type (categorized as esophageal adenocarcinoma and squamous cell carcinoma), number of different genotypes in cases and controls, Hardy-Weinberg equilibrium (HWE), and minor allele frequency in controls.

Statistical analysis

We assessed the strength of association between XPD Lys751Gln polymorphism and esophageal cancer risk by using ORs with 95% CIs which were obtained from the data given in the eligible studies. We evaluated the risk of codominant model (CC *vs* AA, CA *vs* AA), the dominant model (CA/CC *vs* AA), and recessive model (CC *vs* AA/CA), respectively. The between-study heterogeneity was investigated by Chi-square based Q-test[24], and it was considered significant if $P < 0.05$. The random-effects model (DerSimonian and Laird method) was then selected to pool the data[25]. Otherwise, the fixed-effects model (Mantel-Haenszel method) was used[26]. If heterogeneity was absent, these two models provided similar results. We used the funnel plot and the Egger weighted regression method ($P < 0.05$ was considered representative of statistical significance) to test possible publication bias in this meta-analysis[27]. All statistical analyses were performed in Statistical Analysis System software (v.9.13; SAS Institute, Cary, NC), and Review Manage (v.4.2; Oxford, England). All the tests were two-sided and the significant level was 0.05.

RESULTS

Eligible studies

A total of 12 potential relevant studies that described the association between the XPD genetic polymorphisms and esophageal cancer were retrieved through PubMed. After reading the full articles, one study by Liu *et al*[17] was excluded since the subjects had also been included in a study by Tse *et al*[20]. One other study was excluded because it did not list data clearly enough for further analysis[23]. Finally, we identified 10 eligible studies including 2288 cases and 4096 controls in total. As summarized in Table 1, four studies were conducted in Asians, four studies in Europeans, and two in mixed subjects. In terms of histology type, there were 4 studies of esophageal adenocarcinoma (EADC), 4 studies of esophageal squamous cell carcinoma (ESCC) and 2 of both EADC and ESCC. Diverse genotyping methods including PCR-RFLP, TaqMan and iPLEX™ were used. The classic PCR-RFLP assay was used in 60% (6/10) studies. Six studies mentioned the quality control. The genotype distributions in the controls of all the included studies were in accordance with HWE.

Meta-analysis

The main results of the meta-analysis on the association between XPD Lys751Gln polymorphism and esophageal cancer risk are shown in Table 2. Overall, no significant association was found between XPD Lys751Gln polymorphism and esophageal cancer risk (for CC *vs* AA: OR = 1.19, 95% CI = 0.84-1.69, $P = 0.01$ for heterogeneity, Figure 1; for CA *vs* AA: OR = 1.03, 95% CI = 0.83-1.27, $P =$

Table 1 Characteristics of case-control studies included in the meta-analysis

First author	Year	Country	Racial descent	Source of controls	Genotyping method	Histological type	Genotype distribution						*P* for HWE[1]	C
							Case			Control				
							AA	AC	CC	AA	AC	CC		
Xing	2002	China	Asian	Age matched	PCR-RFLP	ESCC[3]	367	63	3	451	70	3	0.87	0.07
Yu	2004	China	Asian	Age matched	PCR-RFLP	ESCC	108	16	11	133	17	2	0.11	0.07
Casson	2005	Canada	European	Randomly selected	PCR-RFLP	EADC[4]	31	21	4	34	46	15	0.93	0.40
Ye[2]	2006	Sweden	European	Age matched	PCR-RFLP	EADC	27	51	18	198	203	71	0.11	0.37
Ye[2]	2006	Sweden	European	Age matched	PCR-RFLP	ESCC	23	44	14	198	203	71	0.11	0.37
Sobti	2007	Indian	Asian	Age matched	PCR-RFLP	ESCC	52	61	7	63	77	20	0.64	0.37
Doecke	2008	Australia	Mixed	Age matched	iPLEXTM	EADC	108	124	31	575	588	174	0.22	0.35
Ferguson	2008	Ireland	European	Randomly selected	TaqMan	EADC	80	94	34	91	121	35	0.61	0.39
Tse	2008	America	Mixed	Age matched	TaqMan	EADC	104	159	49	193	208	52	0.72	0.34
Pan[2]	2009	America	European	Age matched	TaqMan	EADC	137	153	56	187	216	53	0.43	0.24
Pan[2]	2009	America	European	Age matched	TaqMan	ESCC	17	18	3	187	216	53	0.43	0.24
Zhai	2009	China	Asian	Age matched	PCR-RFLP	ESCC	167	31	2	148	51	1	0.12	0.13

[1]Hardy-Weinberg equilibrium (HWE) in controls; [2]The studies included esophageal adenocarcinoma (EADC), and esophageal squamous cell carcinoma (ESCC) for cases group, but controls were same; [3]Esophageal squamous cell carcinoma; [4]Esophageal adenocarcinoma. PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism.

Table 2 Summary odds ratios and 95% confidence interval of xeroderma pigmentosum group D Lys751Gln polymorphism and esophageal cancer risk

	CC *vs* AA		CA *vs* AA		CA/CC *vs* AA		CC *vs* CA/AA	
	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]
Total	1.19 (0.84-1.69)	0.01	1.03 (0.83-1.27)	0.01	1.05 (0.85-1.32)	0.01	1.16 (0.97-1.39)	0.06[2]
Ethnicity								
European	1.26 (0.95-1.65)	0.05[2]	1.00 (0.64-1.54)	0.01	1.01 (0.65-1.56)	0.01	1.20 (0.94-1.55)	0.28[2]
Asian	1.44 (0.37-5.66)	0.02	0.91 (0.71-1.15)	0.12[2]	0.96 (0.63-1.45)	0.03	1.47 (0.38-5.71)	0.02
Histological type								
EADC	1.25 (1.01-1.55)	0.05[2]	1.13 (0.85-1.52)	0.02	0.98 (0.68-1.41)	0.01	1.18 (0.97-1.44)	0.21[2]
ESCC	1.24 (0.56-2.70)	0.04	1.02 (0.73-1.41)	0.04	1.05 (0.74-1.50)	0.01	1.06 (0.71-1.58)	0.06[2]

[1]*P* value for heterogeneity; [2]Estimates for fixed-effects model. OR: Odds ratio; CI: Confidence interval.

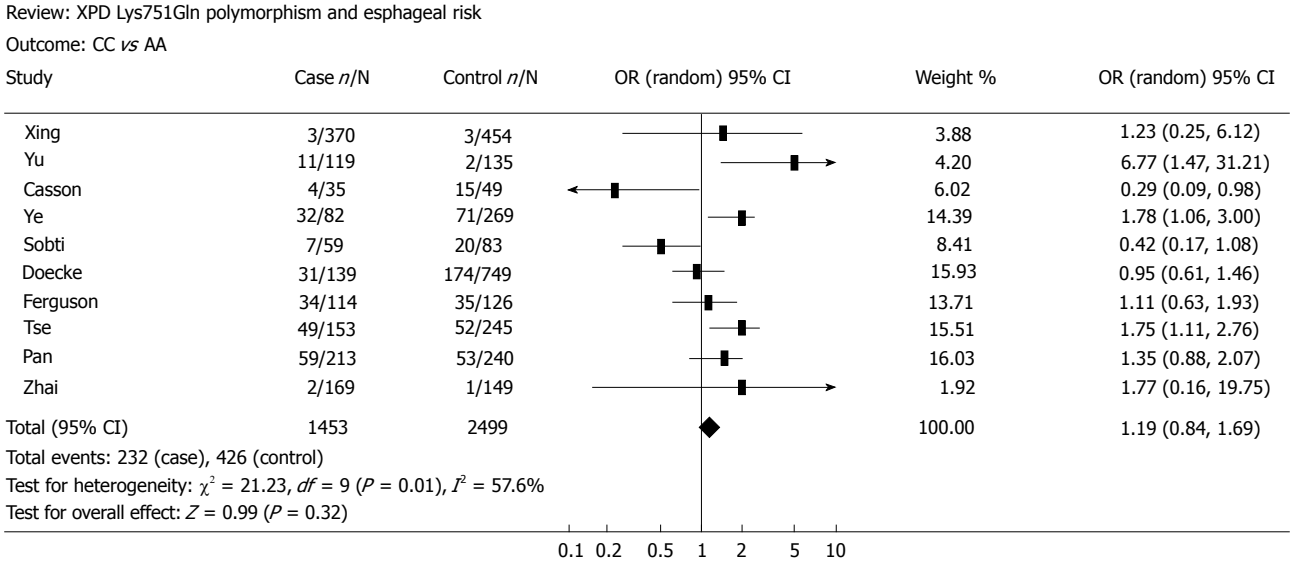

Figure 1 Odds ratio of esophageal cancer associated with xeroderma pigmentosum group D Lys751Gln polymorphism for the CC genotype compared with the AA genotype. XPD: Xeroderma pigmentosum group D; OR: Odds ratio.

0.01 for heterogeneity; for the dominant model CA/CC *vs* AA: OR = 1.05, 95% CI = 0.85-1.32, *P* = 0.01 for heterogeneity; for the recessive model CC *vs* CA/AA: OR = 1.16, 95% CI = 0.97-1.39, *P* = 0.06 for heterogeneity, Figure 2). In subgroup analysis by ethnicity, we also did not detect any significant association in all genetic models. However,

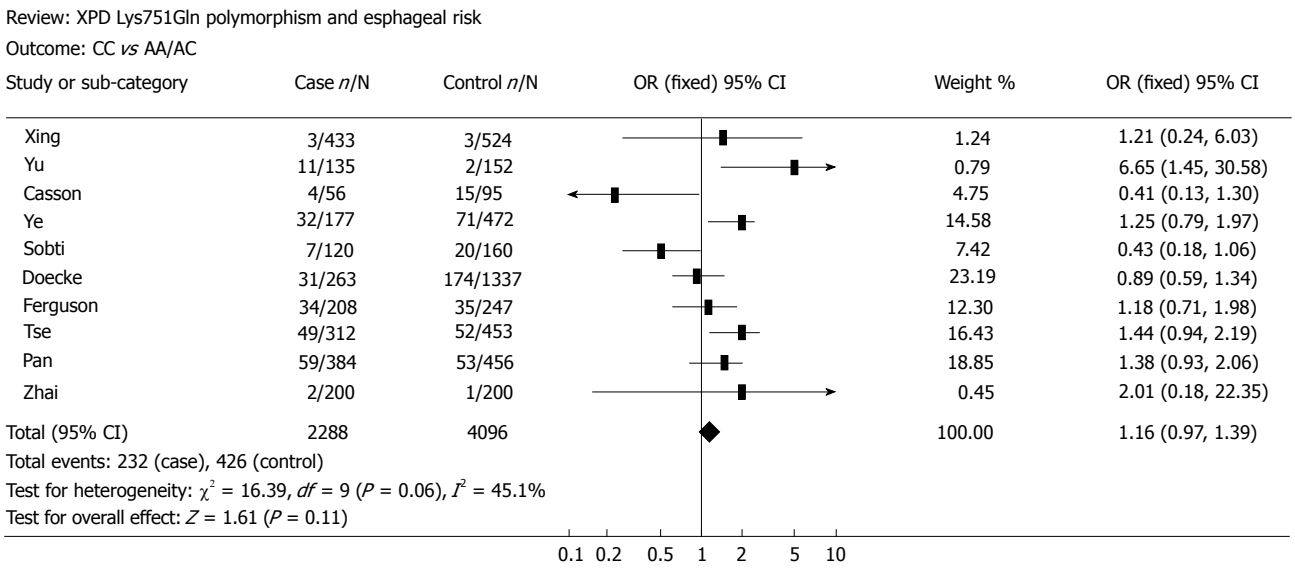

Figure 2 Odds ratio of esophageal cancer associated with xeroderma pigmentosum group D Lys751Gln polymorphism for the CC genotype compared with the AA/AC genotypes. XPD: Xeroderma pigmentosum group D; OR: Odds ratio.

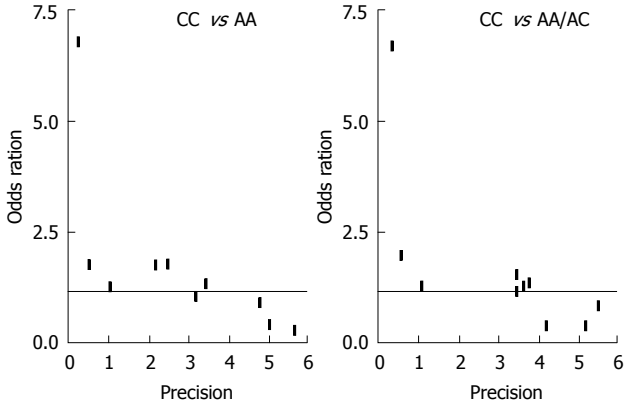

Figure 3 Funnel plot analysis to detect publication bias. Each point represents a separate study for the indicated association. The Odds ration is plotted on a logarithmic scale against the precision (the reciprocal of the SE).

further analysis by histological type revealed that individuals carrying the variant homozygote CC genotype showed an elevated risk to EADC compared to those with the wild-type AA genotype (OR = 1.25, 95% CI = 1.01-1.55, $P = 0.05$ for heterogeneity).

Publication bias

Funnel plot and the Egger's test were performed to assess possible publication bias. As shown in Figure 3, no publication bias was revealed by the funnel plots, which was approximately symmetrical for the codominant model CC *vs* AA and the recessive model CC *vs* CA/AA. Statistical evidence from the results of Egger's test confirmed the funnel plot symmetry (for CC *vs* AA: $t = 2.23$, $P = 0.06$; for CA *vs* AA: $t = 2.03$, $P = 0.08$; for CA/CC *vs* AA: $t = 1.48$, $P = 0.18$; for CC *vs* CA/AA: $t = 2.33$, $P = 0.05$).

DISCUSSION

Through analyzing data from the 10 eligible studies on relationship between XPD Lys751Gln polymorphism and esophageal cancer risk, we found no significant association between XPD Lys751Gln polymorphism and esophageal cancer risk in overall population. However, in the stratified analysis according to histological type, positive association were observed between XPD Lys751Gln polymorphism and elevated susceptibility to EADC.

The XPD gene has been mapped in chromosome 19q13.3. It spans over 20 kb, contains 23 exons and encodes the 761-amino acid protein. The XPD protein possesses both single-strand DNA-dependant ATPase and 5'-3' DNA helicase activities, which is essential for NER pathway and transcription[28]. The NER pathway generally removes bulky adducts caused by exogenous carcinogens, especially from cigarette smoking which is a well defined risk factor for EADC[29]. Any functional variation in NER pathway such as SNPs of key repair genes may lead to a deficiency in the DNA repair capacity (DRC) which is associated with a higher risk of cancer[28,30-32]. Benhamou *et al*[33] found that the single nucleotide substitution from A to C at codon 751 in the XPD gene leads to a complete change in the electronic configuration of the resulting amino acid, and reduces DNA repair efficiency. Many epidemiological studies have investigated the association between XPD Lys751Gln polymorphism and esophageal cancer, but the results were inconclusive. Xing *et al*[12] first explored the polymorphisms of DNA repair gene XPD and their associations with risk of esophageal squamous cell carcinoma in a Chinese population, but a Lys751Gln polymorphism in the XPD gene did not influence risk of ESCC in this study. However, two other studies on the relationship between XPD Lys751Gln polymorphism and ESCC revealed a contradictive result which suggested an increased risk of ESCC in association with the XPD 751 Gln/Gln genotype[13,15]. The more interesting finding revealed by Zhai *et al*[22] suggested an inverse association, which indicated that the XPD codon 751Gln allele was a protective factor rather than a risk factor to ESCC (OR = 0.628, 95% CI = 0.400-0.986). The first study

on the association between XPD 751 codon polymorphism and EADC was conducted by Casson, who observed the protective effect of the homozygous variant of XPD Lys-751Gln for EADC (OR = 0.24, 95% CI = 0.07-0.88)[14]. However, this result has not been supported by more studies. Both studies of Ye *et al* and Tse *et al* suggested that the XPD 751Gln allele was associated with an elevated risk for esophageal adenocarcinoma which is consistent with the result of our meta-analysis.

Some limitations of our meta-analysis should be acknowledged. Firstly, though it is known that the XPD gene has more polymorphisms than just Lys751Gln, we focused our meta-analysis on the most studied Lys-751Gln polymorphism due to limited evidence on others. Secondly, some studies on this relationship were modified by some other potentially suspected factors such as BMI, smoking status, alcohol consumption, history of gastroesophageal reflux disease and lifestyle; however, our results were based on unadjusted estimates due to a lack of the original data. Finally, the XPD gene may influence susceptibility to esophageal cancer with other genes, but we did not conduct the gene-gene interactions analysis in our study.

In conclusion, our meta-analysis suggested that XPD Lys751Gln polymorphism may be a risk factor for esophageal adenocarcinoma. Large and well designed epidemiological studies will be necessary to combine genetic factors together with other potential risk factor such as smoking status, alcohol consumption and history of gastroesophageal reflux disease in order to validate the relationship between XPD Lys751Gln polymorphism and esophageal cancer risk.

ACKNOWLEDGMENTS

We thank Dr. Zhai, Henan University of Science and Technology, Luoyang, China, for providing her full article.

COMMENTS

Background

Esophageal cancer is one of the most deadly malignancies. Many studies have explored the association between the Xeroderma pigmentosum group D (XPD) genetic polymorphism Lys751Gln and esophageal cancer risk, but the results are inconclusive and even controversial. It is, therefore, necessary to perform a meta-analysis in order to get a more precise evaluation of the relationship between the XPD Lys751Gln polymorphism and esophageal cancer risk.

Research frontiers

The XPD gene is responsible for bulky adducts and repair of UV-induced DNA damage. To date, there have been many case-control studies on the association between XPD Lys751Gln and esophageal cancer risk, but few meta-analyses were conducted on this topic.

Innovations and breakthroughs

Our meta-analysis suggested that XPD Lys751Gln polymorphism might alter individuals' susceptibility to esophageal adenocarcinoma. Further studies are needed to confirm it.

Applications

The finding that individuals carrying the variant homozygote CC genotype showed an elevated risk to esophageal adenocarcinoma indicates that genetic variations in the DNA repair protein may contribute to the risk of EADC. This meta-analysis gave a structured and systematic integration of information on the etiology of esophageal cancer, and the result may provide valuable information for researchers and clinicians.

Peer review

This is a review of the ten previously published association studies and extracted the importance of esophageal adenocarcinoma. This information is worthwhile.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2349

World J Gastroenterol 2011 May 14; 17(18): 2349-2352
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Lapatinib-induced hepatitis: A case report

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Received: August 16, 2010 Revised: September 16, 2010
Accepted: September 23, 2010
Published online: May 14, 2011

Abstract

Lapatinib is an inhibitor of the tyrosine kinases of human epidermal growth factor receptor type 2 (HER2) and epidermal growth factor receptor type 1, with clinical activity in HER2-positive metastatic breast cancer. We present here a 60 year-old patient with metastatic breast cancer who presented with jaundice and increased serum aminotransferase levels and who had been treated with lapatinib for the previous 14 days. Laboratory tests excluded other causes of acute liver injury. Liver biopsy revealed lesions compatible with drug-induced hepatotoxicity. Bilirubin and liver enzymes returned to normal within three months of lapatinib discontinuation. Lapatinib should be included among the causes of drug-induced hepatitis.

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Key words: Lapatinib; Hepatitis; Hepatotoxicity; Breast cancer; Human epidermal growth factor receptor type 2

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Peroukides S, Makatsoris T, Koutras A, Tsamandas A, Onyenadum A, Labropoulou-Karatza C, Kalofonos H. Lapatinib-induced hepatitis: A case report. *World J Gastroenterol* 2011; 17(18): 2349-2352 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2349.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2349

INTRODUCTION

Metastatic breast cancer is the leading cause of death from cancer among women worldwide[1]. The overexpression of human epidermal growth factor receptor type 2 (HER2) predisposes patients to a greater risk for disease progression and death than women whose tumors do not overexpress HER2[2]. Therapeutic approaches to block HER2 signaling pathways include both trastuzumab (a recombinant, humanized, monoclonal antibody that binds to the extracellular domain of the HER2) and lapatinib. Lapatinib is an orally administered small molecule that inhibits the tyrosine kinases of HER2 and epidermal growth factor receptor type 1 (EGFR). A number of studies have shown that lapatinib has clinical activity in patients with HER2-positive breast cancer, with a significant reduction in the risk of disease progression[3]. Lapatinib is generally well tolerated and the most common treatment-related adverse events include rash, diarrhea, and nausea[4].

We report here a case of advanced breast cancer treated with lapatinib with drug induced hepatitis. Upon discontinuation of lapatinib, levels of serum aspartate amino transferase (SGOT) and serum alanine amino transferase (SGPT) declined progressively.

CASE REPORT

A 60 year-old woman presented with metastatic breast cancer in the lung. Three years earlier she was diagnosed with invasive ductal adenocarcinoma of the right breast

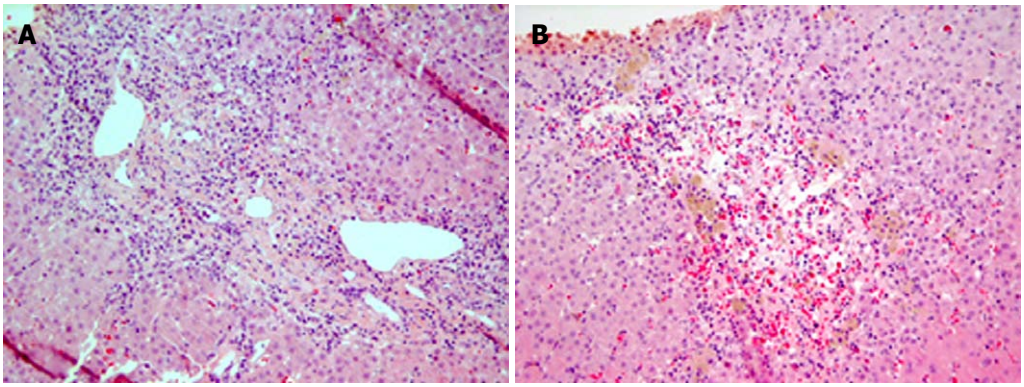

Figure 1 Photomicrograph. A: Areas of bridging necrosis (HE x 200); B: Areas of centrilobular hepatocellular dropout, hemorrhage and macrophages (HE x 200).

(pT1cN0M0). Immunohistochemistry was positive for estrogen (70% positive cells), progesterone (2% positive cells) receptors and for HER-2. She was treated with lumpectomy and axillary lymph node dissection. Subsequently she received adjuvant chemotherapy (four cycles of epirubicin/cyclophosphamide followed by 4 cycles of paclitaxel) and treatment with the monoclonal antibody trastuzumab for one year. Furthermore, radiotherapy was given to the entire breast as an adjunct to breast conservation treatment and hormonal therapy with the aromatase inhibitor exemestane was started. She remained free of disease for three years.

In terms of periodic reassessment, the patient had a computed tomography (CT) of the chest in January 2010 and was found to have three nodules in the right lung compatible with metastatic sites. A CT of the brain and abdomen showed no abnormalities and a mammogram of both breasts and a bone scan were also normal. According to laboratory data, there were no abnormal values.

The patient commenced therapy with capecitabine (1000 mg/m^2 twice daily, day 1-14) and lapatinib (1250 mg/d), while exemestane was discontinued after two and a half years of continuous administration. After ten days, capecitabine was discontinued due to grade 2 diarrhea and the patient continued to receive lapatinib only.

Two weeks later, the patient developed jaundice without any other clinical signs such as asthenia or pruritus and she was evaluated in the Department of Clinical Oncology.

Physical examination was unremarkable except for jaundice and mild hepatomegaly. Laboratory results showed: SGPT 583 U/L units (normal range: 5-45), SGOT 457 U/L (normal range: 5-40), ALP 348 U/L (normal limits < 270), g-glutamyl transpeptidase 213 U/L (normal range: 10-55), total bilirubin 4.1 mg/dL (normal range: 0-1.5), LDH 305 units (normal range: 100-240), total protein 6.4 g/dL (normal range: 6-8.4), albumin 3.7 g/dL (normal range: 3.4-5), white blood cells 7500/mm^3, Hb 12.8 g/dL, Hct 38.8%, platelets 118000/mm^3 and international normalized ratio-1.14 (normal range: 0.8-1.2). Other blood chemistry results, including glucose, cholesterol, triglycerides, serum amylase, uric acid, creatinine, BUN, Na, K, Ca as well as urinalysis were normal.

Serology tests for hepatitis A, B, and C viruses were negative. Immune serology was also negative for ANA, anti-DNA, c-ANCA, p-ANCA, antismooth muscle, and antimitochondrial antibodies. Furthermore, thyroid hormone tests, a1-antitrypsin, IgG, IgA, IgM and blood ceruloplasmin levels were within normal limits.

Abdominal ultrasound demonstrated an increased-sized liver with non-homogeneous and diffuse echogenicity, without biliary tract abnormalities. Normal blood flow was seen in the portal vein, hepatic artery, hepatic veins, and inferior vena cava. A new abdominal CT showed hepatomegaly without biliary tract obstruction.

Fifteen days after the clinical evolution, a liver biopsy was performed and showed necrosis of contiguous hepatocytes in portal-to-portal and portal-to-central fashion (bridging necrosis). Foci of severe hemorrhage and hepatocellular dropout around the centrilobular areas were also demonstrated (Figure 1). Eosinophils were observed in more than twelve portal spaces without granulomas.

The patient was not taking other concomitant hepatotoxic medications, herbal or dietary supplements and did not have any underlying liver dysfunction, chronic hepatitis or alcohol use history.

Lapatinib was discontinued and both the patient's jaundice and liver function tests gradually improved and returned to normal range within 3 mo (Table 1).

DISCUSSION

Lapatinib combined with capecitabine has been approved for the treatment of patients with HER-2 positive metastatic breast cancer improving median time to disease progression[3].

Furthermore, recent studies suggest that single-agent lapatinib has clinical activity with manageable toxicity in HER2-overexpressing breast cancer that progressed on trastuzumab-containing therapy[4,5]. The fact that lapatinib inhibits EGFR signaling may also contribute to its activity in the context of refractory HER2-positive breast cancer.

No significant liver dysfunction has been recorded to lapatinib administration in any daily dose (500-1600 mg) in many phase I and II studies[5,6]. Hence, grade 3 and 4 liver toxicity with elevations in transaminases are uncommon after single agent lapatinib administration and only one out

Table 1 Evolution of laboratory tests after lapatinib discontinuation

	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	GGT (U/L)	TBIL (mg/dL)
Baseline (before initiation of capecitabine/lapatinib)	24	21	120	11	0.8
Ten days later (capecitabine discontinuation due to diarrhea)	30	22	110	20	0.7
Two weeks after capecitabine discontinuation (lapatinib discontinuation)	583	457	348	213	4.1
Two weeks after lapatinib discontinuation	481	589	310	294	11.8
1 mo after lapatinib discontinuation	260	238	318	160	2.7
2 mo after lapatinib discontinuation	229	180	298	140	2.3
3 mo after lapatinib discontinuation	44	36	246	42	0.9

SGPT: Serum glutamic pyruvic transaminase; SGOT: Serum glutamic oxaloacetic transaminase; ALP: Alcaline phosphatase; GGT: Gamma-glutamyl transpeptidase; TBIL: Total Bilirubin.

of 37 patients experienced grade 3 elevation of transaminases in a phase Ⅱ trial in patients with brain metastases from HER-2 positive breast cancer[7].

Lapatinib is predominantly metabolized in the liver *via* the cytochrome P450 system, by the enzyme P450 3A4 and < 2% of the drug is excreted unchanged in urine.

Concurrent administration of the strong CYP3A4 inhibitor ketoconazole increases the lapatinib area under the curve and prolongs the $t_{1/2}$. For this reason, strong inhibitors of CYP3A4, including grapefruit juice, should be avoided, as they may increase plasma concentrations of lapatinib and thus lapatinib toxicity[8].

Furthermore, it can be assumed that polymorphisms of the CYP3A4 gene may affect lapatinib disposition[9]. However, pharmacogenomical studies on the same gene did not show any correlation between gene polymorphisms and commonly observed toxicities such as exanthema or diarrhea caused by the use of erlotinib, an epidermal growth factor receptor tyrosine-kinase inhibitor[10].

The patient described in our case developed severe hepatic enzyme disturbances during lapatinib treatment and liver biopsy showed acute drug-induced hepatitis.

The patient did not take any other drug except lapatinib and recovered when the treatment was discontinued. There was no evidence of viral or autoimmune hepatitis or any other cause of hepatitis. Hepatic enzymes normalized rapidly after discontinuation of lapatinib.

The time to onset of jaundice and laboratory test abnormalities as well as the time and course of recovery, while the patient did not receive other medications, led us to support the idea that lapatinib is the most likely cause of hepatic injury. Exemestane appears not to be an important risk factor for the development of hepatitis because this medication was administered for two and a half years, without any clinical symptoms or abnormal laboratory tests.

Based on the fact that capecitabine was stopped only two weeks before the onset of symptoms, it was necessary to assesss the likelihood of the agent being causative. Capecitabine has not been implicated in hepatocellular injury and hepatitis with the specific histological features. Furthermore in order to confirm that liver injury was due to lapatinib, we used the Roussel Uclaf Causality Assesment Method, a scoring system which assigns attribution for drug-induced liver injury[11]. The summed points grouped lapatinib into highly probable category, confirming our suggestion.

Our case demonstrates the potential risk of developing toxic hepatitis during treatment with lapatinib.

To conclude, to our knowledge this is the first case of acute drug-induced hepatitis secondary to lapatinib. Given that lapatinib is now often used in the treatment of HER-2 positive advanced breast cancer, it is mandatory for medical oncologists to be aware of this potential side effect in clinical practice.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2353

World J Gastroenterol 2011 May 14; 17(18): 2353-2355
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Transarterial injection of H101 in combination with chemoembolization overcomes recurrent hepatocellular carcinoma

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Received: December 9, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: May 14, 2011

Abstract

Transcatheter arterial chemoembolization (TACE) has become the standard treatment modality for unresectable hepatocellular carcinoma (HCC). Nonetheless, the clinical outcomes in patients with unresectable HCC are often unsatisfactory, especially in those with recurrent HCC. H101, an E1B gene deleted adenovirus, is known to have a significant antitumor activity. In addition, local injection of H101 can enhance the effect of antitumor therapies (chemotherapy and radiotherapy). Transarterial H101 gene injection in combination with TACE may help to control refractory and recurrent HCC. In this study, we report a 55-year-old patient with recurrent HCC which was treated with transarterial injection of H101 in combination with TACE, leading to a good clinical prognosis of the patient.

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Key words: Hepatocellular carcinoma; H101; Transcatheter arterial chemoembolization; Therapy

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He Q, Liu Y, Zou Q, Guan YS. Transarterial injection of H101 in combination with chemoembolization overcomes recurrent hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(18): 2353-2355 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2353.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2353

INTRODUCTION

It is difficult to eradicate hepatocellular carcinoma (HCC) because of its repeated recurrence in the liver. Surgical resection and transcatheter arterial chemoembolization (TACE) are the mostly used effective modalities for HCC, but it still frequently recurs after treatment with such modalities[1,2]. Gene therapy for tumors is a new hope in the 21st century. It is coming to be widely used in clinic and has achieved relatively well results especially when it is directly injected into the tumor[3-9]. Since combined TACE and gene injection may help to manage recurrent HCC, we tried to use the H101 gene in combination with TACE to treat our patient with recurrent HCC and achieved a good clinical prognosis.

CASE REPORT

A 55-year-old man was diagnosed with HCC in the right liver lobe, which was histologically proven (Figure 1A) in June 2006. The patient underwent six rounds of TACE from June 2006 to September 2008 (Figure 1B). His α-fetoprotein (AFP) level was 595.4 ng/mL after the six rounds of TACE, and decreased to 10.99 ng/mL after a partial hepatectomy. In February 2009, a recurrent nodule was found in the remnant liver at a routine postoperative computed tomography (CT) scan. At that time, his AFP level was 724 ng/mL. Because the effect of TACE was poor on HCC (Figure 2A), we decided to treat the patient with combined TACE and H101, a recombinant human type-5 adenovirus (Ad5), in which the E1B-55 kDs gene

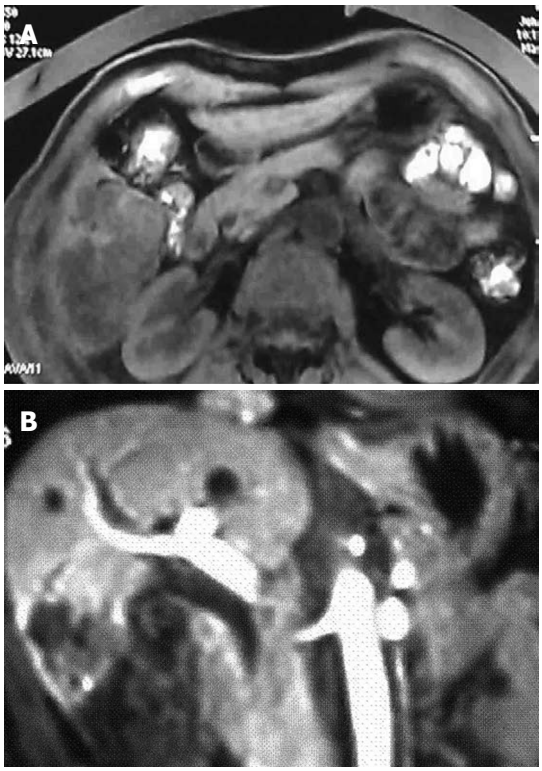

Figure 1 Magnetic resonance imaging. A: A hypodense nodule and hepatocellular carcinoma before therapy; B: A hypodense nodule with partial contrast enhancement before hepatectomy.

Table 1 Schedule of therapy and change in follow-up α-fetoprotein

Time (yr-mo)	APF (ng/mL)	TACE (yes/no)	H101 (yes/no)	Resection (yes/no)
2006-06	1210.00	Yes		
2006-07	143.20	Yes		
2007-09	70.00	Yes		
2007-11	21.67	No		
2008-03	355.60	Yes		
2008-04	491.16	No		
2008-06	1390.00	Yes		
2008-07	955.10	No		
2008-08	1072.00	Yes		
2008-09	595.40	No		Yes
2008-10	10.99	No		
2009-02	352.30	Yes		
2009-03	724.00	Yes	Yes	
2009-05	338.40	Yes	Yes	
2009-07	4.56	Yes	Yes	
2010-12	2.28	No		

APF: α-fetoprotein; TACE: Transcatheter arterial chemoembolization.

is totally deleted (Oncorine, Shanghai Sunway Biotech, China). First, we injected 5-fluoro-2-deoxyuridine (1.0 g), vinorelbine (40 mg), and cisplatin (80 mg) into the celiac trunk. Then, H101 and iodized oil were injected into the artery that supplies the tumor. A total of 1×10^{12} virus particles (VP) and 10 mL iodized oil were administered.

The patient had no discomfort after the procedure. Two months later, routine follow-up CT showed a fairly good result (Figure 2B) and the AFP level in the patient decreased to 338.4 ng/mL from 724 ng/mL before the treatment. We repeated this therapy 2 times at 2-mo interval. At the last admission, abdominal CT demonstrated complete deposit of oil with no signs of recurrence (Figure 2C). Furthermore, his AFP was within the normal reference range (4.56 ng/mL).

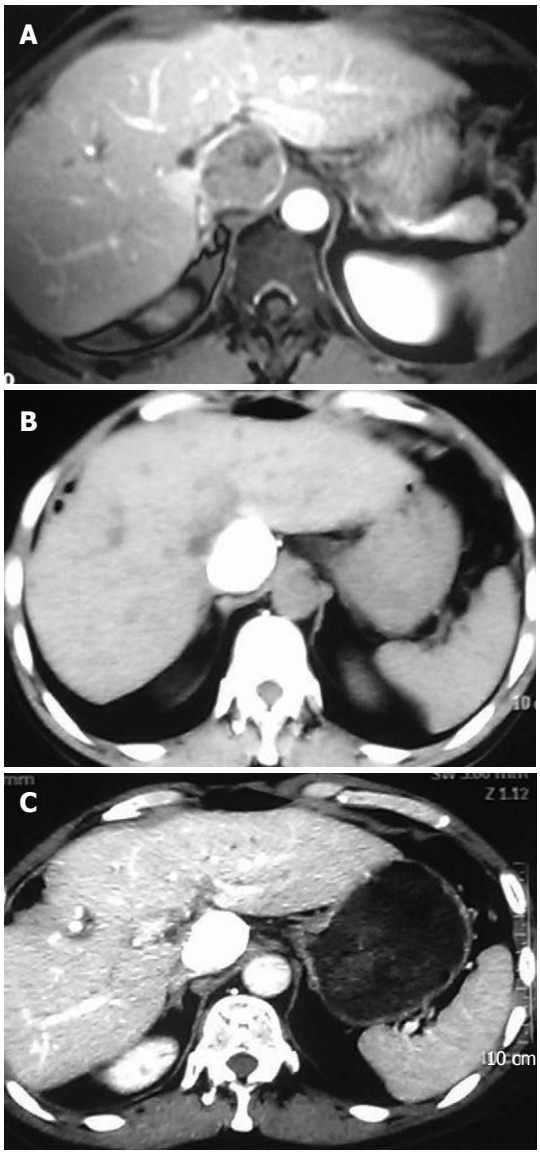

Figure 2 Contrast magnetic resonance imaging. A: A hypodense nodule with circle contrast manifestation after hepatectomy and the seventh Transcatheter arterial chemoembolization; B: Contrast computed tomography showing homogeneous dense retention of lipiodol within the entire tumor mass two months after treatment; C: Contrast computed tomography no recurrent mass 4 mo after treatment.

Eighteen months following the last H101/TACE treatment, the patient showed no evidence of recurrence and no abnormal liver function, but had a normal serum AFP level. The AFP levels in the patient during the therapy are listed in Table 1.

DISCUSSION

Although TACE has become the standard treatment modality for unresectable HCC, it is frequently unsuccessful[1,2].

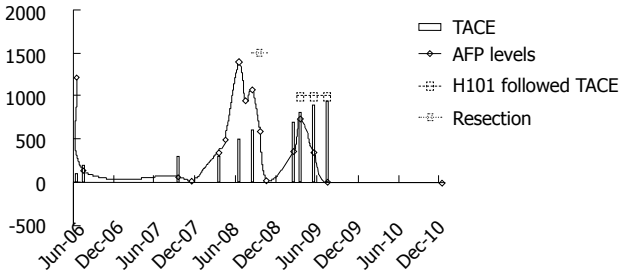

Figure 3 Changes in α-fetoprotein levels during and after treatment.

Similarly, HCC recurs frequently following its resection. Although TACE was effective in our patient, HCC recurred and failed the seventh TACE. The recurred HCC was unresectable because it occupied the entire caudate lobe and was close to the vena cava and abdominal aorta. Thus, H101 in combination with TACE was attempted for the patient with a fairly good outcome without any complications. From Table 1 and Figure 3, we can see that the AFP level in the patient decreased significantly following treatment with TACE plus H101. Both the initial series of TACE and tumor resection failed to prevent recurrent HCC although AFP was controlled, whereas combined H101 and TACE appeared to be effective and well-tolerated.

H101 is a recombinant human type-5 adenovirus in which E1B-55 kDs gene is totally deleted. The H101 virus produced by Shanghai Sunway Biotech also contains a deletion in the E3 region with a significant antitumor activity. This recombinant adenovirus has a replication-selective property and replicates only in tumor cells. Before modification, the E1B region of the wild adenovirus type 5 expresses early gene products that bind to and inhibit the function of p53, a tumor suppressor. Deletion or mutation of the E1B region confers p53-selective replication of oncolytic viruses which infect tumor cells and induce massive accumulation of normal p53. By this way, the adenovirus causes direct cytotoxicity only to tumor cells during replication. The E3 region is related to the inhibition of host immunity, which enhances the virus replication and spread in tumors[3,4]. The virus replication and spread can be enhanced by repeated injection of H101. By sacrificing the spread ability, the virus may activate the host immune response to virus-infected tumor cells and help the host immune system to recognize tumor cells, thus benefiting patients under such a therapy. Metastasis is prevalent in patients with malignant tumors, leading to treatment failure and death of patients. Moreover, patients may have more than one tumor lesion, and some of these lesions may be hard to reach in order to be injected with H101. Therefore, the ability of H101 to activate the host immune response seems crucial. Treatment with the E3 region deleted adenovirus, H101, may have additional benefits to patients.

H101 is formulated as a sterile viral solution in phosphate buffered saline and kept at -20℃. Each vial contains 0.5 mL virus solution with 5×10^{11} viral particles and titered less than 1/60 of 50% tissue culture infectious dose. Sterile and purified viruses were produced for clinical use by Shanghai Sunway Biotech (Shanghai, China), and tested for the titer, sterility, and general safety by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Its safety has been demonstrated in a number of clinical trials[5-8].

Although the anticancer activity of H101 has been proved in a wide type of advanced cancer by intra-tumor injection, its clinical efficacy against liver cancer has been rarely reported[9-11]. Transcatheter arterial injection of H101 was effective against liver cancer in our patient, suggesting that H101 in combination with TACE is useful for recurrent HCC. However, subsequent large, multi-center randomized, controlled studies are needed to facilitate the introduction of genetically engineered and reinforced viruses as novel therapeutic platforms for the treatment of cancers.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2356

World J Gastroenterol 2011 May 14; 17(18): 2356
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Prophylactic antibiotics for variceal hemorrhage: Clostridium difficile infection still can be a risk

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Received: January 6, 2011 Revised: January 29, 2011
Accepted: February 5, 2011
Published online: May 14, 2011

Abstract

Bron *et al* presented a retrospective study regarding the prophylactic use of antibiotics for variceal hemorrhage. Antibiotics appeared to improve the survival rate of patients without increasing clostridium difficile infection (CDI). We argue against the conclusion of the authors and consider that this result may be simply due to concurrent use of metronidazole, a therapeutic agent against CDI.

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Key words: Variceal hemorrhage; Prophylactic antibiotics; Clostridium difficile infection

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Okano N, Iwata K. Prophylactic antibiotics for variceal hemorrhage: Clostridium difficile infection still can be a risk. *World J Gastroenterol* 2011; 17(18): 2356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2356.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2356

TO THE EDITOR

Brown *et al*[1] presented a retrospective study regarding prophylactic use of antibiotics in treatment of variceal hemorrhage. The data show that antibiotics appear to improve the survival rate of patients without increasing Clostridium difficile infection (CDI). However, 70.3% of the patients who were given antibiotics received metronidazole, a therapeutic agent against CDI. No apparent increase in CDI may be simply due to the suppression of the organism by metronidazole. As pointed out in the article, currently recommended antibiotic for this purpose is ceftriaxone[2,3], which is known to predispose to CDI[4]. The result of the article should not be interpreted as the currently recommended use of ceftriaxone posing a low risk of CDI.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 May 14; 17(18): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 May 14; 17(18): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, 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 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 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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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 May 14; 17(18): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Name of journal

World Journal of Gastroenterology

ISSN and EISSN
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, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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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.

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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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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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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 υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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

World Journal of *Gastroenterology*

World J Gastroenterol 2011 May 21; 17(19): 2357-2454

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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 19 May 21, 2011

EDITORIAL

2357 Targeting voltage-gated sodium channels for treatment for chronic visceral pain
Qi FH, Zhou YL, Xu GY

TOPIC HIGHLIGHT

2365 Review of screening for pancreatic cancer in high risk individuals
Stoita A, Penman ID, Williams DB

REVIEW

2372 Surgicopathological classification of hepatic space-occupying lesions: A single-center experience with literature review
Cong WM, Dong H, Tan L, Sun XX, Wu MC

ORIGINAL ARTICLE

2379 Inhibitory effect of schisandrin B on free fatty acid-induced steatosis in L-02 cells
Chu JH, Wang H, Ye Y, Chan PK, Pan SY, Fong WF, Yu ZL

2389 CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma
Zhao BC, Wang ZJ, Mao WZ, Ma HC, Han JG, Zhao B, Xu HM

2397 Protective effects of ACLF sera on metabolic functions and proliferation of hepatocytes co-cultured with bone marrow MSCs *in vitro*
Shi XL, Gu JY, Zhang Y, Han B, Xiao JQ, Yuan XW, Zhang N, Ding YT

BRIEF ARTICLE

2407 Incidence of brain metastasis in patients with esophageal carcinoma
Smith RS, Miller RC

2411 Resected specimen evaluation, anorectal manometry, endoanal ultrasonography and clinical follow-up after STARR procedures
Naldini G, Cerullo G, Menconi C, Martellucci J, Orlandi S, Romano N, Rossi M

2417 p53 antibodies, metallothioneins, and oxidative stress markers in chronic ulcerative colitis with dysplasia
Hamouda HE, Zakaria SS, Ismail SA, Khedr MA, Mayah WW

2424 Monoclonal immunoscintigraphy for detection of metastasis and recurrence of colorectal cancer
Artiko V, Marković AK, Šobić-Šaranović D, Petrović M, Antić A, Stojković M, Žuvela M, Šaranović D, Stojković M, Radovanović N, Galun D, Milovanović A, Milovanović J, Bobić-Radovanović A, Krivokapic Z, Obradović V

2431 Long-term outcome and efficacy of endoscopic hemorrhoid ligation for symptomatic internal hemorrhoids
Su MY, Chiu CT, Lin WP, Hsu CM, Chen PC

2437 Effects of appendectomy and oral tolerance on dextran sulfate sodium colitis
Yue M, Shen Z, Yu CH, Ye H, Ye YF, Li YM

CASE REPORT

2446 A case of steroid-dependent myeloid granulocytic sarcoma masquerading as Crohn's disease
Kwan LY, Targan SR, Shih DQ

2450 Optimized management of advanced hepatocellular carcinoma: Four long-lasting responses to sorafenib
Abbadessa G, Rimassa L, Pressiani T, Carrillo Infante C, Cucchi E, Santoro A

LETTERS TO THE EDITOR

2454 Severe alcoholic hepatitis: Glucocorticoid saves lives and transplantation is promising
Braillon A

World Journal of Gastroenterology
Volume 17 Number 19 May 21, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Kwan LY, Targan SR, Shih DQ. A case of steroid-dependent myeloid granulocytic sarcoma masquerading as Crohn's disease. *World J Gastroenterol 2011*; 17(19): 2446-2449 http://www.wjgnet.com/1007-9327/full/v17/i19/2446.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

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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
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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
May 21, 2011

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

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2357

World J Gastroenterol 2011 May 21; 17(19): 2357-2364
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Targeting voltage-gated sodium channels for treatment for chronic visceral pain

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Supported by an NIH grant AT005158
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Received: December 14, 2010 Revised: March 16, 2011
Accepted: March 23, 2011
Published online: May 21, 2011

Abstract

Voltage-gated sodium channels (VGSCs) play a fundamental role in controlling cellular excitability, and their abnormal activity is related to several pathological processes, including cardiac arrhythmias, epilepsy, neurodegenerative diseases, spasticity and chronic pain. In particular, chronic visceral pain, the central symptom of functional gastrointestinal disorders such as irritable bowel syndrome, is a serious clinical problem that affects a high percentage of the world population. In spite of intense research efforts and after the dedicated decade of pain control and research, there are not many options to treat chronic pain conditions. However, there is a wealth of evidence emerging to give hope that a more refined approach may be achievable. By using electronic databases, available data on structural and functional properties of VGSCs in chronic pain, particularly functional gastrointestinal hypersensitivity, were reviewed. We summarize the involvement and molecular bases of action of VGSCs in the pathophysiology of several organic and functional gastrointestinal disorders. We also describe the efficacy of VGSC blockers in the treatment of these neurological diseases, and outline future developments that may extend the therapeutic use of compounds that target VGSCs. Overall, clinical and experimental data indicate that isoform-specific blockers of these channels or targeting of their modulators may provide effective and novel approaches for visceral pain therapy.

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Key words: Voltage-gated sodium channel; Dorsal root ganglion; Visceral pain; Functional gastrointestinal disorders; Treatment

Peer reviewer: Dan L Dumitrascu, Professor, President, Romanian Society of Neurogastroenterology, 2nd Medical Department University of Medicine and Pharmacy Iuliu Hatieganu Cluj, Romania

Qi FH, Zhou YL, Xu GY. Targeting voltage-gated sodium channels for treatment for chronic visceral pain. *World J Gastroenterol* 2011; 17(19): 2357-2364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2357.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2357

INTRODUCTION

Treating chronic pain remains a significant clinical challenge, and in particular, current treatments for visceral pain conditions are still based mainly on empirical rather than mechanistic evidence. Search for a consistently effective pharmacological agent to treat these pain conditions remain elusive. Functional gastrointestinal (GI) pain, one of the most prevalent forms of chronic visceral pain, is commonly interpreted as a consequence of hypersensitivity of GI nociceptive pathways, either of the peripheral sensory nociceptors in the gut or of the central neurons[1,2]. Human and animal studies have identified several channels as pivotal for enhanced signal transmission along

the pain axis, including voltage-gated sodium channels (VGSCs) $Na_v1.3$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$, with the latter three preferentially expressed in peripheral sensory neurons[3-5]. VGSCs are integral membrane glycoproteins that are essential for generation and conduction of electrical impulses in excitable cells. Many of the most common neurological disorders, such as epilepsy, migraine, neurodegenerative diseases, and chronic pain involve abnormalities of neuronal excitability[6,7]. There is a growing body of evidence that implicates abnormal expression and function of VGSCs in these disorders[8-12]. Although there is an incomplete understanding of these channels and/or primary site of action at which these compounds exert their effects and significant side effects are often encountered, there is enough evidence emerging to give hope that a more refined approach may be achievable[13]. This article does not aim to provide an exhaustive list of potential targets, but to focus on some of the most recent advances in the field of pain research and medicine and to highlight where therapeutic strategies for relieving visceral pain may emerge in the near future.

BIOPHYSICAL AND MOLECULAR PROPERTIES OF VGSCS

VGSCs are integral membrane glycoproteins that consist of a central α-subunit of 260 kDa associated with one or more auxiliary β-subunits of about 35 kDa (Figure 1A)[5,14]. Nine α-subunit subtypes have been cloned and functionally expressed. These subtypes are designated $Na_v1.1$-$Na_v1.9$ for the proteins and *SCN1A*-*SCN5A* and *SCN8A*-*SCN11A* for the genes; *SCN6A*/*SCN7A* codifies the related protein Na_x. The VGSC α-subunit, which forms the ion-conducting pore and the channel gate for activation and inactivation, contains four domains (D Ⅰ-D Ⅳ), each with six α-helical transmembrane segments labeled S1-S6 (Figure 1A). Pore loops between S5 and S6 (highlighted in red) in each of the four domains form the selectivity filter of the channel. Each pore loop contributes a single amino acid (aspartate from D Ⅰ, glutamate from D Ⅱ, lysine from D Ⅲ, and alanine from D Ⅳ), which together form a narrow ring that is mainly responsible for conferring Na^+ selectivity. The four S6 segments form the cytoplasmic end of the pore, which binds various types of therapeutically important pore-blocking compounds, including local anesthetics, and antiarrhythmic drugs. The S4 segments (highlighted in green) in each of the four domains contain regularly spaced, positively charged amino acid residues and serve as voltage-sensors, coupling membrane depolarization to channel activation. The intracellular loop between D Ⅲ and D Ⅳ forms the fast-inactivation gate that occludes the cytoplasmic end of the pore when the channel inactivates. The C-terminal cytoplasmic domain is important for setting some of the properties of fast inactivation and contains binding sites for interacting proteins. In addition to fast inactivation, a distinct process called slow inactivation develops during prolonged depolarizing plateaus and during high frequency repetitive firing. The

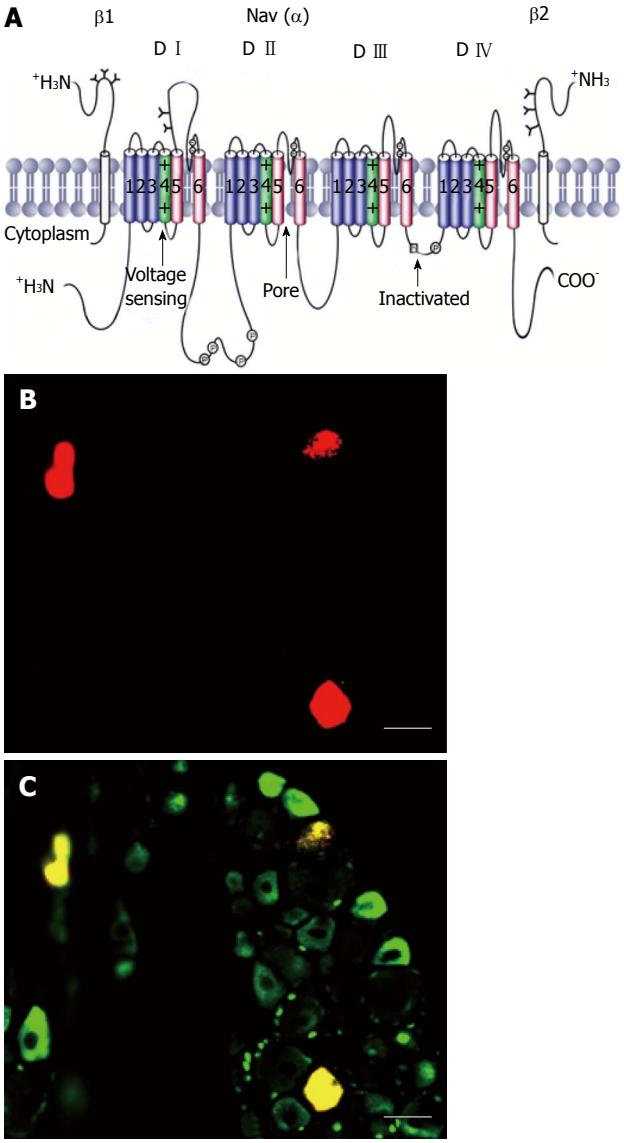

Figure 1 Schematic of a voltage-gated sodium channel polypeptide and Nav1.8 positive colon specific dorsal root ganglion neurons. A: The transmembrane organization includes four domains (D Ⅰ-D Ⅳ) joined by three intracellular loops (L1-L3) and the intracellular N and C termini. Cylinders represent probable α-helical segments. P in circles, sites of demonstrated protein phosphorylation by protein kinase A (PKA) or PKC; green, S4 voltage sensors; h in which square, inactivation particle in the inactivation gate loop (modified, with permission, from Catterall[14]); B: Colon specific dorsal root ganglion neurons labeled by DiI (red); C: Nav1.8 positive neurons (green). Colon specific Nav1.8 positive neurons are stained yellow. Bar = 50 μm for both B and C.

kinetics of onset and recovery of slow inactivation are about four orders of magnitude slower than those of fast inactivation. Slow inactivation does not depend on the fast inactivation gate formed by the intracellular loop between D Ⅲ and D Ⅳ, but instead mainly involves rearrangements of the pore of the channel[15].

VGSCS IN PRIMARY SENSORY NEURONS INNERVATING VISCERAL ORGANS

Primary sensory neurons, with their cell bodies located in the dorsal root ganglion (DRG), are the first link in the so-

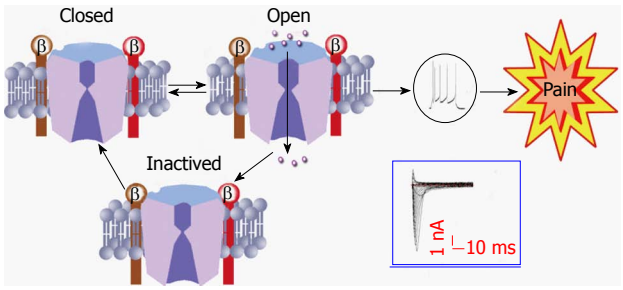

Figure 2 A model showing the three states of the sodium channel. Tissue damage/inflammation leads to activation of the sodium channel from a closed state. Once activated, sodium ions entered through the channel and evoke the action potential of the cell membrane, eventually producing the sensation of pain. Once the channel becomes inactivated, it must go to a closed state before being activated again. Action potentials and inward currents were recorded from a colon specific dorsal root ganglion neuron from a healthy rat.

matosensory pathway. They encode their messages in the form of a series of action potentials whose depolarizing upstroke is produced by sodium channels[3-5,16]. Most neuroscientists and neurologists are familiar with the textbook description of VGSC function (Figure 2). VGSCs contribute to the control of membrane excitability and underlie action potential generation. They are closed at resting membrane potentials characteristic of quiescent neurons. In response to membrane depolarization, they open within a few hundred microseconds (a process termed activation), resulting in an inward sodium ion (Na^+) current, and then convert within a few milliseconds to a non-conducting inactivated state through a process called fast inactivation. Transient Na^+ influx through thousands of rapidly opening and inactivating VGSCs results in the familiar transient macroscopic Na^+ current detected in whole-cell voltage clamp studies[16]. This transient current gives rise to the depolarizing phase of the action potential in neurons, and eventually causes the sensation of pain (Figure 2).

Distinct distribution

Expression of the different subtypes of VGSCs is tissue- and/or cell-specific. The main subtypes expressed in adult brain neurons are $Na_v1.6$. $Na_v1.2$ in unmyelinated axons and in myelinated axons early in development (before being replaced by $Na_v1.6$), and $Na_v1.1$ in the neuronal somata. In rodents, $Na_v1.3$ is expressed primarily in embryonic neurons; however, expression in the human central nervous system remains comparatively high into adulthood. $Na_v1.4$ is the main subtype in adult skeletal muscle, whereas $Na_v1.5$ is expressed in cardiac muscle, and it is also expressed in some neurons. In DRG neurons, $Na_v1.6$ is the major sodium channel isoform at the nodes of Ranvier[17], is present in unmyelinated fibers in the sciatic nerve[18,19] and is preferentially expressed by TrkC (tyrosine receptor kinase C) neurons. $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ are found in peripheral primary sensory afferents. They are more abundant in C-fiber neurons[16,20]. Small diameter DRG neurons can be broadly classified into isolectin B4 (IB4)(+) and IB4(-) neurons. James Brock had reviewed sensory and biophysical properties differences between IB4(+) and IB4(-) neurons[21]. Our preliminary experiments showed that most colon specific DRG neurons are $Na_v1.8$ positive (Figure 1B and C).

Developmentally regulated expression

Expression of the different subtypes is developmentally regulated in DRG neurons[22-24]. $Na_v1.3$ is overexpressed at embryonic day E1, peaks at E17, is reduced by postnatal day P15, and is not detectable by P30. $Na_v1.6$ is upregulated from E17 only in large neurons. Expression of both $Na_v1.8$ and $Na_v1.9$ increases with age, beginning at E15 and E17, respectively, and reaching adult levels by P7. Their distribution is restricted mainly to those subpopulations of primary sensory neurons in developing and adult DRGs that give rise to unmyelinated C-fibers (neurofilament 200 negative). $Na_v1.8$ is expressed in a higher proportion of neuronal profiles than $Na_v1.9$ at all stages during development, as in the adult[24]. It is worthy of note that nerve injury would evoke expression of some of these channels that are not present in the sensory nerve system in the adult, and may contribute to abnormal pain sensation[20,25,26].

Expression in glial cells

Na-G, a distinct type of "glial" Na channel expressed at high levels in Schwann cells *in vivo*[27,28], was thought to play a role in regulation of cytoplasmic Na^+ homoeostasis. However, recent studies show that action potential-like events have been recorded in glial precursor cells, astrocytes and a subset of oligodendrocyte precursor cells. More recently, Frieboes reported *de novo* expression of $Na_v1.8$ in endoneurial Schwann cells following injury[10]. This is surprising since $Na_v1.8$ expression is thought to be restricted to sensory neurons. No similar studies have been reported under pathophysiological conditions in visceral organs. Nevertheless, these data indicate a sodium channel in glial cells is linked to the development of neuropathic pain. Further investigation of the underlying molecular basis of VGSCs in glial cells and neural-glial interaction could yield promising targets for treatment of chronic pain.

VGSCS AND CHRONIC VISCERAL PAIN

There are relatively few published studies on roles of sodium channels in visceral pain and a uniform theme has not emerged. Saito *et al*[29] demonstrated a mutation in the cardiac sodium channel gene *SCN5A* in interstitial cells of Cajal (ICC) in irritable bowel syndrome (IBS) patients, suggesting *SCN5A* as a candidate gene in the pathophysiology of IBS. Additionally, Verne *et al*[30] showed there was a reduction in abdominal pain in IBS patients receiving intrarectal lidocaine, indicating a possible normalization of abnormal sodium channels in IBS patients. Yiangou *et al*[31] carried out an immunohistochemistry study on biopsies from patients with idiopathic rectal hypersensitivity which showed that the nerve fibers immunoreactive to the sodium channel $Na_v1.7$ were significantly increased in this group compared with controls. Collectively, these clinical data suggest that voltage-

gated sodium channels appear to play an important role in visceral pain[5,9].

Since no pharmacological tools to separate sodium currents are available, it is hard to relate the channel current to its molecular subtype. However, tetrodotoxin (TTX) is the most used drug to separate the TTX-S (sensitive) and TTX-R (resistant) sodium channel currents. Whole-cell patch-clamp recording of fast blue-labeled bladder afferent neurons demonstrated that the majority (70%) of bladder neurons that were small in size had capsaicin-sensitive TTX-R sodium channels[32,33]. More than 80% of gastric DRG neurons displayed TTX-R action potentials[34]. These data demonstrated that visceral sensory neurons expressed the TTX-R sodium channels that are thought to contribute to pain processing.

Sensitization of VGSCs

Several animal models have been used to determine the role for VGSCs under visceral pain conditions. These included the trinitrobenzenesulphonic acid (TNBS)-induced colitis, painful chronic pancreatitis, and acetic acid-induced gastric ulcers. TNBS-induced colitis significantly enhanced neuronal excitability of colon specific DRG neurons, which is associated with a significant potentiation of slow inactivating TTX-R Na^+ current density in mice[35] and in guinea pigs[36]. Changes in expression of Na_v1.7, 1.8 and 1.9 protein and mRNA during TNBS colitis were measured using Western blotting and quantitative polymerase chain reaction. On day 7 of colitis, there was a 3-fold increase in Na_v1.8 protein in ganglia from $T_{9\text{-}13}$, but Na_v1.7 and 1.9 levels remained unchanged. Surprisingly, there was no corresponding change in the Na_v1.8 α-subunit mRNA levels[37], indicating that the TNBS specifically evoked an increase in the numbers of Na_v1.8 channels, which contributed to an increased current. In a rat model of gastric hyperalgesia, Gebhart and colleagues reported that ingestion of iodoacetamide increased the peak TTX-R sodium current which was associated with a left-shifted voltage-dependent activation in DRG neurons[38]. In a rat model of painful chronic pancreatitis, however, no change in sodium peak current density was observed in pancreas-specific DRG neurons (Xu *et al*[39], unpublished data) although there was an alteration in voltage-gated potassium channel function in the same model. Although the data from different models are complicated and somehow controversial, they point out the fact that changes in function and expression of VGSCs are organ or/and disease specific.

As mentioned previously, no pharmacological tools to separate TTX-R currents are available. However, researchers have employed antisense oligodeoxynucleotide or Na_v1.8 and Na_v1.9 null mice to evaluate their current roles in animal models of visceral hypersensitivity. Inhibition of expression of Na_v1.8 (PN3/SNS) sodium channels by an antisense oligodeoxynucleotide injected intrathecally suppressed bladder hyperactivity and c-fos expression in the spinal cord induced by chemical irritation of the urinary bladder in rats[32]. In *Nippostrongylus brasiliensis* infection-induced transient jejunitis, hyperexcitability was absent in $Na_v1.8^{-/-}$ mice[40]. Further experiments showed that Na_v1.8-null mice displayed normal nociceptive behavior provoked by acute noxious stimulation of abdominal viscera. Na_v1.8-null mice also showed weak pain and no referred hyperalgesia to intracolonic capsaicin and mustard oil[41]. In a model of cyclophosphamide-induced bladder cystitis, Na_v1.8 null mice showed normal responses. These data suggest that Na_v1.8 plays an essential role in mediating spontaneous activity in sensitized nociceptors. However, the role for Na_v1.9 is inconsistent. Hillsley *et al*[40] reported that $Na_v1.9^{-/-}$ mice maintained hyperexcitability in *Nippostrongylus brasiliensis* infection-induced transient jejunitis. On the other hand, Martinez reported that R-848 (the toll-like receptor 7 activator)-induced hypersensitivity was blunt in Na_v1.9-null mice although their normal pain responses were similar to those of wild-type mice[42]. Hence, they suggested that Na_v1.9 channels do not significantly contribute to normal visceral pain responses to acute colonic mechanical stimulation but may be important for the development of inflammation-related visceral hyperalgesic responses. This idea was further supported by a recent report that peripheral nerve recordings from pelvic afferents in Na_v1.9-null mice revealed a lack of sensitization to intravesicularly applied prostaglandin (PG) E2[43]. Again, this points out that the role for Na_v1.9 may be organ and/or disease specific.

Modulation of VGSCs

There is growing evidence to support the hypothesis that inflammatory mediators are involved in the modulation of VGSCs function and expression under pathophysiological states (Figure 3). Among these are well-studied nerve growth factor (NGF) and PGE2. Blockade of NGF and tyrosine kinase receptors inhibits peripheral mechanical sensitivity accompanying cystitis in rats[44]. NGF continuously infused into the intrathecal space at the L6-S1 level of the spinal cord for 1 or 2 wk using osmotic pumps (0.5 μL/h) significantly lowered the threshold for spike activation. In addition, the number of TTX-R action potentials during 600 ms depolarizing pulses was significantly and time-dependently increased[33]. Another inflammatory messenger PGE2 induced a rapid (< 15 s) increase in TTX-R I_{Na} that was associated with a hyperpolarizing shift in the conductance-voltage curve (3.4 ± 0.7 mV), an increase in the rate of inactivation (4.21 ± 0.7 ms at 0 mV), and no change in steady-state availability. These results do suggest that modulation of TTX-R I_{Na} by NGF and PGE2 in colonic afferents is an underlying mechanism of hyperalgesia associated with inflammation of the colon, and that this current constitutes a novel target for therapeutic relief of visceral inflammatory pain[45-47]. Other modulators include, but are not limited to, aquaporins (AQP-1), interleukin (IL)-1β and gaseous modulators/transmitters. AQP-1 may directly interact with Na_v1.8 channels in DRG neurons[48], while IL-1β may act *via* a p38 mitogen-activated protein kinase pathway[49]. Hydrogen sulfide (H_2S), as an endogenous novel third gaseous modulator/transmitter, might directly or indirectly modulate the function of VGSCs since it enhanced excitability of primary sensory neurons,

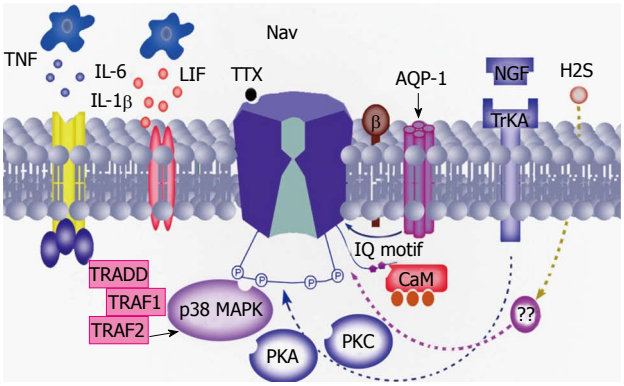

Figure 3 Possible mechanisms underlying the modulation of voltage-gated sodium channel activity. Inflammatory mediators such as nerve growth factor (NGF), prostaglandin (PG) E2, interleukin (IL)-1β may sensitize sodium channels by different intracellular signal transduction pathways such as protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase and calmodulin (CaM). In addition, a recent study showed that aquaporin (AQP)-1 directly interacted with the sodium channel, thus contributing to the perception of pain[48]. TTX: Tetrodotoxin; LIF: leukemia inhibitory factor; Trk: Tyrosine receptor kinase; TNF: Tumor necrosis factor; TRAF: Tumor necrosis factor receptor-associated factor; TRADD: TNF receptor-associated death domain protein.

and its endogenous generating enzyme was upregulated under visceral pain conditions[50].

The regulation of VGSCs by intracellular protein kinases is another important mechanism for maintenance of persistent behavioral hypernociception. Direct phosphorylation of $Na_v1.7$ by pERK1/2 and modulation of $Na_v1.8$ by p38 regulated the gating properties of these channels[49,51]. In addition, functional regulation of the $Na_v1.8$ by PKA and PKCε in the primary sensory neuron is important for the development of the peripheral pro-nociceptive state induced by repetitive inflammatory stimuli and for the maintenance of behavioral persistent hypernociception[52]. Since the $Na_v1.8$ C-terminus carries a conserved calmodulin-binding isoleucine-glutamine motif, it is reasonable to speculate that these two proteins can interact *in vivo*. Indeed, calmodulin demonstrated a regulation of $Na_v1.8$ currents[53]. Unfortunately, our understanding of regulation of VGSCs in visceral pain conditions lags behind our knowledge of mechanisms of somatic pain. Future experiments are warranted to determine whether and how $Na_v1.8$ channels are modulated by inflammatory and stress mediators in the peripheral nerve system.

MANAGEMENT OF VISCERAL PAIN WITH VGSC BLOCKERS

Treating chronic pain remains a significant clinical challenge, and in particular current treatment options for visceral pain are very limited and marginally effective. Na^+ channel blockade is one of the most powerful and best-proven analgesic principles, beginning with the use of local anesthetics for sensory blockade and then with the discovery that Na_v-blocking anticonvulsants also have benefit as pain therapy clinically[5,13,16,54-57]. Based on their mechanism of action, sodium channel blockers are divided into the following categories: (1) Extracellular blockers, which bind to and occlude the extracellular pore of the channel, e.g. TTX and saxitoxin; (2) Intracellular blockers, which block from the intracellular side of the channel, including local anesthetics, class I antiarrhythmic agents, and some anticonvulsants; and (3) Unknown mechanisms of inhibition of the sodium current in ventricular cells of guinea pigs by e.g. caffeine[58], or A-803467, a specific blocker of the $Na_v1.8$ channel (SCN10A)[59].

Among the neuronal Na^+ channel subtypes, $Na_v1.8$ is believed to be of importance for certain visceral pain states, and $Na_v1.8$-preferring channel blockers should be able to relieve pain without causing severe effects (due to the restricted expression of this channel type). The compounds are lidocaine, mexiletine, benzocaine, and ambroxol, which are clinically used to treat pain after local or systemic administration. Of these compounds, ambroxol has been reported to effectively suppress pain symptoms in animal models of chronic, neuropathic and inflammatory pain[60]. The analgesic effects of ambroxol by either systemic administration to animals, or by topical application in humans can be explained by ambroxol-induced blockade of ion channels in peripheral neurons. However, this has not yet been used to treat visceral pain. Pregabalin effectively inhibits TNBS-induced chronic colonic allodynia in the rat[61]. Pregabalin increased distension sensory thresholds to normal levels in IBS patients with rectal hypersensitivity[62]. Although downregulation of the $\alpha_2\delta$ calcium channel subunit may be one of the mechanisms underlying the analgesic effect of pregablin[63], the precise mechanism of pregabalin action is not fully understood. Nevertheless, these findings support the idea that pharmacological substances that modify the function of VGSCs would be potentially useful in treating pain conditions[11]. Further experiments are needed to provide more evidence for physicians to treat visceral pain with these pharmacological substances.

Numerous natural toxins have evolved to target sodium channels, either by blocking current through the pore or by modifying channel gating. Among well-studied toxins, the peptide conotoxins from marine snail venoms and δ-atracotoxin and scorpion venom toxins (e.g. birtoxin) constitute another promising source of such modulators. These peptide toxins are of considerable interest not only as probes for investigating the functioning of ion channels and receptors but also as potential therapeutics for neurological disorders, including neuropathic pain and epilepsy[64,65]. There are three classes of conopeptides that modulate VGSCs: the pore-blocking μ-conotoxins, the δ-conotoxins which delay or inhibit VGSC inactivation, and the μO-conotoxins which inhibit VGSC Na^+ conductance independent of the TTX binding site. Some of these toxins have been evaluated in animal studies and in preliminary clinical studies, but others are highly novel and may develop into potential drugs for the treatment of pain states by engineering a selective μ-conopeptide that has high affinity and efficacy.

It is noted that until now none of the known neurotoxins or small molecule VGSC inhibitors/modulators is

highly selective for a specific VGSC subtype. However, the recent discovery of a genetic link in inherited pain syndromes has advanced our understanding of the contribution of sodium channels to pain in humans and given us hope that it is possible to generate subunit specific blockers. "Gain-of-function" mutations in SCN9A, the gene which encodes $Na_v1.7$, have been linked to two human-inherited pain syndromes, inherited erythromelalgia and paroxysmal extreme pain disorder, while "loss-of-function" mutations in SCN9A have been linked to complete insensitivity to pain[12,66]. Inhibition of expression of $Na_v1.8$ (PN3/SNS) sodium channels by an antisense oligodeoxynucleotide[52] or by $Na_v1.8$ knockdown[40,41] would be useful tools for treatment of chronic visceral pain in the future.

CONCLUSION

An emerging theme that unifies many supposedly diverse functional GI disorders is altered neuronal excitability, caused by abnormal expression and function of membrane ion channels in organ-specific primary sensory neurons. VGSCs as the main determinants of intrinsic neuronal excitability are particularly appealing targets for pharmacological intervention. Other ion channels such as vanilloid receptor, ATP receptors or potassium channels, which are certainly involved in pain processing, have not been discussed in this review. Nevertheless, the advent of new experimental medicine techniques presents us with an opportunity to test the effectiveness of novel medicines in great detail. The ability to perform hypothesis-driven research with tool compounds in targeted patients with demonstrable visceral hypersensitivity is a stimulating prospect. As VGSCs are expressed in the glial cells, future studies should be directed toward understanding whether glia-neuron interaction is involved in the peripheral sensitization of nociceptors under visceral pain conditions. Since Na^+ channel subtypes are expressed in a regionally and temporally specific pattern and current VGSC blockers offer little discrimination between various VGSC subtypes, the development of selective blockers could increase their clinical usefulness. For example, a drug that selectively inhibits the $Na_v1.8$ VGSC, which appears to be crucially and specifically involved in visceral nociception, would presumably act as a novel and powerful analgesic, with few side effects in most indications.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2365

World J Gastroenterol 2011 May 21; 17(19): 2365-2371
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Review of screening for pancreatic cancer in high risk individuals

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Received: September 17, 2010 Revised: December 17, 2010
Accepted: December 24, 2010
Published online: May 21, 2011

Abstract

Pancreatic cancer is difficult to diagnose at an early stage and is associated with a very poor survival. Ten percent of pancreatic cancers result from genetic susceptibility and/or familial aggregation. Individuals from families with multiple affected first-degree relatives and those with a known cancer-causing genetic mutation have been shown to be at much higher risk of developing pancreatic cancer. Recent efforts have focused on detecting disease at an earlier stage to improve survival in these high-risk groups. This article reviews high-risk groups, screening methods, and current screening programs and their results.

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Key words: Pancreatic cancer; Familial cancer; Cancer screening; Risk factors; Endoscopic ultrasound; Hereditary cancer

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Stoita A, Penman ID, Williams DB. Review of screening for pancreatic cancer in high risk individuals. *World J Gastroenterol* 2011; 17(19): 2365-2371 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2365.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2365

INTRODUCTION

Pancreatic cancer is a deadly disease with more than 277 668 new cases and 266 029 deaths worldwide in 2008[1]. The median age at diagnosis is 72 years, with an age-adjusted incidence rate of 11.7 per 100 000 men and women[2]. Unfortunately, mortality rates for pancreatic cancer have not significantly changed for several decades. For example, in the United States, pancreatic cancer is the fourth commonest cancer killer. Although accounting for only 3% of new cases, pancreatic cancer is responsible for 6% of all cancer deaths each year[3]. Most patients with symptomatic pancreatic cancer have advanced and/or metastatic disease at presentation, and as a result, the overall 5-year survival rate is only 5.6%[2]. Even patients with resected, margin-negative pancreatic cancers have only a 22% chance of being alive at 5 years[2,4].

As patients with pancreatic cancer have a median survival of 6 mo from diagnosis, recent efforts have focused on detecting disease at an earlier stage as a way of improving survival. Because the disease is uncommon, and because we lack a simple, cheap and safe, non-invasive test, general population screening programs are neither feasible nor cost-effective. However, 10% of pancreatic cancers result from genetic susceptibility and/or familial aggregation[5]. Individuals from families with multiple affected first-degree relatives with pancreatic cancer, and those with a known cancer-causing genetic mutation have been clearly shown to be at much higher risk of developing pancreatic cancer. Accumulating data suggests that screening of these high-risk groups might be of benefit.

DEFINITION OF HIGH-RISK INDIVIDUALS

Individuals at high risk of developing pancreatic cancer are either part of inherited pancreatic cancer syndromes with a known genetic mutation or are members of a family with at least two first-degree relatives affected by the disease. At present, screening programs are usually targeted at individuals with 5% or greater lifetime risk of developing pancreatic cancer.

Inherited pancreatic cancer syndromes

Peutz-Jeghers Syndrome (PJS) is an autosomal disorder caused by germline mutations in the STK11/LKB1 tumor suppressor gene. These patients are at risk of esophageal, gastric, small intestine, lung, breast, uterine, ovarian and pancreatic cancer. Giardiello *et al*[6] have demonstrated that the risk for pancreatic cancer in patients with PJS is very high, with a relative risk of 132 and the cumulative life time risk by age 65 years of 36%.

Hereditary pancreatitis is a rare autosomal dominant disorder, caused in more than two-thirds of cases by a mutation in SPINK1 gene, with a high risk of pancreatic cancer. In this population, the cumulative risks of pancreatic cancer at age 50 and 75 years are 11% and 49% for men and 8% and 55% for women[7]. In individuals with a paternal inheritance, the cumulative life time risk for pancreatic cancer is as high as 75%[8]. In patients with hereditary pancreatitis, smoking has been shown to double their risk of cancer and lower the age of onset by approximately 20 years[9].

Familial atypical multiple mole melanoma syndrome is an autosomal dominant syndrome caused by a germline mutation in CDKN2A (or p16) gene on chromosome 9p21, or in a minority of cases, in the CDK4 gene on chromosome 12[10,11]. Patients with this syndrome are predisposed to develop multiple (more than 50) atypical nevi and multiple malignant melanomas. The individuals with this syndrome have a 20%-34% relative risk of developing pancreatic cancer and a 16% lifetime risk of pancreatic cancer[12].

Hereditary breast ovarian cancer syndrome is associated with germline mutation in the BRCA2 and BRCA1 genes. It is estimated that BRCA2 germline mutation carriers have a 5% lifetime risk of pancreatic cancer[13]. The risk of pancreatic cancer in BRCA1 mutation is 2.26 times that of the normal population[14]. A characteristic single mutation of 6174delT in the BRCA2 gene has been discovered in high-risk breast ovarian cancer families with pancreatic cancer of Jewish Ashkenazi origin.

Lynch syndrome is an autosomal dominant condition caused by defects in mismatch repair genes (MLH1, MSH2, MSH6 or PMS2). Recently, it has been shown that, in addition to colorectal and endometrial cancers, these individuals have a ninefold increased risk of developing pancreatic cancer compared with the general population[15]. Data on 6342 individuals from 147 families with mismatch repair gene defects have been analyzed and 21% of families reported at least one case of pancreatic cancer[15]. This study showed that the estimated relative risk of pancreatic cancer was higher for younger individuals (aged between 20 and 49 years), and concluded that individuals with Lynch syndrome and a family history of pancreatic cancer should be included in screening programs.

Familial pancreatic cancer

Individuals from families with a strong history of pancreatic cancer have an inherited predisposition of developing the disease themselves. Familial pancreatic cancer (FPC) families have been defined as families with at least two first-degree relatives, suggesting an autosomal dominant penetrance[16]. In addition, expert opinion recommends a subset of these people to be considered high risk and be included into screening: these are individuals with three or more affected family members; one of whom must be a first-degree relative[17]. Families that do not meet these criteria (i.e. families with only one relative with pancreatic cancer or with multiple pancreatic cancers in more distant relatives) are defined as sporadic pancreatic cancer and are not currently considered for screening. Due to the complex nature of pedigrees in pancreatic cancer, in order to take into account the current age of the subject, the age of onset of pancreatic cancer in the family and the exact relationships between family members, a computer-based risk assessment tool, named PancPRO was developed in 2007. The model has been shown to provide an accurate risk assessment for kindreds with familial pancreatic cancer[18], and further studies are underway to validate this. The program is available free of charge as part of UTSW Medical Centre Dallas at and Bayes Medel Group at John Hopkins Cancer Gene package at John Hopkins (http://www4.utsouthwestern.edu/breasthealth/cagene, accessed 15th June 2010).

Unfortunately, the genetic basis for most cases of FPC is not known but studies using segregation analysis have suggested that it is due to at least one rare novel major gene that has not yet been identified[19]. Germline mutation in the BRCA2 gene is present in 6%-16% of patients with pancreatic cancer[20,21]. The association between BRCA1 and pancreatic cancer is weaker but there are reports of pancreatic cancers in documented BRCA1 patients[22]. A mutation in the paladin gene[23] has been described in the affected members from families with FPC under surveillance at Washington University but this has not been confirmed in other studies. The *4q32-34* region has been previously identified as a potential locus for FPC in a large American family, however, this locus seems unlikely to harbor an FPC gene in European families[24]. Sequence analysis in FPC has revealed six genes previously described in neoplasia/carcinogenesis: *Apolipoprotein A4, CEA, Keratin 19, Stratifin (14-3-3 sigma), Trefoil Factor,* and *Calcium Binding Protein S100 A6*. The pattern of frequency suggests that these genes are associated with conditions that produce significant desmoplastic responses and are difficult to differentiate from chronic inflammatory processes. *Apolipoprotein A4* is preferentially expressed in familial patients, suggesting the importance of fatty acid synthesis in carcinogenesis but this requires further investigations[25]. A more recent study has identified the PALB2 gene as a pancreatic cancer susceptibility gene[26].

Even without knowing the gene that causes FPC, the

risk of pancreatic cancer in these kindreds has been estimated. Prospective analysis of incident pancreatic cancers in Johns Hopkins' National Familial Pancreas Tumor Registry kindreds, performed as part of the Johns Hopkins GI SPORE study, has demonstrated that the relative risk of pancreatic cancer in persons with two affected first-degree relatives is 6.4% and the cumulative life time risk is 8%-12%. In individuals with three affected first-degree relatives, the relative risk for pancreatic cancer is 32% and the cumulative life time risk is 16%-32%[19]. People with a pair of relatives related by first degree have an 18-fold increase in risk and an estimated life time risk of 9%-18%[27].

The age of onset in pancreatic cancer kindreds appears to be important. The risk of pancreatic cancer is higher among members of FPC kindreds with a young onset (age < 50 years)[28]. Risk survival analysis shows that the life time risk of pancreatic cancer in FPC kindreds increases with decreasing age of onset in the kindred. Interestingly, this was not true for sporadic pancreatic cancer. For example, the life time (by age 80 years) risk of pancreatic cancer was 15.7% for individuals with one or two first-degree relatives with pancreatic cancer who came from a family in which one of the members of the family was diagnosed at age 40 years. In this family history group, the cumulative incidence of pancreatic cancer decreased to 7.1% when the youngest age of onset of pancreatic cancer was at 60 years and to 3.1% when the youngest age was at 80 years. The life time risk rose to 38.9% for individuals with three first-degree relatives with pancreatic cancer who came from a family in which one of the members of the family was diagnosed at age 40 years[28]. Smoking is a strong risk factor in FPC kindred, particularly in men and people younger than 50 years of age, because it increases the risk of pancreatic cancer by 2-3.7 times over the inherited predisposition and lowers the age of onset by 10 years[29].

CANCER SEQUENCE

There are three known precursor lesions to pancreatic cancer: intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasia (MCN) and pancreatic intra-epithelial neoplasia (PanIN). Main duct IPMN has a higher frequency of malignancy (range: 60%-92%, mean 70%) compared with branch duct IPMN (range: 6%-46%, mean 25%)[30]. PanIN is by far the commonest lesion and three grades of PanIN have been described as cellular atypia progresses from low-grade dysplasia (PanIN-1) to high-grade dysplasia (PanIN-3). Molecular studies have revealed K-ras activation in PanIN-1, inactivation of p16 in PanIN-2 and inactivation of p53, DPC4, and rarely BRCA2 mutation in PanIN-3 and invasive adenocarcinoma[31,32]. Non-invasive multifocal precursor lesions, such as PanINs and IPMNs, are more common in patients with a strong family history of pancreatic cancer than in patients with sporadic disease, and precursor lesions are of a higher grade in patients with a strong family history of pancreatic cancer[33]. Studies on pancreases resected from patients with FPC have shown that precursor lesions, even the low-grade PanIN-1 lesions, are often directly associated with lobular atrophy of the surrounding pancreatic parenchyma[34]. In addition, animal studies have shown that it is not the atrophy that causes the PanINs to develop, but rather that it is the PanINs which develop first, producing multiple foci of small duct obstruction, which in turn progress to multifocal lobulocentric atrophy[33,35]. The lobular atrophy associated with these precursor lesions provides an explanation for the chronic pancreatitis-like changes seen in these pancreases. The multifocality of the precursor lesions suggests that an inherited mutation of a gatekeeper gene is responsible for some cases of FPC.

Table 1 Potential pancreatic cancer biomarkers under study %

Biomarker	CEACAM-1	Span-1	DUPAN-2	MIC-1	Alpha4GnT	PAM4
Sensitivity	85	81-94	48-80	90	76	77
Specificity	98	75	75-85	62	83	95

MIC-1: Macrophage inhibitory cytokine-1; CEACAM-1: Carcinoembryonic antigen-related cell adhesion molecule 1; SPan-1: Monoclonal antibody; DUPAN 2: Pancreatic cancer associated antigen; Alpha4GnT: alpha1,4- N-acetylglucosaminyltransferase; PAM4: Anti-MUC1 monoclonal antibody.

While main duct IPMN and large MCN can be detected by computer tomography (CT) and magnetic resonance imaging (MRI), small MCN, branch duct IPMN and chronic pancreatitis like changes are best detected by endoscopic ultrasound (EUS).

SCREENING METHODS

As pancreatic cancer has such poor survival, the aim of screening is to detect precursor lesions or early pancreatic cancers. A study from the Japanese National Pancreatic Cancer Registry on operated pancreatic cancers has shown that stage I tumors less than 2 cm have a much better survival (58% alive at 5 years) compared with stage IIb (17% alive)[36]. Even more, another Japanese study showed 100% survival in 79 patients with tumors less than 1 cm undergoing curative resection[37].

Unfortunately, premalignant lesions and small pancreatic cancers are asymptomatic. Therefore, screening programs have tried to use biomarkers and imaging tests to identify these early lesions. There are no validated biomarkers in use for detection of early pancreatic cancer. CA19-9 is neither specific nor sensitive enough for reliable detection of early pancreatic cancer or pancreatic precursor lesions. In a study of 71 000 asymptomatic patients undergoing abdominal ultrasound, CA19-9 was found to have a positive predictive value less than 1% using a cut off value of 37 U/mL[38]. Currently, many biomarkers have been researched but none is yet in routine clinical use (Table 1)[39].

Fasting blood glucose has been shown to be a marker for early cancer in sporadic cases[40] and is currently used by EUROPAC study in high-risk individuals. Molecular analysis of pancreatic juice for K-ras mutation, p53 mutation and p16 promoter methylation is also evaluated in the EUROPAC study.

EUS is the main imaging modality for screening these high-risk individuals. In addition to EUS, other imaging modalities have been used in different protocols such as computed tomography (CT), magnetic resonance imag-

ing (MRI) magnetic resonance cholangiopancreatography (MRCP), MRI with secretin, and endoscopic retrograde cholangiopancreatography (ERCP).

EUS has been shown to be the best modality to stage pancreatic cancers (sensitivity of 84% and specificity of 97%)[41] and for diagnosing small tumors (Figure 1) and IPMN (Figure 2). EUS fine needle aspiration (FNA) allows cytological sampling of abnormal areas and has an accuracy of 92%[42]. Multifocal non-invasive epithelial precursor lesions (PanINs, small IPMN) are associated with chronic pancreatitis-like changes, including abnormalities of the ducts (ectasia, irregularity, saccules) and parenchyma (heterogeneity, lobularity) and are best observed at EUS. Two thirds of patients screened in the CAPS 1 and CAPS 2 studies were found to have these chronic pancreatitis changes by EUS and ERCP[43,44]. These changes correlate with markers of neoplasia including abnormal DNA methylation in the pancreatic juice[45], and were significantly more common and more severe in individuals from FPC kindreds than in controls, even after adjusting for age and alcohol exposure. In addition, Canto *et al*[43] have reported high-grade PanINs and invasive adenocarcinoma arising in patients with benign IPMN, suggesting that in this high-risk cohort, IPMN can be a marker of a field effect of neoplasia.

CT and MRI remain the standard of care modalities to identify pancreatic cancers larger than 2 cm. CT use for screening has been discouraged because it is associated with delivering ionizing radiation (10mSv) to patients who most likely have a DNA repair defect. MRI with secretin has better results than MRI and MRCP but it is still unable to visualize very small lesions and can be associated with movement artefacts and claustrophobia. John Hopkins researchers have compared EUS with CT and MRI/MRCP in detecting FPC. MRI/MRCP is superior to CT particularly for detecting IPMNs (71% *vs* 14%) and EUS detected twice as many neoplastic lesions as CT/MRCP[46]. Some centers have used ERCP to detect saccular dilatation in the pancreatic duct said to be associated with PanIN lesions[44]. However, its use must be weighed against the potential risk of procedure-related acute pancreatitis (3%-5%).

Genetic counseling is an essential part of all current screening programs, and practice recommendations about counseling these people about their individual risk, possible preventive and surveillance measures were made at the Fourth International Symposium of Inherited Diseases of the Pancreas in 2007[17].

REVIEW OF SCREENING PROGRAMS

Currently, there are multiple international programs that screen for pancreatic cancer in high-risk individuals in a research setting. The optimal approach to screening for early pancreatic neoplasia has not yet been established and each protocol is slightly different, but all use EUS as the main imaging modality.

The largest program is led by John Hopkins University and involves 24 American Centers of Excellence (CAPS study). The results from the CAPS 1 and CAPS 2 studies have been published and they show that early pancreatic neoplasia can be detected through a screening program in asymptomatic patients. In the CAPS 1 study, 36 patients were screened using only EUS. Six masses were found and these patients went to surgery. The histology showed that two of them had malignant and premalignant lesions (invasive adenocarcinoma and IPMN). In this study, the diagnostic yield of screening was 5.3% (Table 2). Most importantly, the patient with pancreatic cancer is still alive and disease free more than 5 years after surgery[44].

Table 2 Results of screening programs for pancreatic cancer in high-risk groups

Study	CAPS 1[44]	CAPS 2[43]	Washington[47,48]	FaPaCa[49]	Dutch study[50]
Yield of screening	5.30% (2/38)	10% (8/78)	13% (10/75)	1.30% (1/76)	23%[1] (10/44)

[1]7% diagnostic yield of pancreatic cancer and 16% diagnostic yield of premalignant IPMN lesions. CAPS: Cancer of the Pancreas Screening Study; FaPaCa: Familial Pancreatic Cancer Study Germany.

The CAPS 2 study found a 10% diagnostic yield of screening for pre-invasive malignant lesions. In this study, screening was performed using annual EUS and CT. If an abnormality was detected, ERCP was offered. Seventy-eight high risk patients (72 from FPC kindreds with three or more affected members, six PJS) and 149 control patients were studied. Of these, eight patients had confirmed pancreatic neoplasia by surgery or FNA (10% yield of screening): six patients had IPMN with diffuse multifocal PanIN lesions, one had an IPMN with invasive ductal adenocarcinoma, and one had high-grade pancreatic intraepithelial neoplasia (PanIN-3). Five patients had complications (pancreatitis) following ERCP and one patient developed metastatic pancreatic cancer while under surveillance. EUS and/or CT also diagnosed three patients with extra-pancreatic neoplasms (ovarian cancer, renal cancer, ovarian cystadenoma) suggesting that the diagnostic yield for screening and the potential clinical impact may be even greater[43]. The CAPS 3 study is a multicentric prospective controlled cohort study that involves annual screening using EUS and MRCP, MRI with secretin and a panel of candidate DNA and protein markers in serum and pancreatic juice [CA19-9, macrophage inhibitory cytokine-1 (MIC-1), DNA hypermethylation, and KRAS gene mutations] as indicators of pancreatic neoplasms. The study has recently been completed and the results are awaited.

Another well-established formal screening program in high-risk individuals is at the University of Washington. Subjects are screened using EUS and the screening begins 10 years prior to the earliest pancreatic cancer death in the family. Patients with normal EUS findings are followed up with repeat EUS at 2-3 year intervals depending of the age when the youngest affected relative had pancreatic cancer. The subjects with abnormal EUS are referred for ERCP and if this is abnormal, patients are offered surgery (laparoscopic resection of the tail to look for PanIN)[47]. Patients with abnormal EUS but normal ERCP are offered annual EUS. Out of 75 subjects screened, 15 had abnormalities on EUS and ERCP and went to surgery. The his-

Figure 1 Radial endoscopic ultrasound image of a small (12 mm) early pancreatic cancer, seen as an irregular hypoechoic mass lesion in the pancreatic head.

Figure 2 Endoscopic ultrasound image of 17 mm × 7 mm main-duct type intraductal papillary mucinous neoplasm. The duct is markedly dilated and villous papillary projections can be seen arising from the duct wall. Pathology after surgical resection showed high-grade dysplasia.

tology revealed premalignant lesions in all: PanIN-3 in 10 cases and PanIN-2 in five[48]. This would give a diagnostic yield of 13% for detecting premalignant lesions. Unfortunately, one patient developed unresectable pancreatic cancer while under annual surveillance[16].

In Europe, a European Registry for Familial Pancreatic Cancer and Hereditary Pancreatitis (EUROPAC) has been created specifically to screen high-risk individuals and this uses EUS, ERCP and molecular analysis of the pancreatic juice looking for early mutations (mt p53, K-ras, P16). The results have not yet been published. A German Study (FaPaCa) enrolled 76 patients in a screening program using yearly EUS, MRCP and laboratory tests (genetic analysis of CDKN2a and BRCA2 gene, Ca 19-9 and CEA). Any suspicious lesion had a control EUS ± FNA after 6 wk and a close follow up at 12 wk. If changes were detected, the patient would undergo operative exploration with intraoperative ultrasound, limited pancreatic resection with frozen section, and if cancer was detected, total pancreatectomy. Ten solid lesions were seen on EUS but only seven could be detected by MRCP. Out of the seven patients, six had limited resections and the histology showed PanIN-3 in one patient, PanIN-2 in one, and PanIN-1 in one, and three benign lesions. These results gave a diagnostic yield of 1.3% in detecting PanIN-3, which is much lower than in previous studies[49].

Given the low yield and taking into consideration the high cost and psychological stress, the German researchers concluded that screening is not justified. These results may be explained by the fact that they were screening a large number of patients who had only a moderate risk of pancreatic cancer (58% of the patients) as they had only two affected family members. The study is ongoing as they still have 17 patients with EUS abnormalities under close surveillance. A recent study from The Netherlands that used only EUS as the first screening modality in 44 high-risk asymptomatic subjects showed a 7% diagnostic yield for asymptomatic cancers and 16% diagnostic yield for premalignant lesions (IPMN like lesions)[50].

Most programs continue screening with yearly EUS if no abnormalities are detected. Pancreatic cancer screening programs have reported high compliance rates (64%-94%)[51]. A recent study has found that participation in a pancreatic cancer screening program does not lead to a significant increase in risk perception, cancer worry or general distress[52]. Data from John Hopkins shows that patients have found genetic counseling before screening very helpful and these patients perceive their personal cancer risk to be high and would seek predictive genetic testing if it were available[53].

COST-EFFECTIVENESS

Two studies have looked at cost-effectiveness of screening in high-risk individuals. In the first, a decision analysis was used to compare one time screening for pancreatic dysplasia with EUS to no screening. Abnormal EUS findings were confirmed by ERCP and patients with abnormalities were offered total pancreatectomy. The life time medical costs and life expectancy were compared (assuming a 20% incidence of dysplasia and a 90% sensitivity of EUS and ERCP). The study concluded that screening was cost-effective with an incremental cost-effectiveness ratio of $16 885/life-year saved[54]. Screening remained cost-effective as long as the prevalence of dysplasia was > 16% and the sensitivity of EUS was > 84%. Rubenstein *et al*[55] have performed a clinical and economic evaluation of EUS for patients at risk for familial pancreatic adenocarcinoma using a Markov model. In his model "Do nothing strategy" provided the lowest cost, the greatest remaining years of life and greatest quality adjusted life years, whereas total pancreatectomy provided the longest life expectancy if the lifetime risk of pancreatic was at least 46%.

FUTURE DIRECTIONS

In the near future, screening of high-risk patients may incorporate biomarkers with high specificity and sensitivity in addition to EUS imaging. Contrast-enhanced EUS may also be a promising technique to differentiate between focal pancreatitis and malignancy[56] but it remains to be seen if it will be useful in further characterizing lesions in high-risk individuals. The use of neural network analysis of dynamic sequences of EUS-elastography is a promising new technique

that may provide important additional information in distinguishing benign and malignant pancreatic lesions. Further investigations are required to determine if this technique can guide EUS-FNA to regions most likely to harbor cancer and to exclude cancer in patients with a low probability[57]. More work is required to determine if laparoscopic resection of the pancreatic tail in patients with imaging abnormalities is reliable in detecting PanIN changes, and hence, in establishing real future cancer risk that enables subsequent recommendations for total pancreatectomy.

CONCLUSION

Current screening programs have shown that premalignant lesions and early cancers can be detected by screening but two interval cancers have been missed. The risk of cancer in these groups is sufficiently high to perform screening but methodology needs to be improved. Based on these limited data, the estimated diagnostic yield and potential clinical impact of screening for early pancreatic neoplasms appears to exceed that for other standard indications such as breast cancer screening in high-risk women (95% of women with abnormal mammograms do not have breast cancer)[58]. As we learn more about the natural history of premalignant lesions, studies with long-term follow-up are needed. Given the complexities involved, it seems essential that screening of this cohort is coordinated in a multidisciplinary setting and should take place in a limited number of centers with appropriate expertise and facilities.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2372

World J Gastroenterol 2011 May 21; 17(19): 2372-2378
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Surgicopathological classification of hepatic space-occupying lesions: A single-center experience with literature review

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Received: October 12, 2010 Revised: February 26, 2011
Accepted: March 5, 2011
Published online: May 21, 2011

Abstract

Accompanying rapid developments in hepatic surgery, the number of surgeries and identifications of histological types of primary hepatic space-occupying lesions (PHSOLs) have increased dramatically. This has led to many changes in the surgicopathological spectrum of PHSOLs, and has contributed to a theoretical basis for modern hepatic surgery and oncological pathology. Between 1982 and 2009 at the Eastern Hepatobiliary Surgery Hospital (EHBH) in Shanghai, 31 901 patients underwent surgery and were diagnosed as having a PHSOL. In this paper, we present an analysis of the PHSOL cases at the EHBH for this time period, along with results from a systematic literature review. We describe a surgicopathological spectrum comprising more than 100 types of PHSOLs that can be stratified into three types: tumor-like, benign, and malignant. We also stratified the PHSOLs into six subtypes derived from hepatocytes; cholangiocytes; vascular, lymphoid and hemopoietic tissues; muscular, fibrous and adipose tissues; neural and neuroendocrine tissues; and miscellaneous tissues. The present study provides a new classification system that can be used as a current reference for clinicians and pathologists to make correct diagnoses and differential diagnoses among various PHSOLs.

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Key words: Liver tumors; Tumor-like lesions; Pathology; Immunohistochemistry; Classification

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Cong WM, Dong H, Tan L, Sun XX, Wu MC. Surgicopathological classification of hepatic space-occupying lesions: A single-center experience with literature review. *World J Gastroenterol* 2011; 17(19): 2372-2378 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2372.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2372

INTRODUCTION

Liver neoplasms are one of the most common tumors worldwide, especially in China and other developing countries. Rapid developments in liver surgery and liver pathology have led to many new types of primary hepatic space-occupying lesions (PHSOLs) being surgically resected and pathologically diagnosed, which has greatly increased the surgicopathological spectrum of PHSOLs. Indeed, insights into tumor pathological characteristics have illuminated the need for an improved practical guide for oncological clinicians and pathologists to make correct diagnoses and differential diagnoses among PH-

SOLs[1]. However, to the best of our knowledge, there is no report in the English literature that thoroughly assesses the whole spectrum of PHSOLs.

During the period from January 1982 to December 2009, 31 901 surgically resected PHSOLs were deposited in the archives of the Department of Pathology, Eastern Hepatobiliary Surgery Hospital (EHBH) in Shanghai. In this paper, we present an analysis of the above 31 901 PHSOL cases, along with results from a systematic literature review. To the best of our knowledge, this is the largest series of PHSOLs presented from a single center. Based on the EHBH archival data and literature reviews using MEDLINE and PUBMED, more than 100 types of PHSOLs have been described. In this article, we suggest a surgicopathological classification of PHSOLs comprising three types: tumor-like PHSOLs, benign PHSOLs, and malignant PHSOLs. We also stratified the PHSOLs into six subtypes: lesions derived from hepatocytes; cholangiocytes; vascular, lymphoid and hemopoietic tissues; muscular**,** fibrous and adipose tissues; neural and neuroendocrine tissues; and miscellaneous tissues.

TUMOR-LIKE PHSOLS

Tumor-like PHSOLs are usually a type of space-occupying lesion within the hepatic parenchyma or intrahepatic bile ducts, but without a truly neoplastic nature. At least 31 kinds of tumor-like PHSOLs have been reported, as summarized in Table 1[2-25]. In the EHBH series, tumor-like PHSOLs accounted for 4.3% (n = 1370) of the 31 901 cases. Of the tumor-like PHSOLs, focal nodular hyperplasia (FNH) accounted for 51.5% (n = 705), solitary necrotic nodules accounted for 19.6% (n = 269), and hepatic inflammatory pseudotumors (HIP) accounted for 12.0% (n = 165). These are the three most common tumor-like PHSOLs.

In the latest edition of the World Health Organization (WHO) classification report (2010 Edition), FNH and HIP were grouped as benign liver tumors[26]. However, most scholars, and the present authors, prefer to regard FNH and HIP as a kind of non-neoplastic lesion or a tumor-like lesion[6,27]. FNH is a regenerative hepatocellular nodule that is frequently related to factors that stimulate the hyperperfusion of either the artery or the portal vein. Clonal analysis using the human androgen receptor locus test demonstrated the reactive polyclonal nature in 50%-100% of the FNH cases. Genetic analysis of FNH failed to identify somatic gene mutations that occurred in hepatocellular adenoma (HCA)[28]. Currently, most FNH are considered as polyclonal, and there was neither recurrence nor substantiated malignant transformation in all 705 FNH cases included in the EHBH series after surgery, even though FNH may occasionally coexist with hepatocellular carcinoma (HCC)[29].

Either clinically or pathologically, FNH should be distinguished from other hepatocellular nodules, such as HCA and highly differentiated HCC. Hepatocyte paraffin 1 (Hep Par 1) and polyclonal carcinoembryonic antigen (CEA) are special hepatocellular markers, which cannot, however, differentiate between benign and malignant natures; therefore, we prefer to use CD34 immunostaining to sensitively and specifically outline microvasculatures to differentiate hepatocellular nodules[6,30,31]. FNH usually presents in a focal distribution pattern of microvasculatures around fibrous scars (Figure 1A and B), whereas HCA shows a chaotic distribution pattern, usually with thin-walled vascular staining (Figure 1C and D). HCC presents in a diffuse distribution pattern occupying a greater proportion of the lesion area (Figure 1E and F). Although glypican-3 (GPC-3) has recently been reported to be overexpressed in HCC, the lack of GPC-3 immunostaining could not exclude the diagnosis in at least 25%-30% of HCC[32].

Table 1 Histological classification of tumor-like primary hepatic space-occupying lesions

Hepatocellular lesions
Focal nodular hyperplasia[2]
Nodular regenerative hyperplasia[2]
Partial nodular transformation[3]
Adenomatoid hyperplasia (dysplastic nodules)[2]
Compensatory lobar or segmental hyperplasia[4]
Focal fatty change[2]
Accessory lobe[5]
Bile duct lesions
Biliary microhamartoma (Von Meyenburg complex)[2]
Cyst and polycystic liver[6]
Ciliated foregut cyst[7]
Epidermoid cyst[8]
Endometrial cyst[9]
Intrahepatic peribiliary gland cyst[2]
Mesothelial cyst[10]
Cystic echinococcosis[11]
Biloma[12]
Miscellaneous lesions
Mesenchymal hamartoma[2]
Inflammatory pseudotumor[2]
Pseudolymphoma[13]
Solitary necrotic nodule[14]
Peliosis hepatis[15]
Hereditary hemmorrhagic telangiectasia[16]
Sarcoidosis[17]
Nodular extramedullary hematopoiesis[18]
Abscess[19]
Tuberculoma[20]
Botryomycosis[21]
Malacoplakia[22]
Ectopic tissue[23] and adrenal rest tumor[24]
Pseudolipoma[2]
Granulomas[25]

BENIGN PHSOLS

At least 30 types of benign PHSOLs have been reported, as summarized in Table 2[2,33-56]. In the EHBH series, benign tumors accounted for 12.1% of the cases (n = 3847), among which hepatic cavernous hemangioma (n = 3191, 82.9%), hepatic angiomyolipoma (HAML, n = 153, 4.0%), and HCA (n = 148, 3.8%) were the most frequent types in this group.

Table 2 Histological classification of benign primary hepatic space-occupying lesions

Hepatocellular tumors
Hepatocellular adenoma[2] and hepatic adenomatosis[33]
Intrahepatic bile duct tumors
Bile duct cystadenoma[2]
Intraductal papillary neoplasm[34] and intraductal papillomatosis[2]
Bile duct adenoma[2]
Biliary adenofibroma[35]
Vascular and lymphoid tumors
Cavernous hemangioma[2]
Perivascular epithelioid cell tumor[36]
Hemangioblastoma[37]
Infantile hemangioendothelioma[2]
Lymphangioma and lymphangiomatosis[2]
Muscle, fibrous and adipose tumors
Angiomyolipoma[2]
Leiomyoma[38]
Solitary fibrous tumor[2]
Lipoma[39]
Myelolipoma[40]
Neuronal and neuroendocrine tumors
Neurilemmoma[41]
Plexiform neurofibroma[42] and plexiform neurofibromatosis[43]
Paraganglioma[44]
Pheochromocytoma[45]
Gastrinoma[46]
Vascoactive intestinal peptide tumor[47]
Somatostatinoma[48]
Miscellaneous tumors
Teratoma[49]
Mesothelioma[50]
Endometrioma[51]
Chondroma[52]
Myxoma[53]
Langerhan's cell histiocytosis[54]
Desmoplastic nested spindle cell tumor[55]
Spongiotic pericytoma[56]

In Western countries, patients with HCA or hepatic adenomatosis are mostly estrogen/androgen dependent types, with a female gender bias (> 90%). Among them, 78% have a history of taking contraceptive drugs, and 4% to 4.7% may develop HCC[57]. However, the 148 cases of Chinese HCA in the EHBH series were the spontaneous type with a female:male ratio of 1:2.2. Recently, HCA has been categorized into three molecular subgroups including those with: (1) hepatocyte nuclear factor 1α mutations; (2) β-catenin mutations; and (3) no mutation, with or without inflammatory infiltrates. HCA with a β-catenin mutation has a risk odds of malignant transformation of 46%[58]. It has also been reported that 4% to 17.6% of HCA may have had histologically confirmed malignant transformation[58]. However, after being followed up for more than 5 years after surgery, there was neither a recurrence nor malignant transformation in all 148 cases of HCA in the EHBH series. No case, so far, has had recurrence or tumor canceration, suggesting that Chinese patients with HCA may have differences in etiology, genetics, and HCA-related HCC risk, compared to Western countries.

The above research suggests that the detection of molecular biological or immunohistochemical markers before or after surgery is essential for providing an active radical radiotherapy cure. In addition, more attention should be paid to the careful follow-up of patients with a high potential for transformation of β-catenin activated HCA to prevent HCA transformation or recurrence[26]. Thus, the treatment roadmap based on HCA molecular characteristics has also been described[59].

In 1993, we reported the first case of primary HAML in China. During the last 3 years of the study period, 85 cases of primary HAML and 66 cases of HCA were surgically resected at the EHBH. HAML is generally considered as a miscellaneous benign tumor; however, we find that some cases of HAML can show doubtful growth patterns, such as multi-focus, boundary infiltration along the sinusoids (Figure 2A and B), or even intravascular aggregation of conspicuous HMB45 positive cells (Figure 2C and D), which are similar to malignant behaviors. However, none of the 153 cases of HAML in the EHBH series showed evidence of malignant transformation or postoperative recurrence up to the time of the termination of this study. The presence of malignant HAML or malignant transformation of HAML[60,61] indicates that surgical excision should be considered as a preferred therapeutic, and a long-term follow-up after liver surgery is needed.

MALIGNANT PHSOLS

At least 41 malignant PHSOLs were reported, as summarized in Table 3[2,60-92]. In the EHBH series, malignant PHSOLs accounted for 83.6% (n = 26 684) of the cases, among which, HCC (n = 24 075, 90.2%) and intrahepatic cholangiocarcinoma (ICC, n = 2188, 8.2%) were the two most common malignant tumors. In contrast, undifferentiated embryonal sarcoma (UES, n = 34, 0.1%) and hepatoblastoma (HB, n = 33, 0.1%) ranked third, with a similar incidence.

Histopathologically, HCC, which comprises more than 10 histological varieties[6], is always the central point of differentiated diagnoses among PHSOLs and metastatic tumors. We propose that CD34 immunostaining is one of the most effective methods to distinguish well-differentiated HCC from benign hepatocellular tumors (Figure 1)[6,26]. When HCC appears as a tubular-like arrangement, with solid nest structures and a pseudoglandular pattern, it is difficult to distinguish from ICC or metastatic adenocarcinomas. Based on scanning a panel of immunohistochemical markers, we propose that, for the diagnosis of HCC, Hep Par 1, CD34, and polyclonal CEA are first-line antibodies, and CK19 and MUC-1 are first-line antibodies for ICC[30,31].

UES is a unique hepatic malignant tumor that usually affects the pediatric population. To the best of our knowledge, only 70 cases of UES in adults have been reported worldwide[75,93]. Histologically, UES is characterized by a huge hemorrhagic mass and is composed of pleomorphic cells with eosinophilic cytoplasmic globules entrapped in a loose myxoid stroma[75]. Among 34 cases of UES in

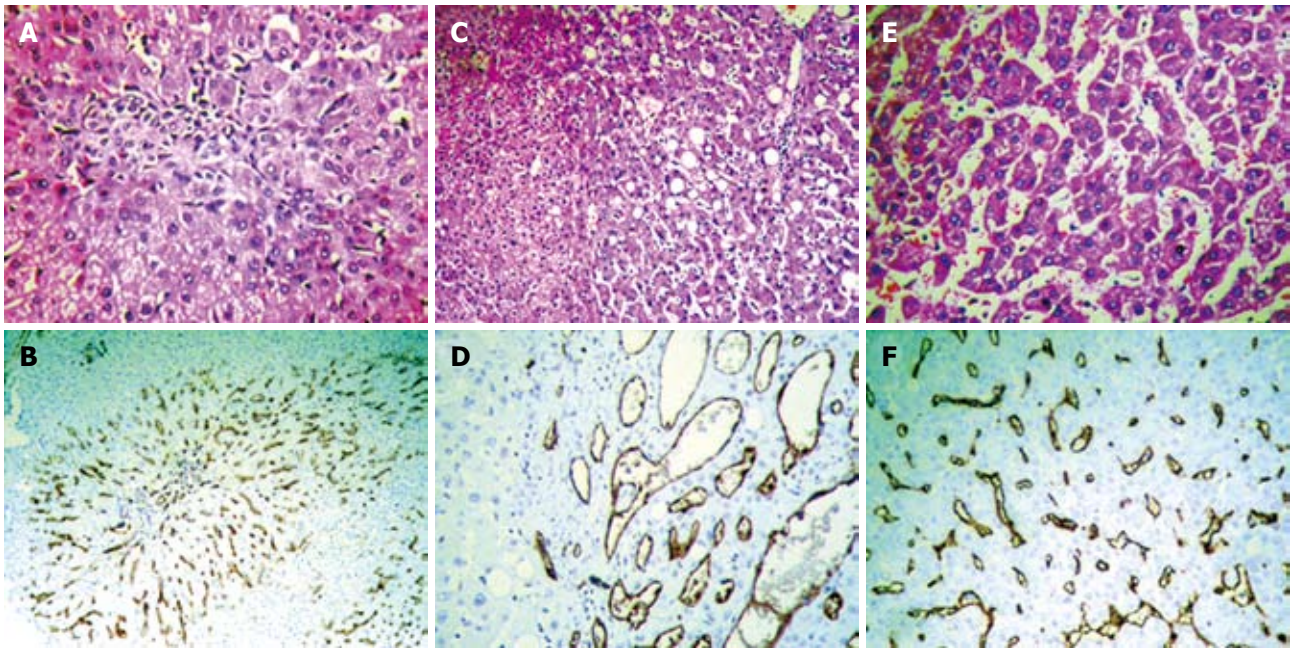

Figure 1 Atypical focal nodular hyperplasia with minimal fibrous septa (A, HE stain, × 200) shows focal microvessels around the periphery of the fibrous septa (B, CD34 immunostaining, × 200). Hepatocellular adenoma is composed of benign-looking hepatocytes with mild steatosis, without a capsule around the periphery (C, HE stain, × 200), and shows a chaotic microvessel distribution pattern with thin-walled vascular staining (D, CD34 immunostaining, × 200). Highly differentiated hepatocellular carcinoma is arranged in a thin trabecular pattern (E, HE stain, × 400) and shows a sinusoidal capillarization pattern (F, CD34 immunostaining, × 200).

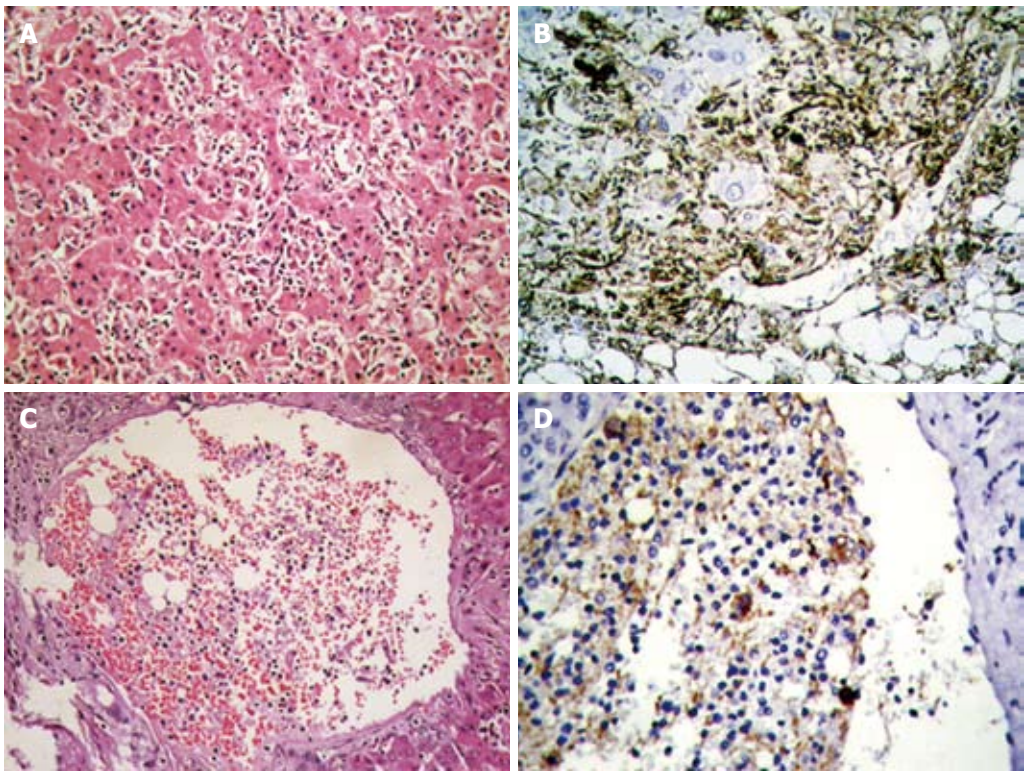

Figure 2 Infiltration of neoplastic cells of hepatic angiomyolipoma within the hepatic sinusoid (A, HE stain, × 200) with strong HMB45 positive staining (B, immunostaining, × 200), and within a branch of the portal vein (C, HE stain, × 100) with strong HMB45 positive staining (D, immunostaining, × 200).

the EHBH series, 32.4% (n = 11) and 29.4% (n = 10) occurred in patients less than 12 and older than 50 years of age (range 5-70 years), respectively, and 32.4% (n = 11) had hepatitis B virus (HBV) infection, suggesting a possible causal link between chronic HBV infection and UES development.

Table 3 Histological classification of malignant primary hepatic space-occupying lesions

Hepatocellular tumors
Hepatocellular carcinoma[2]
Hepatoblastoma[2]
Combined hepatocellular and cholangiocarcinoma[2]
Intrahepatic bile duct tumors
Intrahepatic cholangiocarcinoma[2]
Cholangiolocellular carcinoma[62]
Bile duct cystadenocarcinoma[2]
Biliary rhabdomyosarcoma[63]
Solid-pseudopapilary tumor[64]
Vascular, lymphoid and haemopoietic tumors
Angiosarcoma[2]
Malignant angiomyolipoma[60]/malignant perivascular epithelioid cell tumor[61]
Malignant hemangiopericytoma[65]
Epithelioid hemangioendothelioma[2]
Kaposi's sarcoma[2]
Lymphoma[2]
Follicular dendritic cell sarcoma/tumor[66]
Extramedullary plasmacytoma[67]
Muscle, fibrous and adipose tumors
Leiomyosarcoma[68]
Rhabdomyosarcoma[69]
Fibrosarcoma[70]
Malignant fibrous histocytoma[71]
Liposarcoma[72]
Neuronal and neuroendocrine tumors
Carcinoid tumor[73]
Malignant neurilemmoma[74]
Miscellaneous tumors
Undifferentiated embryonal sarcoma[75]
Undifferentiated carcinoma[76]
Carcinosarcoma[2]
Lymphoepithelioma-like carcinoma[77]
Squamous cell carcinoma[78]
Germ cell tumor[79]
Chorioepithelioma[80]
Yolk sac tumor[81]
Immature teratoma[82]
Malignant rhabdoid tumor[83]
Malignant mesothelioma[84]
Synovial sarcoma[85]
Epithelial-myoepithelial carcinoma[86]
Gastrointestinal stromal tumor[87]
Osteosarcoma[88]
Osteoclast-like giant cell tumor[89]
Desmoplastic small round cell tumor[90]
Nested stromal-epithelial tumor[91]/ossifying stromal epithelial tumor[92]

The incidence of primary hepatic lymphoma (PHL, 0.09%, $n = 23$) was similar to that of UES and HB (0.1%). It has been reported that hepatitis C virus (HCV) plays a role in the pathogenesis of lymphoma, with an HCV prevalence rate of 9% to 42%, especially in Western countries[94]. In contrast, the prevalence of HCV in our patients with PHL was only 4.3% (1 of 23 cases), whereas 56.5% (13 of 23) were positive for HBV, and three of them underwent surgical resections for simultaneous coexistence of PHL with HCC as two independent masses in the liver. Thus, we hypothesize that HBV, as a kind of lymphotropic virus, may play an important pathogenic role in the development of PHL in China.

CONCLUSION

In summary, based on the large number of surgically resected PHSOLs in the EHBH series, we propose a comprehensive surgicopathological classification system that comprises more than 100 kinds of PHSOLs, with three basic types and six subtypes. Our classification system covers all the entities in the new histological classification system generated by the WHO, which included about 30 kinds of PHSOLs, except for microscopic cellular abnormalities[26]. We do not describe details concerning molecular genetics, diagnostic criteria, biological behaviors, treatment strategies, and clinical prognoses for each PHSOL, as they can be found in the given references. Although it is still possible that new types of PHSOLs will be discovered, we think that the above brief summary may provide useful information as a new classification system and current reference for clinicians and pathologists to understand the features of histological spectrum, as well as the differential diagnostic features, of PHSOLs.

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S- Editor Sun H **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2379

World J Gastroenterol 2011 May 21; 17(19): 2379-2388
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Inhibitory effect of schisandrin B on free fatty acid-induced steatosis in L-02 cells

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Supported by The Hong Kong Baptist University, No. FRG/08-09/II-30
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Received: August 12, 2010 Revised: August 13, 2010
Accepted: August 20, 2010
Published online: May 21, 2011

Abstract

AIM: To investigate the effects of schisandrin B (Sch B) on free fatty acid (FFA)-induced steatosis in L-02 cells.

METHODS: Cellular steatosis was induced by incubating L-02 cells with a FFA mixture (oleate and palmitate at the ratio of 2:1) for 24 h. Cytotoxicity and apoptosis were evaluated by 3-(4, 5-dmethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay and Annexin V/propidium iodide staining, respectively. Cellular total lipid was determined using a photocolorimetric method after Nile red staining, and triglyceride content was measured using an enzymatic kit. To study the effects of Sch B on steatosis, L-02 cells were treated with Sch B (1-100 μmol/L) in the absence or presence of 1 mmol/L FFA for 24 h, and cellular total lipid and triglyceride levels were measured. To explore the mechanisms of action of Sch B in the steatotic L-02 cells, mRNA levels of several regulators of hepatic lipid metabolism including adipose differentiation related protein (ADRP), sterol regulatory element binding protein 1 (SREBP-1), peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ were measured by quantitative real-time polymerase chain reaction (PCR), and protein levels of ADRP and SREBP-1 were measured by immunoblotting.

RESULTS: Treatment with 1 mmol/L FFA for 24 h induced intracellular lipid accumulation in L-02 cells comparable to that in human steatotic livers without causing apparent apoptosis and cytotoxicity. Sch B mitigated cellular total lipid and triglyceride accumulations in the steatotic L-02 cells in a dose-dependent manner. Quantitative real-time PCR and Western blot analyses revealed that treatment of L-02 cells with 100 μmol/L Sch B reverted the FFA-stimulated up-regulation of ADRP and SREBP-1.

CONCLUSION: Sch B inhibits FFA-induced steatosis in L-02 cells by, at least in part, reversing the up-regulation of ADRP and SREBP-1.

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Key words: Free fatty acid; Hepatic lipid metabolism; Hepatocellular steatosis; L-02 cells; Schisandrin B

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Chu JH, Wang H, Ye Y, Chan PK, Pan SY, Fong WF, Yu ZL. Inhibitory effect of schisandrin B on free fatty acid-induced steatosis in L-02 cells. *World J Gastroenterol* 2011; 17(19): 2379-2388 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2379.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2379

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has emerged

as a serious and widespread metabolic disease, which entails a wide spectrum of liver disorders and damages ranging from simple steatosis through steatohepatitis and fibrosis to end stage liver diseases including cirrhosis and hepatocellular carcinoma[1]. The clinical significance of NAFLD is largely due to its high prevalence of around 20% in general populations and up to 80% in obese and diabetic individuals worldwide[2]. Although the pathogenesis of NAFLD has not been clearly defined, hepatic steatosis characterized by uncontrolled triglyceride accumulation in hepatocytes and oxidative stress are believed to play a crucial role[3]. Therefore, agents that are capable of lowering hepatic lipid levels and alleviating oxidative stress may be beneficial to the control of NAFLD.

Schisandrin B (Sch B) (Figure 1) is the most abundant and active dibenzocyclooctadiene derivative isolated from the fruits of *Schisandra chinensis*, a traditional Chinese medicinal herb commonly used in treatment of viral and chemical hepatitis. A growing body of evidence has shown that Sch B can protect liver from damage caused by oxidative stress. Sch B may inhibit oxygen free-radical lipoperoxidative damage to plasma membrane of rat hepatocytes *in vitro*[4]. Sch B pretreatment protects mouse livers against tumor necrosis factor α-induced apoptosis in a dose-dependent manner[5]. In addition, Sch B can protect mice against carbon tetrachloride-induced hepatic toxicity by inhibiting lipid peroxidation[6]. Recently, we have reported that Sch B has hepatic lipid lowering effects in mice fed a high-fat diet[7]. These lines of evidence underscore both hepatic lipid-lowering and antioxidant effects of Sch B, making it a promising candidate for the treatment of NAFLD. Although the antioxidant role of Sch B has been well investigated, the mechanism underlying its hepatic lipid-lowering action remains unknown. This study was designed to investigate the anti-hepatosteatotic effects and mechanisms of Sch B using cultured steatotic cells.

NAFLD patients exhibit an elevated lipolysis and high circulating free fatty acid (FFA) levels[8]. High circulating FFA levels can trigger a series of biological changes in hepatic lipid metabolism, thus ultimately leading to hepatic steatosis[9]. Therefore, cellular FFA loading is commonly utilized to develop *in vitro* models of steatosis. These models can reliably reproduce the key features of hepatic steatosis in human beings[10-12], rendering them useful for the identification of potential therapeutic targets and effective intervention approaches against NAFLD. Human hepatocytes in primary culture represent the model closest to human liver tissues. Nevertheless, their use is often greatly hampered due to scarcity of liver samples[13]. HepG2 and Huh-7, two human hepatoma cell lines, are frequently used in establishing *in vitro* steatosis models. However, the validity of cancer cell-based models is concerned because metabolic regulation is often altered in cancer cells. For example, it has been highlighted that cancer cells may carry out an increased fatty acid *de novo* synthesis irrespective of the extracellular lipid levels[13]. Therefore, in this study, we first established FFA-induced steatotic cells using an immortalized normal human hepatocytes-derived cell line L-02[14,15]. Then, we investigated the *in vitro* effects of Sch B on hepatosteatosis in the steatotic L-02 cells, and explored the underlying mechanisms.

MATERIALS AND METHODS

Cell culture and treatment

L-02 (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China) and HepG2 (ATCC) cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and 1% penicillin/streptomycin (P/S, GIBCO, USA) at 37℃ in an atmosphere containing 5% CO_2. When FFA mixture (sodium salts of oleate and palmitate, Sigma, Malaysia) was added, bovine serum albumin (BSA) was supplemented to a final concentration of 1% in the culture medium. Cell cultures were used in experiments when they reached 75% confluence.

Sch B was purchased from Ningli Technology Co. Ltd. (Kunming, China) with a purity of 98% as determined by HPLC. A stock solution of Sch B (100 mmol/L) was prepared in dimethylsulfoxide (DMSO). The concentration of vehicle DMSO was 0.1% in treated cell cultures.

Cell viability assay

Cytotoxicity of FFA to L-02 cells was assessed by 3-(4, 5-dmethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. L-02 cells in 96-well plates were treated with FFA. After incubated for 24 h, 20 μL of MTT solution (5 mg/mL, USB, Austria) was added to each well and the plates were further incubated at 37℃ for 4 h. After medium removal, 100 μL of DMSO was added to each well of the plates which were then gently shaken for 5 min. Optical absorbance was determined at 570 nm with a microplate spectrophotometer (BD Bioscience, USA). Each treatment was performed in triplicate.

Quantification of apoptosis

Early and late phase apoptotic cells were assessed using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit I (BD Bioscience, USA) following the manufacturer's instructions. After treatment with FFA, L-02 cells were harvested and rinsed twice with cold PBS, resuspended in the binding buffer, and incubated with Annexin V-FITC and propidium iodide (PI) staining solution. Samples of 10 000 stained cells were analyzed using a flow cytometer (BD Bioscience, USA).

Nile red staining

L-02 cells in F96 microwell black plates (Nunc, Denmark) were treated with FFA in the presence or absence of Sch B for 24 h. Photocolorimetric measurement of intracellular lipid contents in Nile red stained cells was performed as previously described[16]. Each treatment was performed in triplicate.

Table 1 Primers used for polymerase chain reaction amplification of mRNA

Gene	Forward primer	Reverse primer
SREBP-1	ACGGCAGCCCCTGTAACGACCACTGTGA	TGCCAAGATGGTTCCGCCACTCACCAGG
ADRP	GGGATCCCTGTCTACCAAGC	AGATGTCGCCTGCCATCACC
PPAR-α	CCAGTATTTAGGAAGCTGTCCTG	CGTTGTGTGACATCCCGACAG
PPAR-γ	TGGTGACTTTATGGAGCCCAA	GGCAAACAGCTGTGAGGACTCAG
β-actin	GACTACCTCATGAAGATC	GATCCACATCTGCTGGAA

SREBP-1: Sterol regulatory element binding protein 1; ADRP: Adipose differentiation related protein; PPAR: Peroxisome proliferator-activated receptor.

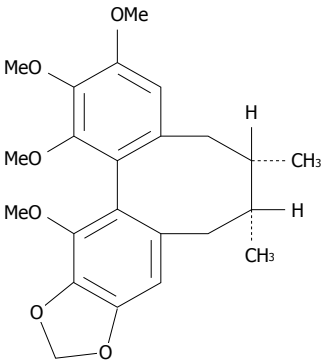

Figure 1 Chemical structure of schisandrin B.

Phase-contrast and fluorescence microscope imaging

L-02 cells in 6-well plates were treated with FFA for 24 h, washed with PBS, stained with 1 μmol/L Nile red in HBSS for 15 min and then examined under phase-contrast (Leica, Germany) and fluorescence (Nikon, Japan) microscopes.

Measurement of intracellular triglyceride content

Cellular triglyceride content was measured using an enzymatic kit (Zhongsheng Beikong Biotechnology and Science Inc, China) following the manufacturer's instructions. Triglyceride content was expressed in microgram of triglycerides per microgram protein. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad, USA). Each treatment was performed in triplicate.

Semi-quantitative and real-time quantitative polymerase chain reaction analyses

Total RNA was extracted with Trizol reagent (Invitrogen, USA), and 2 μg of RNA was reverse-transcribed with oligo-dT using the M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. For semi-quatitative polymerase chain reaction (PCR), the resultant cDNA was subjected to 25-30 cycles of PCR amplification (denaturing at 95℃ for 30 s, annealing at 55-60℃ for 30 s, extension at 72℃ for 60 s). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Quantitative real-time PCR was performed using SYBR green reaction mixture in the ABI 7500 fast real-time PCR system (Applied Biosystems). The PCR conditions were one cycle at 55℃ for 2 min and at 95℃ for 10 min, followed by 40 cycles of amplification at 95℃ for 15 s and at 60℃ for 1 min. The fluorescent signals were detected using the ABI Prism 7500HT sequence detection system (Applied Biosystems). The gene expression data were normalized to the endogenous control β-actin. The relative expression levels of genes were measured according to the formula $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycle values between the targets and β-actin. All samples were analyzed in triplicate. The specific primer pairs used for detecting messenger RNA are listed in Table 1.

Western blot analysis

L-02 cells were harvested and lysed on ice with the RIPA buffer consisting of 50 mmol/L Tris-Cl, 1% NP-40, 0.35% sodium-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, pH 7.4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 1 mmol/L Na_3VO_4 and 10 μg/mL each of aprotinin, leupetin and pepstatin A. Protein concentration in each sample was measured by the Bio-Rad protein assay. Protein samples (each 15 μg) were separated by SDS-PAGE and then electro-transferred onto nitrocellulose membranes (Amersham Biosciences, USA), which were blocked for 30 min with 5% skim milk in the TBST buffer containing 50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl and 0.1% Tween-20 and incubated with specific antibodies against adipose differentiation related protein (ADRP) (Abcam), sterol regulatory element binding protein 1 (SREBP-1) (Santa Cruz) or β-actin (Santa Cruz) overnight at 4℃. The membranes were then incubated with HRP-conjugated secondary antibodies, and immunoreactive bands were visualized using the ECL detection kit (Amersham Biosciences, USA) following the manufacturer's instructions.

Statistical analysis

All results were expressed as mean ± SE. The difference between two groups was analyzed using the Student's *t* test.

RESULTS

Cytotoxic effect of FFA treatment on L-02 cells

L-02 cells were treated with 0.5-2 mmol/L a FFA mixture (oleate and palmitate at the ratio of 2:1) for 24 h and the cytotoxicity of FFA to L-02 cells was detected by MTT

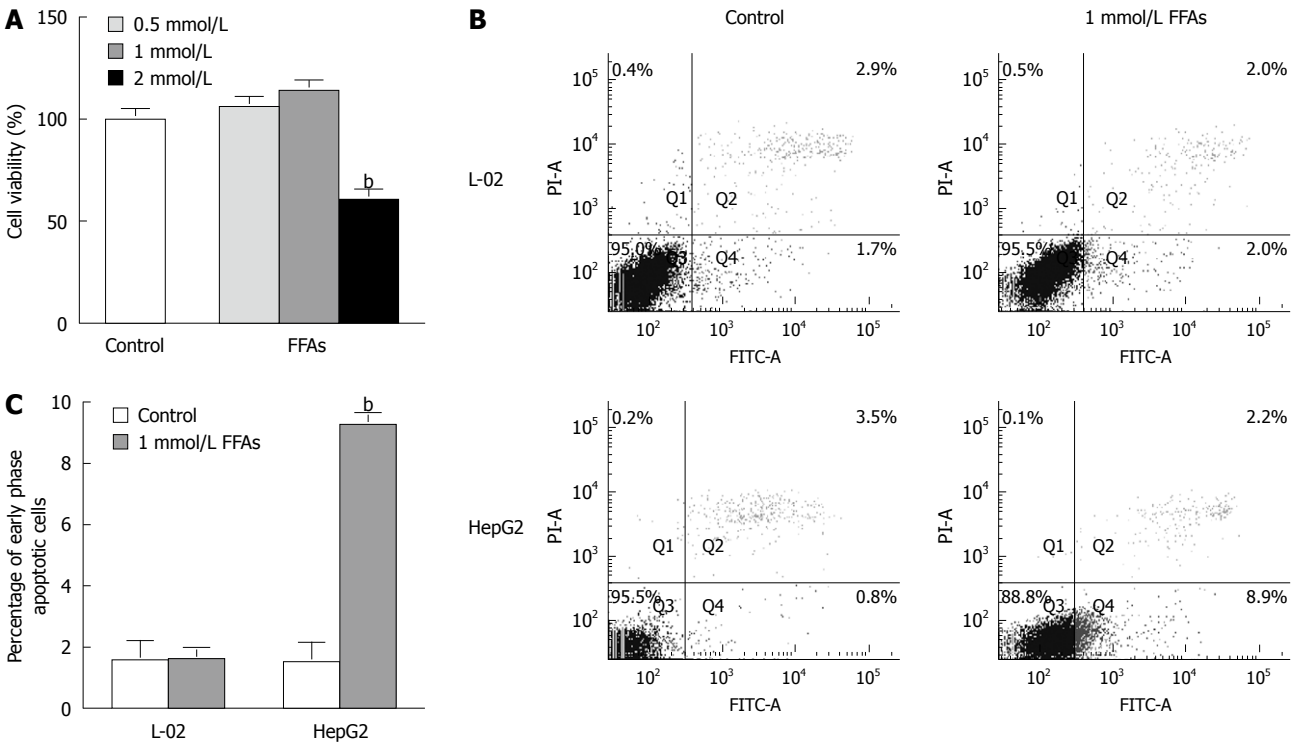

Figure 2 Cytotoxic and apoptotic effects of free fatty acid treatment on cultured cells. A: L-02 cells were treated with a free fatty acid (FFA) mixture (oleate and palmitate at the ratio of 2:1) at various concentrations for 24 h. Cell viability was determined by the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assay. $^{b}P < 0.01$ *vs* control group; B: L-02 and HepG2 cells were treated with 1 mmol/L FFA mixture (oleate and palmitate at the ratio of 2:1) for 24 h and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide. Apoptotic and necrotic cells were monitored by flow cytometry. Normal, early and late apoptotic cells as well as necrotic cells were shown in Q3, Q4, Q2 and Q1 quadrants, respectively. The percentage of cells in each quadrant was displayed. Results were the representative of three independent experiments; C: Quantification of early phase apoptotic cells in response to FFA treatment. $^{b}P < 0.01$ *vs* HepG2 control group.

assay. No apparent cytotoxic effect of FFA was observed on L-02 cells after treatment with FFA at the concentration of 0.5 or 1 mmol/L, while the cell viability was decreased by 40% when L-02 cells were treated with FFA at the concentration of 2 mmol/L (Figure 2A). These results suggest that 0.5 or 1 but not 2 mmol/L FFA can be used to prepare steatotic L-02 cells.

Apoptotic effect of FFA treatment on L-02 cells

To evaluate the apoptotic effect of FFA treatment on L-02 cells, the L-02 cells were treated with 1 mmol/L FFA (oleate and palmitate at the ratio of 2:1) for 24 h, and then stained with Annexin V/PI. Apoptosis of L-02 cells was monitored by flow cytometry. For comparison, apoptosis of HepG2 cells induced by FFA treatment was also analyzed. FFA treatment did not trigger early- or late-stage apoptosis of L-02 cells but significantly induced early-stage apoptosis of HepG2 cells (Figure 2B and C). The percentage of apoptotic HepG2 cells was increased from 1.500% ± 0.473% in control cells to 9.267% ± 0.203% in FFA-treated cells (Figure 2C), which is consistent with previous reports showing that FFA causes apoptosis of HepG2 cells under the same conditions[11,12]. These results suggest that L-02 and HepG2 cell lines do have different responses to FFA treatment.

FFA treatment induced lipid accumulation in L-02 cells

We next examined the cellular lipid accumulation in L-02 cells treated with the FFA mixture for 24 h at the concentration of 0.5 mmol/L or 1 mmol/L using Nile red staining. The results showed that FFA induced lipid accumulation (Figure 3A) in L-02 cells in a dose-dependent manner, which was confirmed by fluorescent microscopy (Figure 3B). When the L-02 cells were treated with FFA mixture at the concentration of 1 mmol/L, the intracellular lipid content was increased by 5.34 ± 0.65-fold in L-02 cells compared to that in FFA-untreated controls (Figure 3A). The cellular lipid accumulation level in L-02 cells treated with FFA at the concentration of 1 mmol/L was comparable to that in human steatotic livers, which is 5.5-fold over non-steatotic livers[13].

We also measured the intracellular triglyceride levels in L-02 cells treated with FFA mixture at the concentration of 1 mmol/L. The triglyceride content was increased by about 2.5-fold from 0.108 ± 0.027 μg/μg protein in control cells to 0.241 ± 0.030 μg/μg protein in FFA-treated cells (Figure 3C), which is similar to the results obtained from human liver samples. The triglyceride content is about 2.7-fold higher in human steatotic livers than in non-steatosis livers[13].

The above data indicate that steatotic cells can be prepared by incubating L-02 cells with a FFA mixture (oleate and palmitate at the ratio of 2:1) at the concentration of 1 mmol/L for 24 h, in which lipid accumulation can reach a level similar to that in human steatotic livers in the absence of apoptosis.

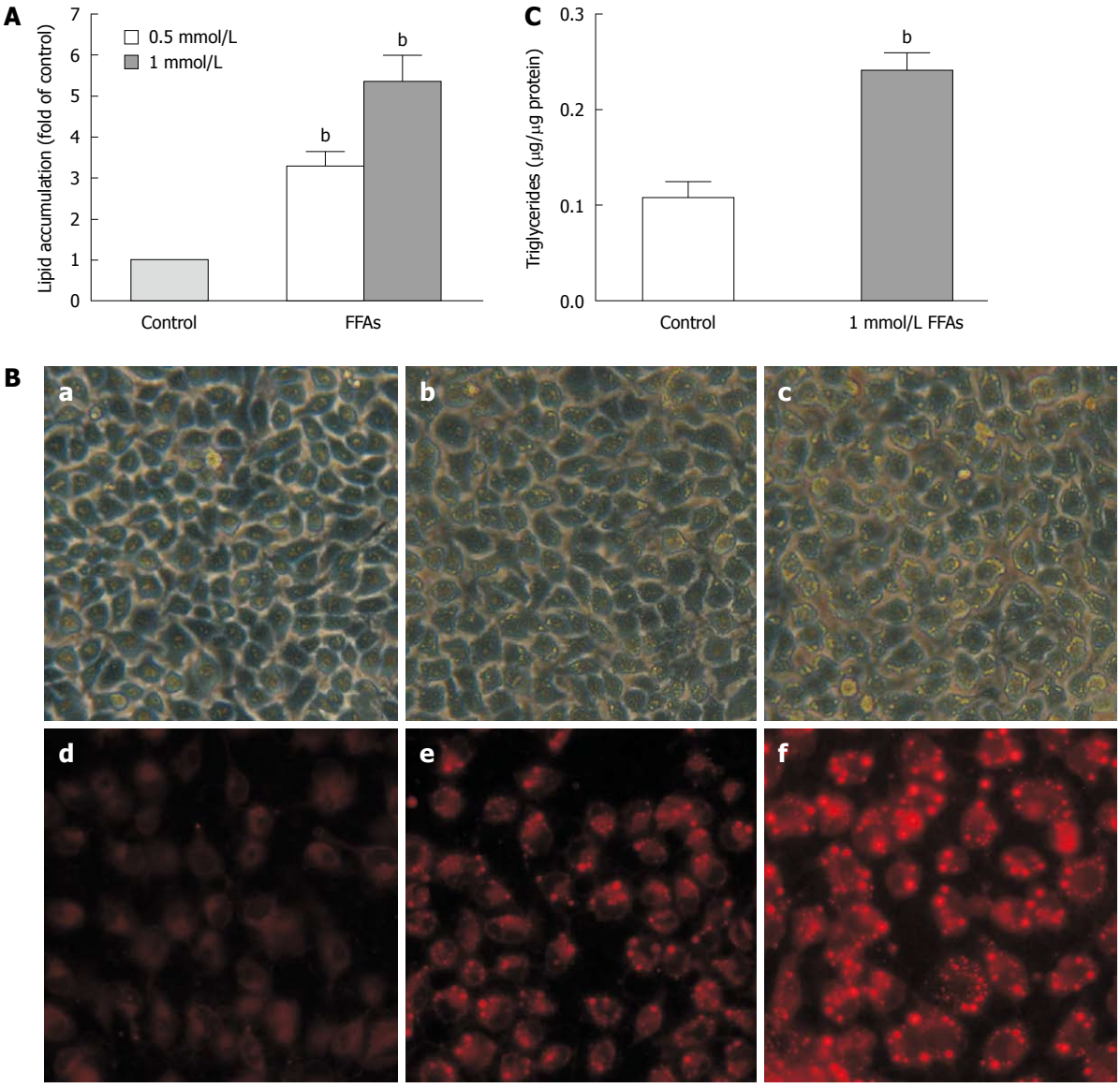

Figure 3 Free fatty acid induced lipid accumulation in L-02 cells. A: L-02 cells were incubated with a free fatty acid (FFA) mixture (oleate and palmitate at the ratio of 2:1) for 24 h. Intracellular lipid accumulation was evaluated after Nile red staining. Results were expressed as mean ± SE of three independent experiments. $^{b}P < 0.01$ *vs* control group; B: Representative micrographs showing intracellular lipid accumulation in L-02 cells as observed by phase-contrast microscopy (panels a-c) and fluorescence microscopy (panels d-f). Panels a/d, b/e and c/f are control cells, cells treated with 0.5 and 1 mmol/L FFA, respectively; C: Triglyceride levels in L-02 cells treated with 1 mmol/L FFA. Results were expressed as mean ± SE of three independent experiments. $^{b}P < 0.01$ *vs* control group.

Sch B treatment alleviated FFA-induced lipid accumulation in L-02 cells

To investigate the anti-steatotic effect of Sch B in L-02 cells, L-02 cells were exposed to various concentrations of Sch B (1-100 μmol/L) in the absence or presence of FFA mixture at the concentration of 1 mmol/L for 24 h. The intracellular total lipid levels in L-02 cells were measured after Nile red staining and the triglyceride contents were assayed using an enzymatic kit. Nile red staining assay showed that Sch B at all concentrations had no significant effect on the cellular lipid content in L-02 cells in the absence of FFA, but substantially ameliorated the lipid accumulation induced by FFA in a dose-dependent manner (Figure 4A). The lipid-lowering effect of Sch B at the concentration of 100 μmol/L was further confirmed by microscopic examination of the fluorescence of Nile red-stained L-02 cells (Figure 4B). The intracellular triglyceride measurements showed that Sch B inhibited the fat accumulation in a dose-dependent manner, and exerted a significant inhibitory effect in L-02 cells treated with FFA at the concentration of 1 mmol/L (Figure 4C). It was noteworthy that Sch B at each tested concentration did not elicit apparent cytotoxicity or apoptosis in L-02 cells in the presence or absence of 1 mmol/L FFA at 24 h (data not shown).

Sch B decreased mRNA and protein expression levels of ADRP and SREBP-1 in FFA-induced steatotic L-02 cells

To explore the mechanisms underlying Sch B-mediated lipid-lowering action in L-02 cells, the mRNA expression lev-

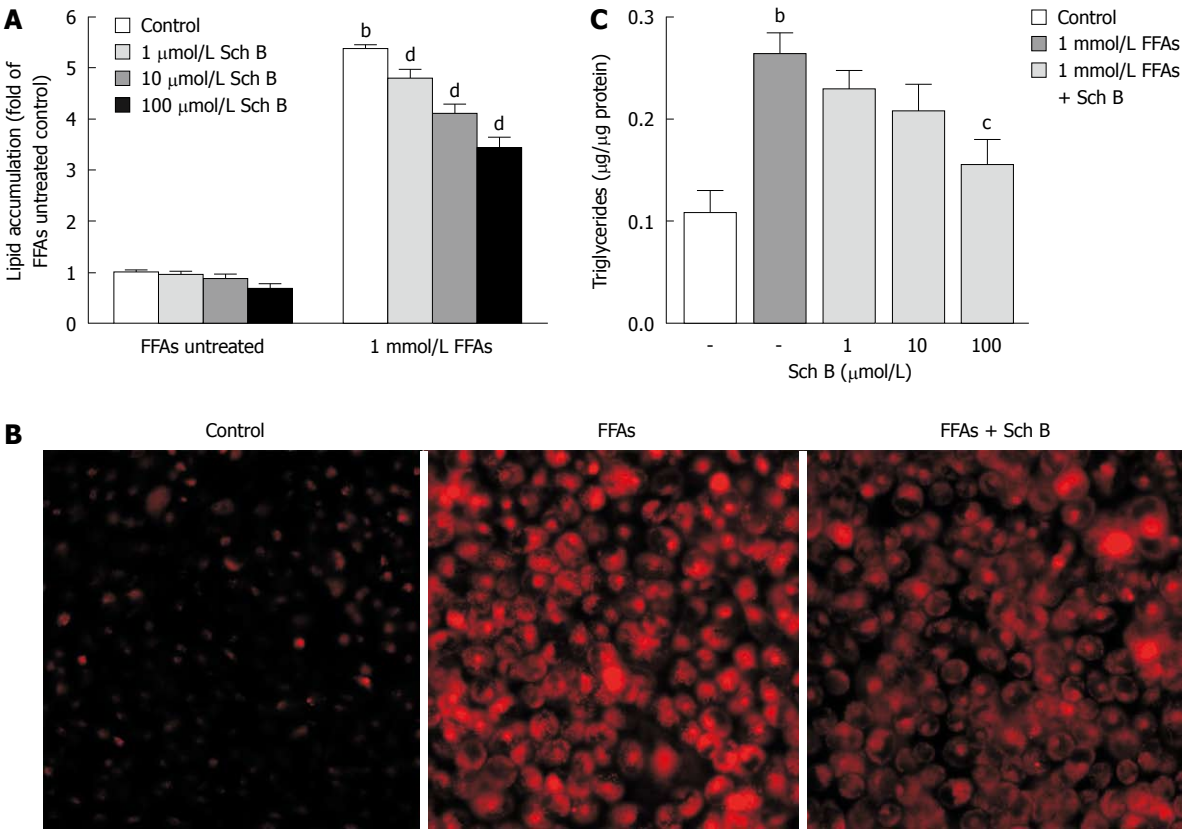

Figure 4 Effect of schisandrin B on free fatty acid-induced fat accumulation in L-02 cells. A: L-02 cells were treated with schisandrin B (Sch B) (1 μmol/L, 10 μmol/L or 100 μmol/L) in the presence or absence of 1 mmol/L free fatty acid (FFA) mixture (oleate and palmitate at the ratio of 2:1) for 24 h. Intracellular total lipid levels were measured after Nile red staining; B: Representative micrographs showing intracellular lipid accumulation in Nile red stained L-02 cells after treatment with 100 μmol/L Sch B in the presence of 1 mmol/L FFA examined by fluorescent microscopy; C: L-02 cells were treated with Sch B at the indicated concentrations for 24 h and cellular triglyceride levels were measured using an enzymatic kit. $^{b}P < 0.01$ *vs* FFA-untreated control groups; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* FFA-treated groups. Data are from three independent experiments.

els of ADRP, SREBP-1, peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ, four important regulators of hepatic lipid metabolism, were first measured by semi-quantitative PCR and quantitative real-time PCR, respectively. Sch B attenuated the FFA-induced fat accumulation most effectively at the concentration of 100 μmol/L, thus this dosage of Sch B was used in this experiment. The mRNA expression level of PPAR-α remained unchanged in FFA-treated L-02 cells, while the mRNA expression levels of the other three genes were up-regulated (Figure 5A and B). Concurrent treatment with Sch B at the concentration of 100 μmol/L for 24 h restored the FFA-upregulated ADRP and SREBP-1 expression to normal levels, but did not affect FFA-stimulated PPAR-γ mRNA expression. In addition, Sch B treatment did not obviously influence the expression of PPAR-α in L-02 cells in the presence of FFA.

Immunoblot analysis showed that the protein levels of ADRP and SREBP-1 were dramatically elevated in L-02 cells in response to FFA treatment, but returned to normal levels after concurrent treatment with Sch B (Figure 5C).

DISCUSSION

FFA-induced hepatocellular steatosis models have been widely applied in studies on NAFLD pathogenesis and anti-NAFLD drugs[13,17]. Although human hepatocytes in primary culture represent the most stringent model of the human liver, they are tedious to prepare and the reproducibility of experimental results is often a big problem[18,19]. On the other hand, the use of liver cancer cell lines including HepG2 and Huh-7 is often questioned about their acquired genetic and epigenetic alterations which may endow them with numerous properties including metabolism regulation distinct from normal hepatocytes[20]. In the present study, we successfully prepared FFA-induced steatotic cells using a normal human hepatocytes-derived cell line L-02. The steatotic L-02 cells behave similarly to human steatotic livers in two aspects. First, 24 h treatment with a FFA mixture (oleate and palmitate at the ratio of 2:1) at the concentration of 1 mmol/L caused no apparent toxicity to L-02 cells. Second, the induced fat accumulation in L-02 cells was comparable to that in human steatotic livers[13]. These aforementioned attributes make FFA-induced steatotic L-02 cells suitable for the investigations of NAFLD pathogenesis and anti-NAFLD agents.

High circulating FFA concentration may aggravate hepatic fat accumulation by disrupting lipid metabolism in NAFLD patients, and thus studying how FFA overload influences metabolic regulators will further improve our

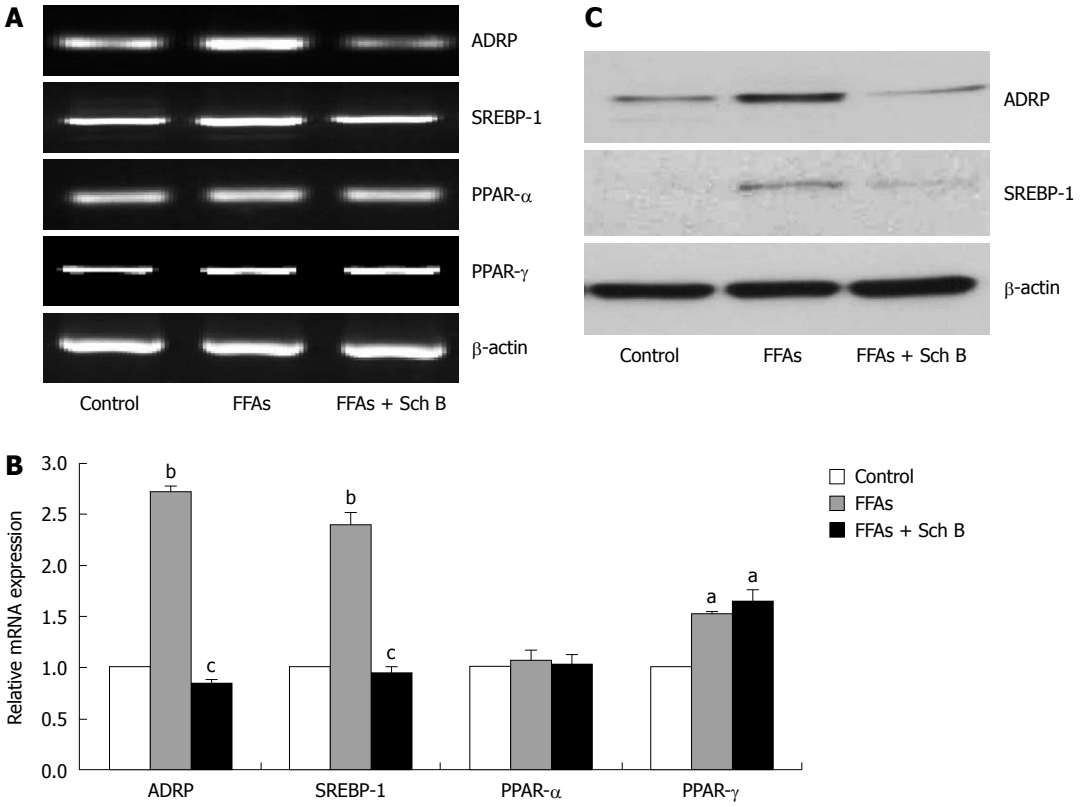

Figure 5 Effect of schisandrin B on mRNA and protein expression levels of several lipid metabolism-related molecules in free fatty acid-treated L-02 cells. A, B: Semi-quantitative polymerase chain reaction (PCR) and real-time quantitative PCR showing mRNA levels of adipose differentiation related protein (ADRP), sterol regulatory element binding protein 1 (SREBP-1), peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ. Results shown are the representative of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* free fatty acid-treated groups; C: Immunoblotting showing expression levels of ADRP and SREBP-1 proteins. The representative blots out of three experiments are shown. FFA: Circulating free fatty acid; Sch B: Schisandrin B.

understanding about the pathogenesis of NAFLD. Given that palmitic and oleic acids are the most abundant fatty acids in liver triglycerides in both normal subjects and NAFLD patients[21], clarification of their effect on hepatocytes is of great importance. In the present study, treatment of L-02 cells with a FFA mixture (oleate and palmitate at the ratio of 2:1) did not significantly affect PPAR-α mRNA expression level but increased the mRNA expression levels of ADRP, SREBP-1 and PPAR-γ. The unchanged PPAR-α mRNA expression in L-02 cells treated with FFA suggests that PPAR-α-mediated mitochondria fatty acid β-oxidation may not been affected by FFA in our experimental conditions. The increased SREBP-1 and PPAR-γ mRNA expression levels in response to FFA treatment are in good accord with a recent report assuming that the up-regulations of SREBP-1 and PPAR-γ are linked to the steatogenic property of oleic acid[17]. Moreover, the ADRP mRNA expression was elevated in L-02 cells challenged with FFA, which is in agreement with the reported observations in other cell lines treated with FFA[22,23]. ADRP is a lipid storage droplet-associated protein found in most cells and tissues, and has been suggested to be a marker of lipid accumulation, because the cellular level of ADRP is proportional to the total mass of neutral lipids within the cells[24]. Fatty acids have been implicated as ligands for PPAR family members including PPAR-α and PPAR-γ, it is thus believed that the stimulation of ADRP gene by FFA is at least in part due to PPAR activation. It has been shown that the activated PPAR can complex with retinoid X receptor and bind to the PPAR response element in the promoter of ADRP gene[25,26]. As both PPAR-α and PPAR-γ were detectable in L-02 cells, further studies are needed to ascertain whether one or both of them are required for the up-regulation of ADRP gene expression induced by FFA treatment. These findings suggest that exposure to exogenous FFA may interfere with lipid metabolism through the modulation of metabolic regulators in L-02 cell line derived from normal human hepatocytes.

We demonstrated that Sch B exerted a drastic inhibitory effect on FFA-induced steatosis in L-02 cells. This finding and the hepatic lipid-lowering action of Sch B observed in high-fat diet-fed mice[7] strongly highlight the anti-steatosis potential of Sch B. Since FFA overloading contributed to hepatic fat accumulation through modulation of lipid metabolism- related genes in our established steatotic L-02 cells, it is conceivable that Sch B may attenuate fat accumulation by counteracting or reversing the unfavorable changes in expression of genes evoked by FFA. In this study, Sch B treatment restored the FFA-induced up-regulation of both mRNA and protein levels of ADRP and SREBP-1 to normal levels, indicating that ADRP and SREBP-1 are the potential targets of Sch B in relation to its lipid-lowering property.

ADRP expression is closely associated with intracellular lipid droplets and up-regulated in hepatic steatosis in human and mouse models[27,28]. It has been reported that ADRP overexpression may promote lipid accumulation in fibroblasts and macrophages without changing the expression of adipogenic genes and genes involved in lipid efflux[29,30]. Intriguingly, ADRP overexpression may facilitate the uptake of long chain FFA in COS-7 cells[31]. Moreover, Edvardsson *et al*[32] have recently proposed that ADRP may enhance cellular triglyceride accumulation in hepatocytes by increasing fatty acid uptake, driving fatty acids to triglyceride formation as well as preventing the use of triglyceride in VLDL assembly. In this connection, the down-regulation of ADRP may contribute to the anti-hepatosteatotic effect of Sch B by inhibiting the uptake of exogenous long chain FFA, decreasing the incorporation of FFA into triglyceride and increasing the availability of triglyceride for VLDL assembly. It has been demonstrated that ADRP-deficient mice produced by either knock-out or anti-sense oligonucleptide technology do not acquire diet-induced hepatic steatosis[26,33,34], raising the possibility that ADRP may become a putative molecular target for the prevention of NAFLD, thus screening for compounds that can repress hepatic ADRP expression may provide a new direction for the identification of potential therapeutic agents against NAFLD. It has been recently demonstrated that pycnogenol, a French maritime pine bark extract, can reduce oleic acid-induced lipid droplet formation in mouse liver epithelial cells MMuLi by inhibiting ADRP expression, and interestingly the suppression of ADRP expression is mediated in part by facilitating mRNA degradation[23]. How Sch B impairs ADRP expression in steatotic L-02 cells remains to be evaluated.

SREBP-1 is the most important transcription factor regulating *de novo* lipogenesis in the liver. There is compelling evidence that supports the involvement of SREBP-1 in NAFLD development. It has been reported that SREBP-1 expression is significantly elevated in livers from NAFLD and obesity patients, and from insulin-resistant and hyperinsulinemic *ob/ob* mice[27,35,36]. Overexpression of SREBP-1 in cultured hepatocytes or mouse livers can increase hepatic triglyceride deposition and mRNA expression of genes involved in lipogenesis[37-39]. Moreover, in $Lep^{ob/ob}$ mice deficient in SREBP-1, hepatic steatosis is markedly attenuated, which is accompanied by decreased mRNA levels of lipogenic enzymes[40]. These lines of evidence strongly suggest that SREBP-1 plays a pivotal role in the regulation of hepatic lipid metabolism, thus pharmacological manipulation of SREBP-1 may be beneficial to the management of NAFLD. In this study, Sch B could reverse FFA-induced up-regulation of SREBP-1. Therefore it is plausible to infer that the down-regulation of SREBP-1 may partly contribute to the lipid-lowering activity of Sch B by inhibiting *de novo* lipogenesis. Since SREBP-1 may transcriptionally activate a variety of genes required for lipogenesis in the liver[41], it is of interest to investigate which SREBP-1 target genes are regulated by Sch B. Another question is how Sch B regulates the expression of SREBP-1. A most recently study showed that resveratrol inhibits palmitate-induced lipid accumulation in HepG2 cells by reducing the up-regulation of SREBP-1 *via* the Sirt1-FOXO1 pathway[42]. Whether the Sirt1-FOXO1 pathway is involved in Sch B-mediated down-regulation of SREBP-1 remains to be clarified.

In summary, Sch B has an inhibitory effect on FFA-induced steatosis in L-02 cells, and the decreased expression of ADRP and SREBP-1 may account for the inhibitory effect of Sch B by reducing FFA uptake, incorporation of FFA into triglycerides and *de novo* fatty acid synthesis, as well as by increasing VLDL assembly. Changes in ADRP and SREBP-1 expression may also provide mechanistic explanations for the hepatic lipid-lowering effect of Sch B in mice fed a high-fat diet as reported previously by us. The results of this study provide the molecular evidence for developing Sch B as a therapeutic agent against NAFLD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD), characterized by fatty infiltration of the liver (hepatic steatosis), is posing a definite threat to global human health. So far, no satisfactory therapeutic agent is available against NAFLD. Schisandrin B (Sch B), a bioactive constituent isolated from Fructus Schisandrae, has been recently reported to exhibit hepatic lipid-lowering effect in mice fed with a high-fat diet. However, the mechanisms of action remain to be elucidated.

Research frontiers

The circulating free fatty acid (FFA) levels are often elevated in NAFLD patients, which may promote the disease progression by interfering with hepatic lipid metabolism through modulating the expression of important metabolic regulators. Cellular FFA loading is commonly utilized to develop *in vitro* models of steatosis valuable for the study of anti-steatosis agents.

Innovations and breakthroughs

The use of human hepatocarcinoma cell lines in developing cellular steatosis models has been questioned due to the altered metabolic regulation in cancerous cells. In this study, FFA-induced steatotic cells were successfully prepared using a normal human hepatocytes-derived cell line L-02. Using this model, the authors demonstrated that Sch B effectively attenuated FFA-induced fat accumulation by abrogating up-regulations of two key metabolic regulators, namely adipose differentiation related protein (ADRP) and sterol regulatory element binding protein 1 (SREBP-1).

Applications

FFA-induced steatotic L-02 cells can be used in investigations of anti-steatosis agents and NAFLD pathogenesis. By understanding how Sch B alleviates cellular steatosis, this study provides the molecular evidence for developing Sch B as a therapeutic agent against NAFLD.

Terminology

ADRP and SREBP-1 are important proteins involved in lipid metabolism in normal livers, and their aberrant expression is believed to contribute to the development of NAFLD.

Peer review

The authors examined the anti-steatotic effects of Sch B on human hepatocytes line treated *in vitro* with FFAs to induce steatosis. Sch B reduced cellular total lipid and triglyceride levels in a dose dependent manner, which is consistent with the earlier findings. The novel aspect of this study is that the expression of ADRP and SREBP-1, two lipid metabolism regulators, is reduced by Sch B, which provides some insights into the mechanism of action of Sch B.

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S- Editor Wang JL **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2389

World J Gastroenterol 2011 May 21; 17(19): 2389-2396
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma

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Supported by The National Natural Science Foundation of China, No. 30772542
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Received: August 19, 2010 Revised: November 13, 2010
Accepted: November 20, 2010
Published online: May 21, 2011

Abstract

AIM: To investigate the role of CXC chemokine receptor-4 (CXCR4) and stromal cell-derived factor-1 (SDF-1) in lymph node metastasis of gastric carcinoma.

METHODS: In 40 cases of gastric cancer, expression of CXCR4 mRNA in cancer and normal mucous membrane and SDF-1 mRNA in lymph nodes around the stomach was detected using quantitative polymerase chain reaction (PCR) (TaqMan) and immunohistochemistric assay. SGC-7901 and MGC80-3 cancer cells were used to investigate the effect of SDF-1 on cell proliferation and migration.

RESULTS: Quantitative reverse transcription PCR and immunohistochemistry revealed that the expression level of CXCR4 in gastric cancer was significantly higher than that in normal mucous membrane (1.6244 ± 1.3801 *vs* 1.0715 ± 0.5243, $P < 0.05$). The expression level of CXCR4 mRNA in gastric cancer with lymph node metastasis was also significantly higher than that without lymph node metastasis (0.823 ± 0.551 *vs* 0.392 ± 0.338, $P < 0.05$). CXCR4 expression was significantly related to poorly differentiated, high tumor stage and lymph node metastasis. Significant differences in the expression level of SDF-1 mRNA were found between lymph nodes in metastatic gastric cancer and normal nodes (0.5432 ± 0.4907 *vs* 0.2640 ± 0.2601, $P < 0.05$). The positive expression of SDF-1 mRNA in lymph nodes of metastatic gastric cancer was consistent with the positive expression of CXCR4 mRNA in gastric cancer ($r = 0.776$, $P < 0.01$). Additionally, human gastric cancer cell lines expressed CXCR4 and showed vigorous proliferation and migratory responses to SDF-1. AMD3100 (a specific CXCR4 antagonist) was also found to effectively reduce the migration of gastric cancer cells.

CONCLUSION: The CXCR4/SDF-1 axis is involved in the lymph node metastasis of gastric cancer. CXCR4 is considered as a potential therapeutic target in the treatment of gastric cancer.

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Key words: Gastric carcinoma; Chemokines; Stromal cell-derived factor-1; CXC chemokine receptor-4; Lymph node metastasis

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Zhao BC, Wang ZJ, Mao WZ, Ma HC, Han JG, Zhao B, Xu HM. CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma. *World J Gastroenterol* 2011; 17(19): 2389-2396 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2389.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2389

INTRODUCTION

Gastric cancer is one of the most commonly diagnosed malignancies and the main cause of cancer-related deaths in Asian populations. Most deaths from gastric cancer are caused by metastasis, of which lymph node metastasis is the most common cause, which leads to the failure of surgery, chemotherapy or radiotherapy. Therefore, inhibition of metastatic gastric cancer is an important therapeutic strategy. However, the molecular mechanisms involved in this process have not been fully elucidated.

Chemokines are a family of small heparin-binding and secretory proteins, and through interactions with their corresponding receptors, they can control and activate many types of cells. According to the position of the four conserved cysteine residues in the amino acid sequence, they are classified into four groups (CXC, CX3C, CC and C). Stromal cell-derived factor (SDF)-1 is a member of the CXC subfamily, which was first cloned from murine bone marrow[1]. SDF-1 exerts an effect through interaction with its specific receptor CXC chemokine receptor-4 (CXCR4). Many studies have proven that CXCR4 is the major chemokine receptor expressed in many types of cancer cells[2,3]. and demonstrated that the CXCR4/SDF-1 axis plays a major role in cell survival, proliferation, migration and adhesion of several tumor cells, including those from colon cancer[4], breast cancer[5,6], non-small cell lung cancer[7], prostate cancer[8], melanoma[9,10], cholangiocarcinoma[11], and oral squamous cell carcinoma[12], However, most of the studies about SDF-1 and CXCR4 have been conducted *in vitro*, and the definitive pathophysiological functions of the CXCR4/SDF-1 axis in human diseases, especially cancer, require further research.

Recently, it has been suggested that the interaction between CXCR4 and SDF-1 plays an important role in the development of peritoneal carcinomatosis from gastric cancer[13]. We hypothesize that the CXCR4/SDF-1 axis also participates in lymph node metastasis of gastric cancer. To verify the hypothesis, we examined the expression of CXCR4 and SDF-1 in gastric cancers, normal mucous membranes, and their related lymph nodes. We also investigated the relationship between CXCR4 expression and clinicopathological features, and determined whether CXCR4 expression influenced the proliferation and migration of gastric cancer cells *in vitro*.

MATERIALS AND METHODS

Patients and tissue samples

A total of 40 patients with gastric cancer who underwent surgery at the Department of General Surgery, Beijing Chaoyang Hospital, between 2008 and 2009 were enrolled. The patient population consisted of 31 men and nine women, with a mean age of 55 years (range, 31-76 years). Patients who were receiving preoperative chemotherapy and/or radiotherapy were not included. The specimens included the tumor tissue, normal mucous membranes (5 cm away from the tumor), and the lymph nodes around the stomach. All the specimens were collected within 30 min after resection and each specimen was divided into two parts: one was fixed in 4% formalin and embedded in paraffin; and the other was snap-frozen in liquid nitrogen and kept at -80℃. Tumor stage was determined according to the TNM classification system of the International Union against Cancer. Histological diagnosis was confirmed for each specimen. Informed consent was obtained from all patients.

Cell culture

The SGC-7901 and MGC-803 gastric cancer cell lines were grown in RPMI 1640 medium (Sigma, USA) that contained 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma). The suspension was placed into T25 flasks and allowed to incubate at 37℃ in a humidified chamber that contained 5% CO_2. The adherent cells were then cultured with medium changed at a 3-d interval. Cells at passage 1-6 were used for all experiments.

Cell proliferation assay

Gastric cancer cells (SGC-7901 and MGC-803) were seeded into 96-well plates at a density of 5×10^3 cells per well without FBS. After 24 h, the cultures were washed and re-fed with medium alone (control) or with medium that contained SDF-1 at various concentrations. After 3 d, the number of viable cells was counted using an MTT assay (Beyotime, China) according to the manufacturer's instructions. The quantity of formazan product measured at 490 nm was proportional to the number of live cells in the culture. The experiments were repeated in triplicate.

Cell migration assays

The invasion potential of cancer cells was assayed using 24-well chemotaxis chambers (Corning, Corning, NY, USA). The upper and lower cultures were separated by 8-μm-pore-size polyvinylpyrrolidone-coated polycarbonate filters. Gastric cancer cells were suspended at 1×10^5 cells/mL in serum-free medium, and 0.2 mL cell suspension was added to the upper chamber. Then 0.5 mL serum-free medium with various concentrations of SDF-1 was added to the lower chamber. In another set of experiments, 0.5 mL serum-free medium with 10 nmol/L SDF-1 (fixed concentration) plus various concentrations of AMD3100 (Sigma) was added to the lower chamber. The chambers were incubated for 12 h at 37℃ in a humid atmosphere of 5% CO_2. After incubation, non-migrated cells were removed from the upper surface of the filters, and the migrated cells adherent to the filters were fixed with ethanol and stained with Giemsa solution. Each experiment was done in triplicate, and cells migrated to the underside of the filter were counted in five fields (10 × magnification) in each well under light microscope.

Immunohistochemistry

Immunohistochemistry was performed using the Histostain-SP kits (Boster, Wuhan, China) according to the manufacturer's recommendations. Sections (4 μm thick) were de-

paraffinized, placed in 0.01 mol/L citrate buffer (pH 6.0), and treated by microwave heating for 15 min. The sections were then placed in a solution of 97% methanol and 3% hydrogen peroxide for 10 min at room temperature, to quench endogenous peroxidase activity. Subsequently, the slides were pretreated with 1% bovine serum albumin in phosphate-buffered saline (PBS) and incubated with anti-SDF-1 antibody (Boster; dilution 1:100) and anti-CXCR4 antibody (Boster; dilution 1:50) for 1 h at room temperature. The primary antibody was washed away with PBS, and the biotinylated secondary antibody was used. After 20 min, the sections were washed with PBS, and treated with peroxidase-conjugated streptavidin for 20 min. Finally, the slides were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min and counterstained with Carazzi's hematoxylin, dehydrated and mounted.

Evaluation of immunostaining

The slides were examined blindly by three pathologists who had no clinicopathological knowledge of the patients. The intensity of staining and percentage of positive cells were determined by the three observers. The intensity, staining percentage, and pattern of staining (nuclear and cytoplasmic) were assessed for CXCR4 and SDF-1. The intensity of staining (brown color) was scored semi-quantitatively as follows: +, weak; ++, medium; +++, strong; and ++++, very strong. The immunostained sections were scanned under light microscope. Samples with a score of ++ or greater were considered CXCR4 or SDF-1-positive.

Determination of CXCR4 and SDF-1 mRNA expression

Total RNA (500 ng) was isolated from frozen tissues and cell pellets using RNArose reagent (Fulin, Qingdao, China) according to the manufacturer's instructions. Reverse transcription was performed in a final volume of 10 μL that contained 5 × PrimeScript™ Buffer (2 μL), PrimeScript™ RT Enzyme Mix (2 μL), Oligo dT Primer (50 μmol/L) (0.5 μL), Random 6 mers (100 μmol/L) (0.5 μL), RNase Free dH_20 (4.5 μL) using a Reverse Transcription System kit (Takara, Japan). The reverse transcription reaction was performed at 37℃ for 15 min, and 85℃ for 5 s. Gene expression of CXCR4 and SDF-1 was detected by quantitative real-time polymerase chain reaction (PCR) (TaqMan) using the 7500 sequence detector (AB Applied Biosystems, USA) and SDS analysis software. The primers and fluorescent probe for human CXCR4, SDF-1 and GAPDH are shown in Table 1. GAPDH served as a control for efficiency of the amplification in the reactions. Thermal cycle conditions were 95℃ for 10 s for one cycle, followed by 40 cycles of 95℃ for 5 s, and 60℃ for 45 s. The expression level of CXCR4 mRNA and SDF-1 mRNA was obtained by $2^{-\Delta\Delta CT}$ calculation. All PCR products were analyzed on a 2% agarose gel with ethidium bromide staining.

Statistical analysis

The SPSS version 12.0 software was used for statistical analysis. The Pearson χ^2 test or Fisher exact test was used to compare qualitative variables. Quantitative variables were analyzed using Student's *t* test. Results were presented as mean ± SE. Pearson correlation analysis was used for correlation analysis. Probability values < 0.05 were considered significant. All experiments were repeated two or three times with triplicate samples, and similar results were obtained.

Table 1 Primers and fluorescent probe for human CXC chemokine receptor-4, stromal cell-derived factor-1 and GAPDH

Primers	5'-3'	Product (bp)
CXCR4	Forward: TGGCCTTATCCTGCCTGGTAT	173
	Reverse: GGAGTCGATGCTGATCCCAAT	
	Taqman: AGAAGCGCAAGGCCCTCAAGACCA	
SDF-1	Forward: GAGCCAACGTCAAGCATCTCA	103
	Reverse: TTCGGGTCAATGCACACTTGT	
	Taqman: CTGTGCCCTTCAGATTGTAGCCCGG	
GAPDH	Forward: TCATGGGTGTGAACCATGAGAA	146
	Reverse: GGCATGGACTGTGGTCATGAG	
	Taqman: TCATCAGCAATGCCTCCTGCACCA	

CXCR4: CXC chemokine receptor-4; SDF-1: Stromal cell-derived factor-1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

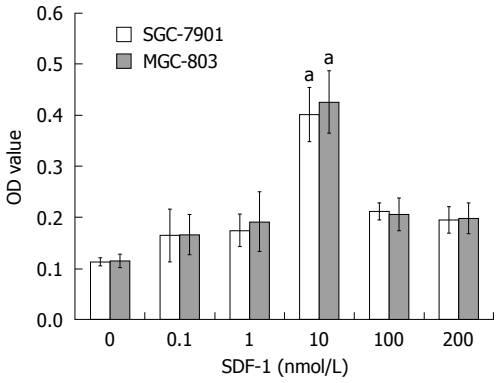

Figure 1 Effect of stromal cell-derived factor-1 on proliferation of gastric cancer cells. Gastric cancer cells were grown in serum-free medium with or without the indicated doses of stromal cell-derived factor-1 (SDF-1). SDF-1 significantly increased the number of SGC-7901 and MGC-803 cells. Maximum effect was observed with 10 nmol/L SDF-1 ($^aP < 0.05$).

RESULTS

Effect of SDF-1 on gastric cancer cell proliferation

The effect of SDF-1 on cell proliferation was examined in gastric cancer cell lines SGC-7901and MGC-803. After incubation for 72 h, cell proliferation was significantly and dose-dependently enhanced by SDF-1 at concentrations from 0.1 to 200 nmol/L (Figure 1).

Effect of AMD3100 on SDF-1-induced migration of gastric cancer cells

SDF-1 stimulated migration of gastric cancer cells (Figure 2). Maximal effect was observed at 10 nmol/L SDF-1 in all gastric cancer cell lines. The inhibitory effect of AMD3100 on SDF-1-induced migration was tested. The migration induced by SDF-1 at 10 nmol/L was inhibited by AMD3100 in SGC-7901 and MGC-803 cells (Figure 3).

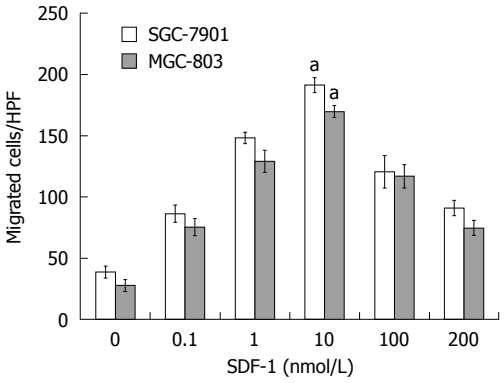

Figure 2 Effect of stromal cell-derived factor-1 on migration of gastric cancer cells. Stromal cell-derived factor-1 (SDF-1) stimulated migration of cancer cells. Maximum effect was observed at 10 nmol/L of SDF-1 ($^aP < 0.05$).

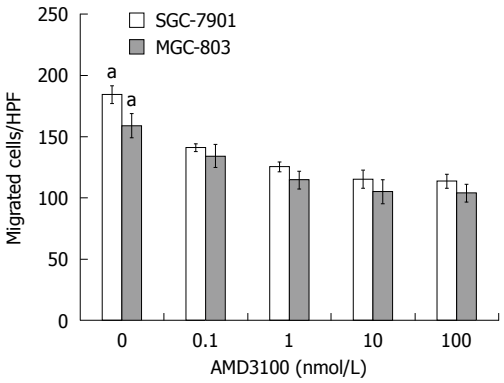

Figure 3 Effect of AMD3100 on stromal cell-derived factor-1-stimulated migration of gastric cancer cells. Gastric cancer cells were stimulated by stromal cell-derived factor-1 (SDF-1) at 10 nmol/L and various concentrations of AMD3100. Cell migration was decreased as the concentration of AMD3100 increased ($^aP < 0.05$).

Expression of CXCR4 in gastric cancer tissues and paired normal samples

In the normal gastric epithelium adjacent to the tumor, weak immunoreactivity for CXCR4 was detected in the non-neoplastic epithelial cells. In gastric cancer tissues, CXCR4 immunoreactivity was strong in cancer cells. Staining was observed predominantly in the cytoplasm and plasma membrane of tumor cells (Figure 4A). Twenty (50%) of the 40 gastric cancers were positive for CXCR4 expression at the invasive front, whereas only three (7.5%) of 40 normal mucous membranes were positive for CXCR4. The levels of CXCR4 mRNA were significantly higher in gastric cancers (1.624 ± 1.380) than in its normal counterpart (1.072 ± 0.524, $P = 0.015$) (Figure 5A and Table 2). The levels of CXCR4 mRNA were significantly higher in gastric cancers with lymph node metastasis (32/40) (0.823 ± 0.551) than in those without (8/40) (0.392 ± 0.338, $P = 0.042$) (Table 3).

Localization of CXCR4 proteins in gastric cancer cell lines

Total RNA from the gastric cancer cell lines SGC-7901 and MGC-803 was isolated using RNArose reagent (Fulin, Qingdao, China) according to the manufacturer's instructions. Reverse transcription PCR was carried out as described above. The PCR products were analyzed on a 2% agarose gel with ethidium bromide staining. We found that both SGC-7901 and MGC-803 cells expressed CXCR4 protein (Figure 5B).

Table 2 Expression of CXC chemokine receptor-4 in primary gastric carcinoma and normal mucous membrane (mean ± SE)

Tissues	No.	CXCR4-mRNA	*t*	*P*
Gastric cancer	40	1.6244 ± 1.3801	2.554	0.015
Normal mucous membrane	40	1.0715 ± 0.5243		

CXCR4: CXC chemokine receptor-4.

Table 3 Expression of CXC chemokine receptor-4 in primary gastric carcinoma with or without lymph nodes metastasis (mean ± SE)

Lymph node metastasis	No.	CXCR4-mRNA	*t*	*P*
Present	32	0.823 ± 0.551	2.101	0.042
Absent	8	0.392 ± 0.338		

CXCR4: CXC chemokine receptor-4.

Relationship between CXCR4 expression and clinicopathological features in gastric cancer

CXCR4 expression was significantly positive in gastric cancer with poor differentiation, high tumor stage and lymph node metastasis. However, other parameters, age, sex, tumor location and tumor size, had no significant relationship with CXCR4 expression. Clinical and pathological characteristics of patients are listed in Table 4.

Expression of SDF-1 in lymph nodes with or without cancer cell metastasis

Among the 40 lymph nodes that we collected, 24 (60%) had cancer cell metastasis and the remaining nodes were normal. Sixteen (66.7%) of 24 lymph nodes with cancer cell metastasis were positive for SDF-1 expression, whereas only 5/16 (31.3%) were positive in the lymph nodes without metastasis (Figure 4C and D). The levels of SDF-1 mRNA were also significantly higher in the lymph nodes with metastasis (0.5432 ± 0.4907) than in their normal counterparts (0.2640 ± 0.2601, $P = 0.025$) (Figure 5C and Table 5).

Correlation analysis of SDF-1 expression in lymph nodes and CXCR4 expression in gastric cancer

Pearson correlation analysis showed that the positive expression of SDF-1 mRNA in lymph node metastasis of gastric cancer was consistent with the positive expression of CXCR4 mRNA in gastric cancer ($r = 0.776$, $P < 0.01$).

DISCUSSION

The mechanisms of lymph node metastasis in gastric

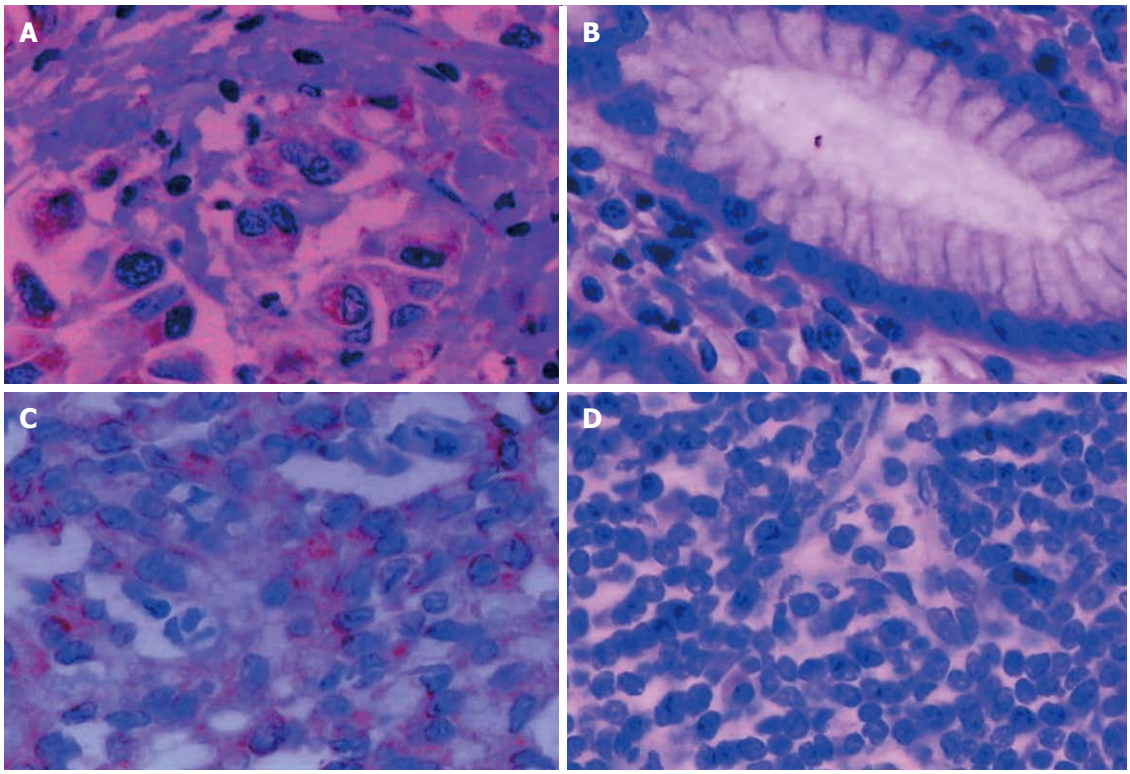

Figure 4 Expression of CXC chemokine receptor-4 in gastric carcinoma tissues and stromal cell-derived factor-1 in lymph nodes. A: CXC chemokine receptor-4 (CXCR4) protein was detected by immunohistochemistry in primary gastric carcinoma tissues; B: CXCR4 protein was not detected in normal mucous membrane; C: Stromal cell-derived factor-1 (SDF-1) protein was detected by immunohistochemistry in lymph nodes with gastric cancer cell metastasis; D: SDF-1 protein was not detected in normal lymph nodes (400 ×).

Table 4 Relationship between CXC chemokine receptor-4 expression and clinicopathological features in gastric cancer

Clinicopathologic parameters	No.	CXC chemokine receptor-4			
		Positive	Negative	χ^2	*P* value
Sex					
Male	31	18	13	0.006	0.938[2]
Female	9	6	3		
Age (yr)					
> 56	19	12	7	0.007	0.935[1]
≤ 56	21	13	8		
Tumor size (cm)					
≥ 5	25	16	9	0.064	0.800[1]
< 5	15	9	6		
Tumor location					
Cardia of stomach	2	2	0	-	0.811[3]
Fundus of stomach	1	1	0		
Body of stomach	6	4	2		
Antrum of stomach	31	18	13		
Differentiation					
Moderate/well	6	1	5	-	0.021[3]
Poor	34	24	10		
Lymph node metastasis					
Present	32	26	6	4.146	0.042[2]
Absent	8	3	5		
Stage					
Ⅱ and Ⅲa	23	8	15	5.013	0.025[1]
Ⅲb and Ⅳ	17	12	5		

[1]χ^2 test; [2]Modified χ^2 test; [3]Fisher exact test.

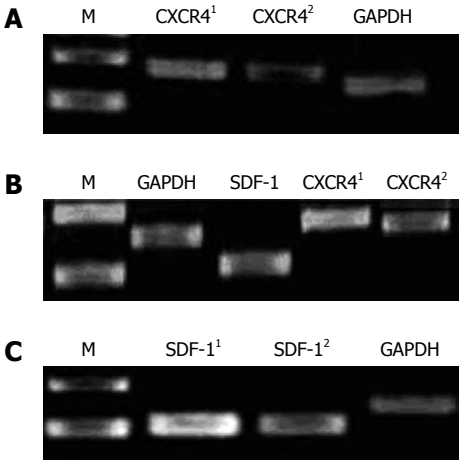

Figure 5 mRNA expression of CXC chemokine receptor-4 in gastric cancer cells, tumors and normal mucous membranes and of stromal cell-derived factor-1 in lymph nodes. A: CXCR[1]: The expression of CXC chemokine receptor-4 (CXCR4) in gastric carcinoma tissues; CXCR[2]: Expression of CXCR4 in normal mucous membrane; B: Stromal cell-derived factor-1 (SDF-1): Expression of SDF-1 in lymph nodes; CXCR[1]: Expression of CXCR4 in gastric cancer cell line SGC-7901; CXCR[2]: Expression of CXCR4 in gastric cancer cell line MGC-803; C: SDF-1[1]: Expression of SDF-1 in lymph nodes with gastric cancer cell metastasis; SDF-1[2]: Expression of SDF-1.

cancer are complex. The process involves the proteolysis of extracellular matrix, altered cancer cell adhesiveness, local invasion, angiogenesis, vascular dissemination, immune evasion and cancer cell survival in a new microenvironment. Some types of tumors show an organ-specific pattern of metastasis, and the "seed (cancer cells) and soil (factors in the organ environment)" hypothesis has been

Table 5 Expression of stromal cell-derived factor-1 in lymph nodes with or without gastric cancer cell metastasis (mean ± SE)

Lymph node metastasis	No.	SDF-1-mRNA	*t*	*P*
With	24	0.5432 ± 0.4907	2.338	0.025
Without	16	0.2640 ± 0.2601		

SDF-1: Stromal cell-derived factor-1.

introduced[14,15]. To date, the role of the CXCR4/SDF-1 signaling axis in the process of tumor metastasis has been extensively investigated. Most results have confirmed that increased expression of CXCR4 is mainly found in cancers, whereas SDF-1 tends to be overexpressed in normal tissues[16-18]. It has been reported that the signaling axis is involved in lymph node metastasis of breast cancer[13], colorectal cancer[19], nasopharyngeal cancer[20] and thyroid carcinoma[21], and also mediates melanoma metastasis to the lungs[22], prostate cancer metastasis to the bone[23], neuroblastoma metastasis to bone marrow[24], hepatocellular cancer metastasis to the bone[25], non-small cell lung cancer metastasis to the pleural space[26], and gastric cancer metastasis to the peritoneum[13]. Therefore, the CXCR4/SDF-1 signaling axis is essential for organ-specific metastasis, and has become a key determinant of tumor metastasis. The lymph nodes might also serve as the soil to promote the survival and proliferation of cancer cells that then cause lymph node metastasis.

Taking all of these results together, we hypothesize that CXCR4/SDF-1 interaction is generally important for lymph node metastasis of gastric cancer. In the present study, we found that CXCR4 was expressed in 50% of gastric cancers and CXCR4 was upregulated more in gastric cancer than in normal gastric tissues, which confirmed the previous data[13]. We also found a significant increase in SDF-1 mRNA in lymph nodes with cancer cell metastasis in comparison with normal lymph nodes, which confirmed that cancer cells can migrate towards an SDF-1 gradient established in specific target organs. It has been shown that higher levels of SDF-1 in target organs such as liver or lymph nodes attract and recruit cancer cells, which subsequently form lymph node metastases[16]. Therefore, these studies strongly support our hypothesis that the CXCR4/SDF-1 signaling axis plays an important role in the process of lymph nodes metastasis of gastric cancer. In supporting this idea, our clinicopathological study revealed that CXCR4 expression was significantly positive in gastric cancers with a high tumor stage and lymph nodes metastasis. No significant correlation between CXCR4 expression and other clinicopathological factors was found. Our study involved a limited group of patients, and more studies with a larger number of cases are necessary to determine the exact role of the CXCR4/SDF-1 axis in the development of lymph node metastasis of gastric cancer.

In our *in vitro* studies, expression of CXCR4 was also found in the gastric cancer cell lines SGC-7901 and MGC-803. The two cell lines showed significant chemotactic responses to SDF-1 in a dose-dependent manner and the chemotactic responses were significantly blocked by neutralizing anti-CXCR4 antibody. SDF-1 also significantly and dose-dependently enhanced cancer cell proliferation. A similar result has been found in several other tumor cell lines, including small cell lung cancer[27], prostate cancer[28], and squamous cell carcinoma of the neck[29]. In contrast, some studies have demonstrated that SDF-1 has no proliferative effects on pancreatic cancer cell lines[30], rhabdomyosarcoma[31], and lymphohematopoietic cells[32]. These differences may be due to the different culture system or the different target cells used.

It has been shown that chemokine receptor CCR7-positive carcinoma cells were detected in 42 (66%) of 64 cases, and that there was a significant difference in lymph node metastasis and lymphatic invasion between CCR7-positive and CCR7-negative cases, which indicates that CCR7 and its ligands interaction are associated with preferential lymph node metastasis of gastric carcinoma[33]. Arigami *et al*[34] have found recently that levels of combined CCR7 and CXCR4 expression are significantly correlated with lymph node metastasis. Similar results have been also observed in esophageal squamous cell carcinoma[35] and oral[36] squamous cell carcinoma. Additionally, nuclear factor-κB[37], c-erbB-2[38], hypoxia-inducible factor 1[39] or nitric oxide[40] can induce CXCR4 expression, which then plays an important role in lymph node metastasis. Therefore, there are certainly many more factors and/or signaling pathways than we thought that are involved in the process of lymph node metastasis and the exact mechanisms need further studies.

In conclusion, the results in this study indicate that the CXCR4/SDF-1 signaling axis appears to be involved in lymph node metastasis of gastric cancer. CXCR4 overexpression in primary gastric cancers might be an independent risk factor for lymph node metastasis. CXCR4 receptor antagonists can inhibit chemotactic behavior of gastric cancer cells. Based on these results, specific therapies with chemokine receptor antagonists could be helpful in the treatment of patients with gastric cancer metastasis.

COMMENTS

Background

Gastric cancer is one of the most commonly diagnosed malignant tumors. Most deaths from gastric cancer are caused by metastasis, of which lymph node metastasis is the most common cause, which leads to treatment failure. Therefore, inhibition of gastric cancer metastasis is thought to be an important therapeutic strategy. However, the molecular mechanisms involved in this process have not been fully elucidated.

Research frontiers

Many researchers have shown that CXC chemokine receptor-4 (CXCR4) seems to be the major chemokine receptor that is expressed in many types of cancer cells. The CXCR4/ and stromal cell-derived factor-1 (SDF-1) axis plays a major role in survival, proliferation, migration and adhesion of many kinds of tumor cells. However, most of the studies about SDF-1 and CXCR4 have been conducted *in vitro*, and the definitive pathophysiological function of the CXCR4/SDF-1 axis in lymph node metastasis of gastric cancer needs further research.

Innovations and breakthroughs

Recent reports have highlighted the importance of the CXCR4/SDF-1 axis in

cancer metastasis. This study has found that the CXCR4/SDF-1 axis is also involved in lymph node metastasis of gastric cancer. Furthermore, this *in vitro* study has suggested that CXCR4 receptor antagonists could suppress the proliferation and migration of gastric cancer cells.

Applications

By understanding how the CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric cancer, this study could represent a future strategy for therapeutic intervention in patients with lymph node metastasis from gastric cancer.

Terminology

Chemokines are a family of small heparin-binding and secretory proteins, and through interactions with their corresponding receptors, they can control and activate many types of cells. According to the position of the four conserved cysteine residues in the amino acid sequence, they are classified into four groups: CXC, CX3C, CC and C. SDF-1 is a member of the CXC subfamily. CXCR4 is the only receptor of SDF-1 and is expressed in many kinds of tumor cells.

Peer review

This is a nice manuscript with good data. The conclusions fit the data.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2397

World J Gastroenterol 2011 May 21; 17(19): 2397-2406
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Protective effects of ACLF sera on metabolic functions and proliferation of hepatocytes co-cultured with bone marrow MSCs *in vitro*

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Supported by the National Natural Science Foundation of China, No.30772129 and Jiangsu Provincial Key Medical Center for Hepatobiliary Disease, No. ZX200605
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Received: November 2, 2010 Revised: January 13, 2011
Accepted: January 20, 2011
Published online: May 21, 2011

Abstract

AIM: To investigate whether the function of hepatocytes co-cultured with bone marrow mesenchymal stem cells (MSCs) could be maintained in serum from acute-on-chronic liver failure (ACLF) patients.

METHODS: Hepatocyte supportive functions and cytotoxicity of sera from 18 patients with viral hepatitis B-induced ACLF and 18 healthy volunteers were evaluated for porcine hepatocytes co-cultured with MSCs and hepatocyte mono-layered culture, respectively. Chemokine profile was also examined for the normal serum and liver failure serum.

RESULTS: Hepatocyte growth factor (HGF) and Tumor necrosis factor; tumor necrosis factor (TNF)-α were remarkably elevated in response to ACLF while epidermal growth factor (EGF) and VEGF levels were significantly decreased. Liver failure serum samples induced a higher detachment rate, lower viability and decreased liver support functions in the homo-hepatocyte culture. Hepatocytes co-cultured with MSCs could tolerate the cytotoxicity of the serum from ACLF patients and had similar liver support functions compared with the hepatocytes cultured with healthy human serum *in vitro*. In addition, co-cultured hepatocytes maintained a proliferative capability despite of the insult from liver failure serum.

CONCLUSION: ACLF serum does not impair the cell morphology, viability, proliferation and overall metabolic capacities of hepatocyte co-cultured with MSCs *in vitro.*

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Key words: Acute-on-chronic liver failure serum; Primary hepatocytes; Bone marrow marrow mesenchymal stem cells; Co-culture; Hepatocyte-based modality

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Shi XL, Gu JY, Zhang Y, Han B, Xiao JQ, Yuan XW, Zhang N, Ding YT. Protective effects of ACLF sera on metabolic functions and proliferation of hepatocytes co-cultured with bone marrow

MSCs *in vitro*. *World J Gastroenterol* 2011; 17(19): 2397-2406
Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2397.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2397

INTRODUCTION

Patients with fulminant hepatic failure (FHF) or acute-on-chronic liver failure (ACLF) represent a major challenge with a high mortality rate despite advances in critical care[1]. Orthotopic liver transplantation (OLT) is one of the choices for the treatment of acute liver failure and end-stage liver diseases although the donors are still insufficient[2]. In addition, OLT is not suitable for the rapidly deteriorating patients due to the irreversible brain damage caused by the cerebral edema and intracranial hypertension. Therefore, alternative therapies are needed to stabilize such patients until an organ is available for transplantation[3]. In the last few years, the isolated, viable and functional cells have been used as a promising therapeutic tool[3]. Applications of cell-based therapy in liver diseases include the development of extracorporeal liver support devices, commonly known as bioartificial livers (BAL), and hepatocyte transplantation, which would ideally serve as a bridge to transplantation or liver regeneration via providing temporary hepatic support[4,5].

To date, several designs for BAL and hepatocyte transplantation have been completed or are being assessed in animal models and human trials[6-8]. It is generally agreed that none of the cell-based therapy currently available can be used as a well defined and practical option in clinical settings, although convincing evidences have validated the usefulness of these modalities in some biochemical parameters and clinical manifestations[6-8]. This is primarily due to the accumulation of a wide range of putative toxic substances within the circulation of hepatic failure patients, which potentially diminish the efficacy of BAL devices or cell transplant[9-10]. *In vitro* studies have demonstrated that serum of patients with acute hepatic failure or FHF can inhibit DNA, RNA and protein synthesis and disrupt cellular integrity, ion transport and metabolic functions in hepatocytes[9-13]. Given that most of the studies concerning the effects of patients' plasma or serum on liver cells use monolayer-cultured hepatocytes or transformed cell lines, and direct contact exists between the patient's serum, plasma or blood and the exogenous hepatocytes in the current cell-based therapy, it is necessary to investigate whether three-dimensional (3-D) hepatocyte culture system could resist the cytotoxicity of circulating inhibitory factors in the serum or plasma of the liver failure patients[14-15].

In our previous studies, we reported a new-brand BAL configuration for the development of a 3-D porcine hepatocyte culture system by co-culturing with bone marrow mesenchymal stem cells (MSCs) *in vitro*[16-18]. Inductions of albumin production, urea synthesis and cytochrome P4503A1 activities were maximally achieved at a seeding ratio of 2/1 for hepatocytes versus MSCs[17]. Hepatocytes co-cultured with MSCs were largely accumulated in the G2-S phase, while less accumulated in the G0-G1 phase compared with the control[17]. In addition, high levels of diversified extracellular matrix (ECM) and soluble cytokines synthesis were confirmed within the distinct cells in hepatocyte homeostasis[17,18]. The aim of the present study was to investigate the *in vitro* effects of the sera from patients with viral hepatitis B-induced ACLF in China on the metabolic functions and proliferation of 3-D hepatocytes spheroids by co-culturing with bone marrow MSCs, which to a great extent approaches to the clinical reality when extracorporeal BAL and hepatocyte transplantation occur.

MATERIALS AND METHODS

Animals and reagents

Three outbred white female pigs (15-20 kg) received humane care and all animal procedures were approved by the Animal Care Ethics Committee of Nanjing Drum Tower Hospital, and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes for Health (NIH Publication 85-23 revised 1996). All chemicals were of analytical grade and purchased from GIBCO (Grand Island, NY) unless otherwise stated.

Human plasma samples

Serum samples were obtained from 18 male patients with ACLF due to severe viral hepatitis B at the onset of plasmapheresis or plasma exchange. Diagnosis of these patients was based on the criteria of severe hepatitis described in the Viral Hepatitis Protection, and the Cure Guideline established by the Chinese Society of Infection and Hepatology. Informed consent was obtained from all the study subjects. A sample of blood was collected from the patients for measurement of biochemical parameters (Table 1), and normal serum was collected from 18 healthy volunteers.

Chemokine assays

Chemokines in human serum were quantified using the following commercially available enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's instructions: hepatocyte growth factor (HGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and tumor necrosis factor-α (TNF-α) (Biosource International, Camarillo, CA).

Porcine MSCs isolation and characterization

Porcine MSCs were isolated by bone marrow aspirates from the iliac crest of pigs as described previously[16-18]. The surface marker of the cultured MSCs was determined by fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against CD45 (Antigenix America, Huntington Station, NY) and phycoerythrin (PE)-conjugated antibodies against CD29 (VMRD, Pullman, WA), CD44 and CD90 (Becton Dickinson) using a FACScan (Becton Dickinson, San Diego, CA). Isotypic antibodies served as controls.

Porcine hepatocyte isolation and culture

Primary hepatocytes were harvested by a two-step *in situ* collagenase perfusion technique[16-18]. The viability of the isolated hepatocytes determined by Trypan blue exclusion

Table 1 Biochemical parameters in plasma samples of 18 patients with acute-on-chronic liver failure

Case No.	Tbil (μ mol/L)	Dbil (μ mol/L)	ALB (g/L)	ALT (U/L)	AST (U/L)	CHE (U/L)	ALP (U/L)	LDH (U/L)	γ-GT (U/L)
1	306	168	34	218	342	3449	152	218	69
2	489	278	32	96	113	2896	136	288	74
3	456	281	36	202	315	1813	223	278	86
4	889	483	39	156	155	2224	195	279	49
5	400	202	33	65	104	3002	170	202	34
6	342	179	31	184	237	2408	138	197	42
7	456	263	33	261	158	1565	148	222	58
8	324	205	28	139	178	2672	125	234	36
9	591	366	32	87	101	2606	189	246	96
10	569	286	38	93	67	4737	121	266	104
11	425	268	43	187	108	5732	131	138	109
12	396	199	32	56	138	2783	138	277	38
13	499	313	34	29	98	4157	294	288	47
14	558	298	31	412	219	2025	104	245	99
15	239	151	28	235	236	1655	136	452	189
16	199	103	40	2343	2773	3858	147	1154	173
17	319	167	33	144	153	1698	116	276	93
18	301	222	33	64	95	3317	99	150	111

ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; CHE: Concentrations of acetylcholine; LDH: Lactate dehydrogenase.

was higher than 95%. The percentage of nonparenchymal cells, as judged by their size (< 10 μm in diameter) and morphology (nonpolygonal or stellate), was less than 1%, which was also verified by immunocytochemical analysis of albumin and cytokeratin 18.

Establishment of homo-culture and co-culture system

To culture the pure primary hepatocytes, serum free DMEM-LG was removed 4 h after seeding 4×10^5 cells per well onto the 6-well culture plates (Corning Incorporated, Corning, NY), and 10% fetal bovine serum (FBS) fresh medium with 1 mm dexamethasone, 5 mg/mL insulin, 5 mg/mL transferrin, 5 mg/mL selenium, 100 IU/mL penicillin and 100 mg/mL streptomycin were added and exchanged every day thereafter. In heterotypic culture system, hepatocytes (4×10^5) were co-cultured with MSCs (2×10^5) during passages 3-5 in the 6-well culture plates.

Intervention with human serum

The culture medium was replaced with fresh FBS-free medium containing pooled normal serum or ACLF serum at concentrations ranging from 10% to 100% for 24 h. To closely simulate BAL intervention and exclude the direct contact between porcine cells and immunoglobulin as well as complements derived from patients, human serum was co-cultured using a 35-mm Millicell culture insert (Millipore, Bedford, MA). Cell morphology, albumin expression levels, and cell cycles in different groups were measured to determine the optimal concentration of human serum for culturing hepatocytes. All the medium samples were subsequently collected and stored at -80℃ for biochemical analysis, which represented the porcine hepatocyte-specific support functions on human serum.

Cell cycle analysis

For cell cycle analysis, MSCs pre-labeled with 10 μm solution of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37℃ were incubated in fresh medium for 30 min prior to co-culture with hepatocytes. Subsequently, cells of homo-culture and co-culture subgroups were stained with the CycleTEST PLUS DNA reagent kit (Becton Dickinson) according to the manufacturer's instructions, respectively. The cell cycle profiles of samples were analyzed by FACScan (Becton Dickinson).

Biochemical parameters

The concentrations of acetylcholine (CHE), glutamine (Gln), superoxide dismutase (SOD) and glucose (Glu) in medium samples were measured by commercial kits (Jiancheng Bioengineering, Nanjing, China) according to the manufacturer's instructions. Secreted albumin in the culture medium was quantified by ELISA using purified goat anti-albumin and horseradish peroxidase-conjugated antibodies (Bethyl Laboratories, Montgomery, TX).

Incubation with FBS

Both hepatocyte homo-culture and co-culture groups with the determined optimal concentration of serum from normal population or liver failure patients were thereafter replaced with fresh DMEM-LG supplemented with 10% FBS and re-incubated for another 24 h.

Transmission electron microscopy

Transmission electron microscopy (TEM) analysis was performed according to the standard protocol.

Hepatocyte viability

A live/dead assay was performed using calcein-AM and SYTOX Orange dye (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) staining was used for visualization

Table 2 Chemokine profiles in liver failure serum *vs* normal serum

	NS group (n = 18)	FS group (n = 18)	P value
HGF (pg/mL)	469.47 ± 134.87	5253.70 ± 1876.35	0.002
EGF (pg/mL)	282.56 ± 41.55	74.63 ± 18.36	0
VEGF (pg/mL)	99.27 ± 19.42	ND	0
TNF-α	66.48 ± 28.56	168.86 ± 38.69	0.001

NS: Normal serum; FS: Liver failure serum; ND: Undetectable; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-alpha.

of nuclei. Cells were visualized under a fluorescence microscope (Zeiss, Jena, Germany).

Cytotoxicity assay

Lactate dehydrogenase (LDH) activities in culture media were measured with a kit (Jiancheng Bioengineering, Nanjing, China) in accordance with the manufacturer's instructions.

Fluorescence-activated cell sorting

To separate hepatocyte from co-cultures, $CD44^-CD45^-$ cell population was sorted out using a fluorescence-activated cell sorting (FACS) caliber flow cytometer (Becton Dickinson). Briefly, cell suspension was simultaneously incubated with FITC-conjugated monoclonal antibody against CD45 and PE-conjugated antibody against CD44 at room temperature for 15 min. CD45 negative cells were gated and then analyzed for red fluorescence. Hepatocytes and MSCs alone served as controls, respectively.

Western blotting

Western blotting was performed using anti-PCNA monoclonal primary antibody (Chemicon, Melbourne, Australia) and HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL) were used for visualization.

Statistical analysis

Means of three independent experiments were reported. The two-tailed unpaired Student's t test or one-way analysis of variance was used to evaluate the statistical differences.

RESULTS

Differential expression of chemokines in normal and liver failure serum

HGF and tumor necrosis factor (TNF)-α level of liver failure serum (5253.70 ± 1876.35 and 168.86 ± 38.69 pg/mL) (mean ± SD) was significantly higher than that of healthy human serum (469.47 ± 134.87 and 66.48 ± 28.56 pg/mL) (P = 0.002 and 0.001). In contrast, other two growth factors, EGF and VEGF, in the serum of ACLF patients (74.63 ± 18.36 pg/mL and undetectable) were lower than those in the normal serum (282.56 ± 41.55 pg/mL and 99.27 ± 19.42 pg/mL) (P = 0.000) (Table 2).

Table 3 Hepatocyte ratio of G2-S phase in Hep-F group and Co-F group

	Concentration of serum %					
	10	20	40	60	80	100
G2-S phase in Hep-F group	9.26	13.47	18.66	23.43	13.99	7.81
G2-S phase in Co-F group	16.02	23.28	28.25	32.08	31.24	9.94

Hep-F: Homo-hepatocytes cultured by various concentrations of liver failure serum; Co-F: Albumin in the medium of both liver failure serum; G2-S: Gap 2-Synthesis.

Optimal concentration of human serum for co-culturing hepatocytes

The morphology of hepatocyte homo-culture underwent a dramatic change (rounding and detachment) after 24 h of culture in the medium containing 10% liver failure serum. With increasing serum concentrations in the culture medium, less cell sheet exposure to 100% liver failure serum could constitute a confluent monolayer and the membranes of the hepatocytes became indistinct (Figure 1A). On the other hand, hepatocytes co-cultured with MSCs maintained anchored spherical multi-cellular aggregates and displayed minor difference when incubated with different concentrations of liver failure serum (Figure 1B), indicating that 3-D hepatocyte spheroids co-cultured with MSCs were not affected by liver failure serum upon 24 h incubation. There was no significant difference of albumin secretion between homo-hepatocytes cultured by various concentrations of liver failure serum (Hep-F) and those cultured by normal serum (Hep-N) (Figure 1C). As for the co-culture system, the albumin in the medium of both liver failure serum (Co-F) and normal serum (Co-N) groups were significantly higher than those of Hep-F and Hep-N (P < 0.01). When the concentration of serum reached or surpassed 40%, albumin in the medium of Co-F group was significantly higher than that in the Co-N group (P < 0.05) (Figure 1C). The largest amount of albumin secretion was observed in the co-cultured hepatocytes with 60% of serum (P < 0.01) (Figure 1C). The ratio of G2-S phase cells in Hep-F group ranged from 7.81% to 23.43%, while larger populations of CFSE-negative cells (hepatocytes) were accumulated in G2-S phase in response to MSCs, peaking in the 60% serum subgroup with 32.08% (Table 3). Taken together, 60% liver failure serum in culture medium was used for the following investigations.

Hepatocyte-specific support functions of MSCs on human serum

To investigate the hepatocyte's supportive functions when incubated with liver failure serum, the metabolic capacity of medium samples from the 60% serum subgroups upon the first 24 h incubation was determined. As shown in Figure 2A, CHE activity in Hep-F and Co-F groups was significantly lower than that in the Hep-N and Co-N groups, respectively (P = 0.000) (Figure 2A). Fortunately, CHE activity in Co-F was 2-folds that in Hep-F (123.90 ± 28.15 U/mL *vs* 60.53 ± 13.97 U/mL, P = 0.043). There was no significant difference of Gln synthesis between Co-N and Co-F

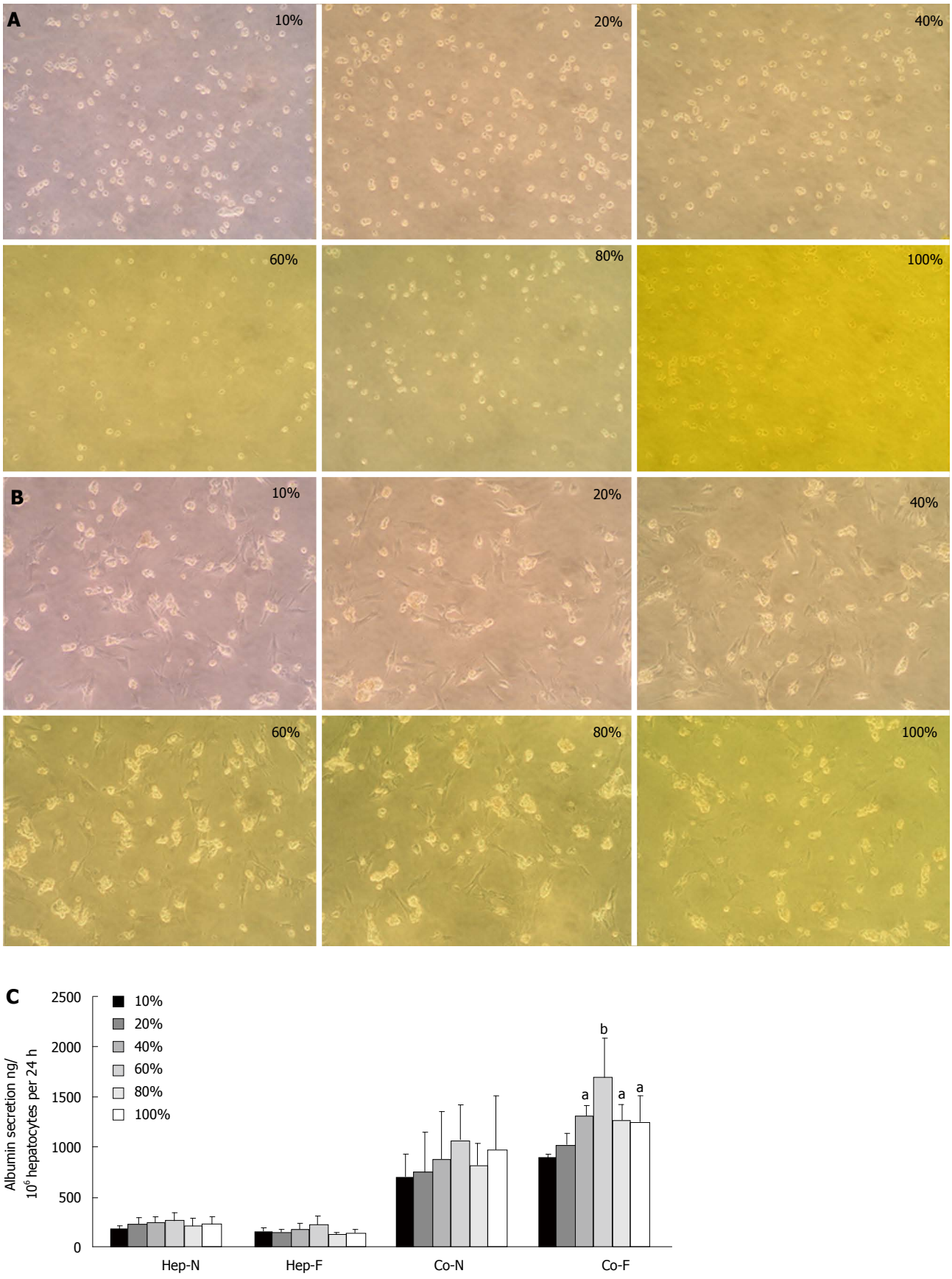

Figure 1 Optimal serum concentrations for co-cultured hepatocytes. A: Morphology of homo-cultured hepatocytes at different concentrations of liver failure serum (magnification x 100); B: Morphology of hepatocytes co-cultured with mesenchymal stem cells (MSCs) at different concentrations of liver failure serum (magnification x 100); C: Albumin secretion in hepatocytes homo-cultured with normal serum (Hep-N), liver failure serum (Hep-F) and hepatocytes co-cultured with normal serum (Co-N) and liver failure serum (Co-F). [a]Represents the significant difference *vs* Co-N group; [b]Represents the largest significance *vs* Co-N group.

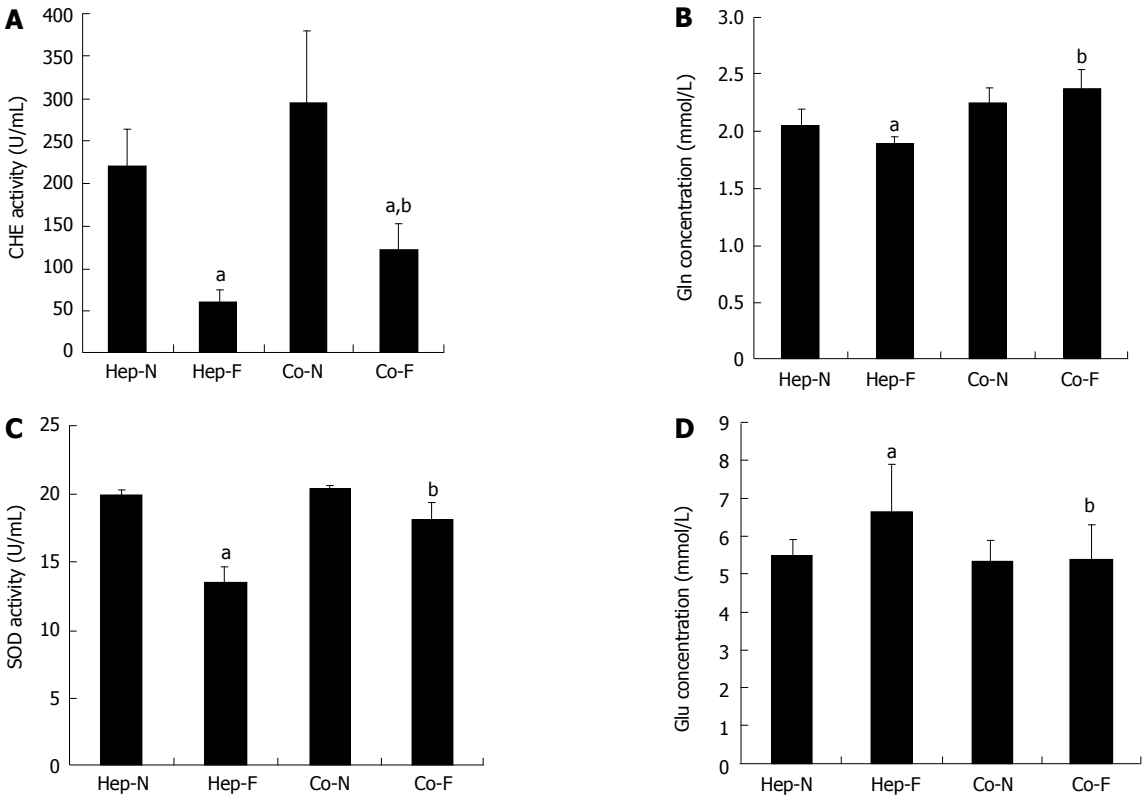

Figure 2 Hepatocyte-specific support functions in human serum. Concentrations of acetylcholine (CHE) activity (A), glutamine (Gln) synthesis (B), superoxide dismutase (SOD) activity (C) and glucose (Glu) concentrations (D) in the medium of each group. [a]Indicates significant difference *vs* Hep-N group; [b]Indicates significant difference *vs* homo-hepatocytes cultured by various concentrations of liver failure serum (Hep-F) group. Hep-N: Hepatocytes homo-cultured with normal serum; Co-N:Hepatocytes co-cultured with normal serum; Co-F: Liver failure serum.

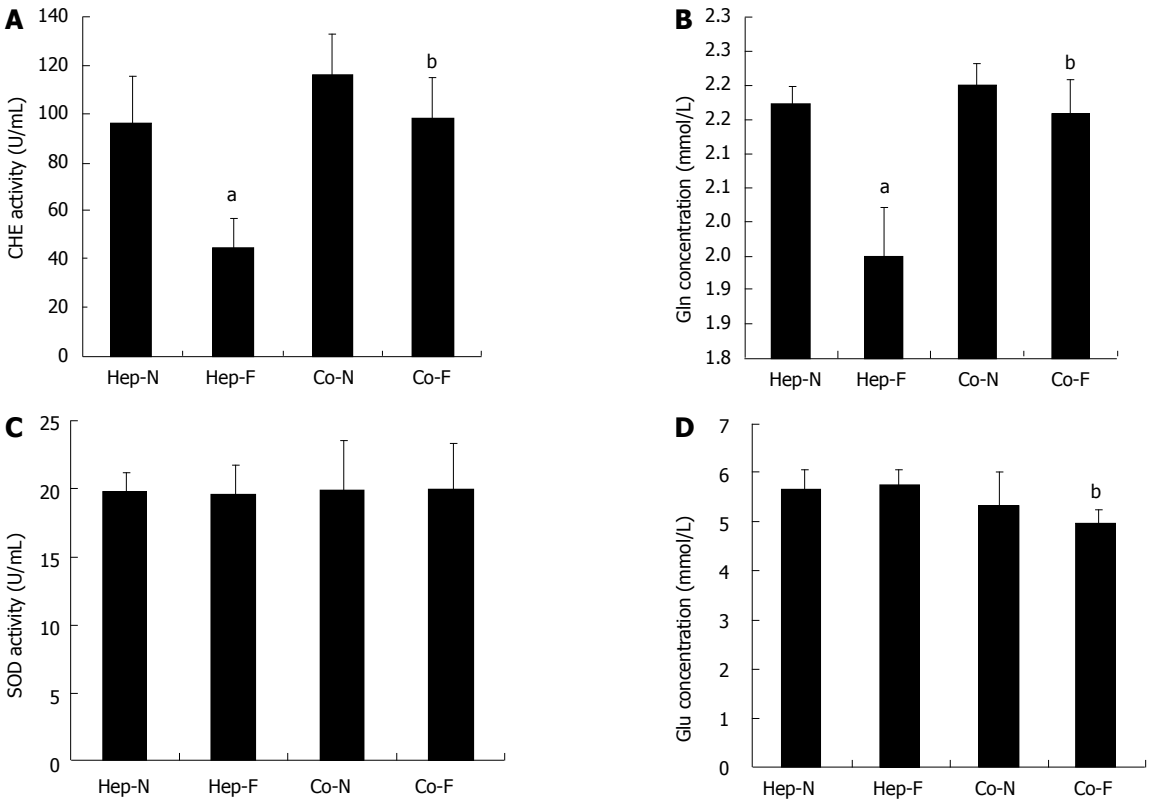

Figure 3 Post-incubation influence of liver failure serum on hepatocyte metabolic functions. Concentrations of acetylcholine (CHE) activity (A), glutamine (Gln) synthesis (B), superoxide dismutase (SOD) activity (C) and glucose (Glu) concentrations (D) in the medium of each group. [a]Indicates significant difference *vs* hepatocytes homo-cultured with normal serum (Hep-N) group; [b]Indicates significant difference *vs* homo-hepatocytes cultured by various concentrations of liver failure serum (Hep-F) group. Co-N:Hepatocytes co-cultured with normal serum; Co-F: Liver failure serum.

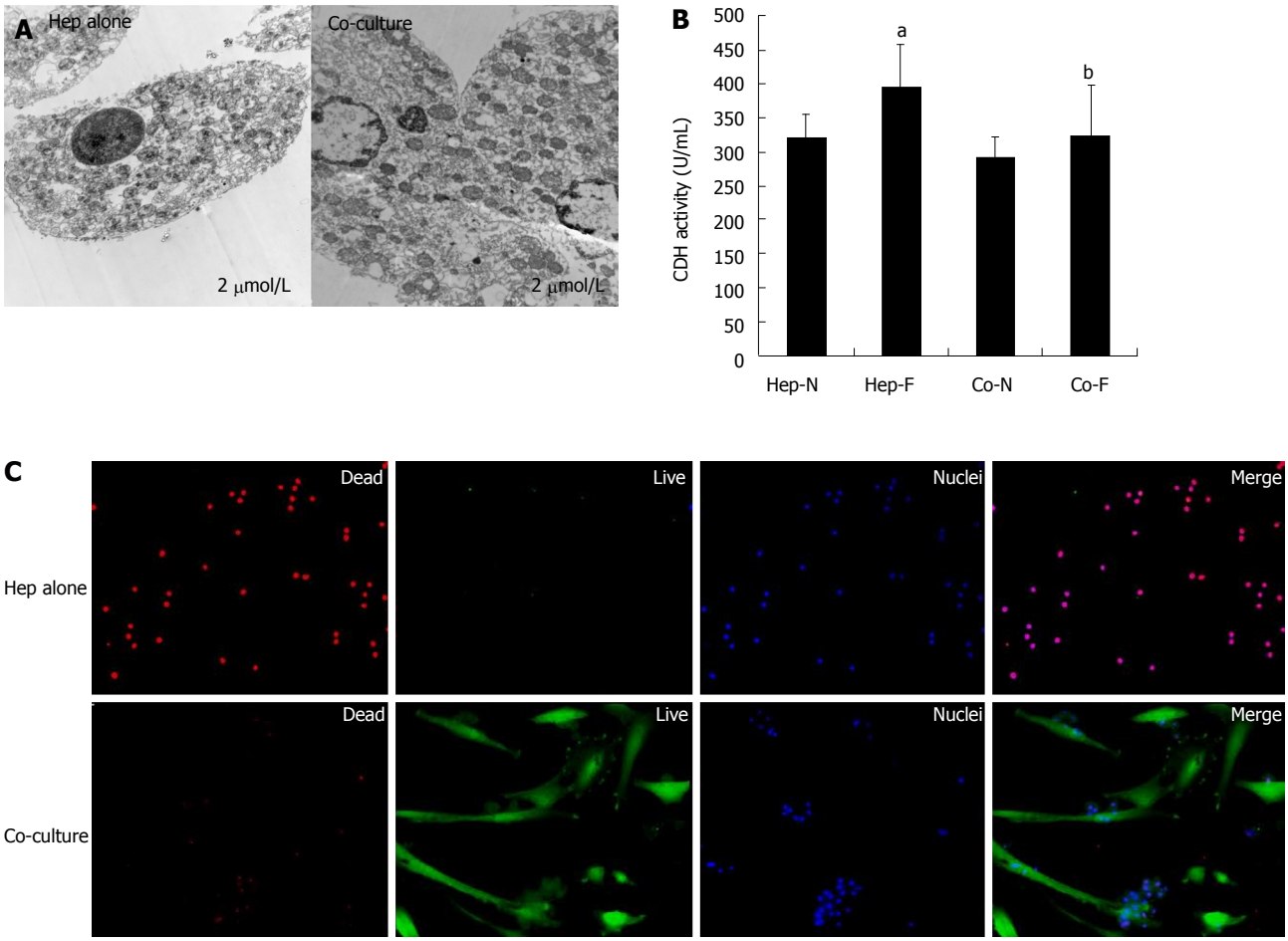

Figure 4 Cytotoxic effects of liver failure serum on hepatocytes cultured alone *vs* co-cultured hepatocytes. A: Transmission electron microscopy (TEM) analysis of the homo-cultured hepatocytes (left panel) and hepatocytes co-cultured with mesenchymal stem cells (MSCs) (righ panel) in the presence of 60% of liver failure serum; B: Lactate dehydrogenase (LDH) release assay of hepatocytes was indicated in each group; C: Live/dead assay of homo-cultured hepatocytes (upper panel) and hepatocytes co-cultured with MSCs (lower panel) in the presence of 60% of liver failure serum. [a]Indicates significant difference *vs* Hep-N group; [b]Indicates significant difference *vs* homo-hepatocytes cultured by various concentrations of liver failure serum (Hep-F) group. Hep-N: Hepatocytes homo-cultured with normal serum; Co-N:Hepatocytes co-cultured with normal serum; Co-F: Liver failure serum.

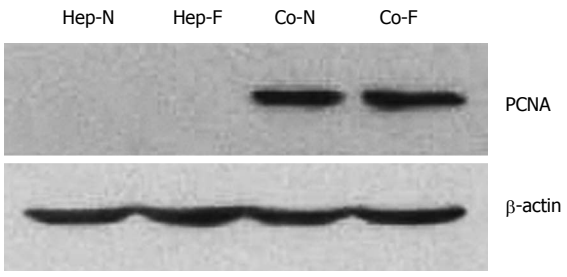

Figure 5 Increased proliferation after incubation with liver failure serum by co-culturing with mesenchymal stem cells. The proliferating cell nuclear antigen (PCNA) levels of hepatocytes cutured alone in Hep-N and Hep-F groups were too low to be detected while a significant increase was observed in the co-cultured groups irrespective of liver serum insult ($P < 0.05$). Statistical analysis with regard to PCNA level showed no significant difference between Co-N and Co-F groups ($P > 0.05$). Hep-N: Hepatocytes homo-cultured with normal serum; Co-N:Hepatocytes co-cultured with normal serum; Co-F: Liver failure serum.

groups ($P = 0.083$) (Figure 2B). Gln synthesis in Hep-F group was significantly lower than that in Hep-N group ($P = 0.033$) (Figure 2B). Gln concentration in Co-F group was significantly higher than in Hep-F group ($P = 0.000$) (Figure 2B). SOD activity in Hep-F group was significantly lower than that in Hep-N group ($P = 0.000$) (Figure 2C). There was no significant difference in the SOD activity between the Co-F group and Co-N group ($P = 0.088$) (Figure 2C). Furthermore, SOD activity in Co-F group was significantly increased compared with that in Hep-F group (18.15 ± 1.25 U/mL *vs* 13.49 ± 1.17 U/mL, $P = 0.001$) (Figure 2C). The concentration of Glu in the medium indirectly represented the Glu consumption by hepatocytes. The results showed that Glu in Hep-F group was significantly higher than that in the Hep-N group ($P = 0.041$) (Figure 2D), suggesting that liver failure serum delayed the glycometabolism of homo-hepatocytes. There was no significant difference of Glu between Co-F and Co-N groups ($P = 0.926$) (Figure 2D), suggesting that liver failure serum does not affect the glycometabolism of hepatocytes co-cultured with MSCs. Importantly, Glu concentration in Co-F group was significantly lower than that in Hep-F group (5.42 ± 0.91 *vs* 6.60 ± 1.28 mmol/L, $P = 0.026$), indicating that Glu consumption rate was improved in response to MSCs treatment.

Post-incubation influence of liver failure serum on hepatocyte metabolic functions

We further determined the effect on the hepatocyte metabolic functions after the second 24 h incubation with 10% FBS instead of human serum, which was defined as the post-incubation influence of liver failure serum on hepatocytes. As shown in Figure 3A, B and D, the pattern of CHE activity, Gln concentration and Glu content in the medium were similar to the effect of the first 24 h incubation with liver failure serum. Surprisingly, there was no significant difference of SOD activity in each group when human serum was changed into FBS ($P > 0.05$) (Figure 3C).

Cytotoxic effects of liver failure serum on homo-hepatocytes and co-cultured hepatocytes

TEM, dead/live assay and LDH leakage were used to determine the cytotoxic effects of liver failure serum on the homo-hepatocytes (Hep-F) and co-cultured hepatocytes (Co-F). Figure 4A displays quite a few occurrences of nuclear pyknosis, dilated endoplasmic reticulum, mitochondrial hydropic degeneration and large peroxisomes in the cytoplasm of hepatocytes in Hep-F group. On the contrary, hepatocytes co-cultured with MSCs maintained distinct membranes and cell ultrastructure, which was similar to that of hepatocytes *in vivo,* irrespective of presence or absence of liver failure serum (Figure 4A). Hepatocyte viability assay revealed that few hepatocytes were labeled with nonviable cell marker SYTOX (red nuclei) in co-culture spheroids and a large population of cells, including hepatocytes and MSCs, was stained with viable cell marker calcein-AM (green cytoplasm) (Figure 4B, lower panel). In contrast, almost all hepatocytes scattering in full field showed a lower viability in Hep-F group (Figure 4B, upper panel). In addition, LDH activity in Hep-F group was significantly higher than that in Hep-N group ($P = 0.034$) (Figure 4C). In contrast, there was no significant difference in the LDH activity between Co-N and Co-F groups ($P = 0.340$) (Figure 4C), indicating that co-cultured hepatocytes did not response to liver failure serum. Finally, LDH activity in Co-F group was significantly decreased compared with that in Hep-F group ($P = 0.036$) (Figure 4C).

Increased proliferation of hepatocytes upon co-culturing with MSCs

Proliferating cell nuclear antigen (PCNA) level is one of the markers of cell proliferation. The PCNA of hepatocyte in Hep-N and Hep-F groups was not detected by Western blot analysis (Figure 5). A significant amount of PCNA was detected in the hepatocytes of Co-F and Co-N groups (Figure 5). Statistical analysis showed that there was no significant difference in the PCNA level between Co-N group and Co-F group ($P > 0.05$).

DISCUSSION

Of the estimated 112 million persons with chronic hepatitis B in China, 15%-40% will eventually develop liver complications including liver cirrhosis, chronic hepatic failure and hepatocellular carcinoma[19]. Ideal treatment is still not available for severe viral hepatitis. Chronic severe hepatitis could cause ACLF with a mortality rate of >70% if liver transplantation cannot be performed[20]. Therefore, more and more attention has been paid to delaying the progression of ACLF by using BAL devices or hepatocyte transplantation in China and other Asian countries[21-23]. Porcine hepatocytes have been serving as the most ideal cell source for both preclinical and clinical liver support due to their physiological similarities with human hepatocytes and their availability[24-25].

A wide range of endogenous toxic metabolites as well as cytokines accumulate in the circulation of patients with liver failure[9-10]. Serum from acute liver failure or FHF patients inhibited the growth, proliferation and metabolic functions of various hepatocytes, e.g, primary hepatocytes[11], Hep G2 cells[9,10] and HHY41 cells[12] by interfering RNA, DNA and protein synthesis and inducing apoptosis. However, recent studies argued that serum from adult patients with acute liver failure did not have detrimental effects on the overall cell metabolic activity, protein synthesis, and cytochrome P450 activity in the freshly isolated human hepatocytes[13]. One of the reasons for interpreting the controversial results of these studies is that several hepatotrophic substances, e.g., cytokines and growth factors are elevated in the sera of patients with liver failure[26,27]. Therefore, the effect of liver failure serum on the liver or hepatocytes is the net result of the balance between circulating stimulatory molecules and inhibitory compounds.

To date, the greatest challenge to the restoration of well-functioning liver cells *in vitro* lies in maintaining the short-term viability and facilitating the rapid phenotypic de-differentiation of primary hepatocytes in standard mono-layered culture[28]. The maintenance of cell phenotype is dependent on the cellular physiological microenvironment *in vivo,* which consists of multiple signals including paracrine cytokines and neighboring cells[29,30]. Recently, hepatocytes are co-cultured with a range of different cell types *in vitro* to mimic the liver-like microenvironment[31]. Our previous studies provided the first *in vitro* 3-D co-culture system in which porcine hepatocytes were co-cultured with bone marrow MSCs. The morphology and functionality of the hepatocytes in this co-culturing system were well maintained and were superior to the hepatocyte homo-culture. In addition, the role of soluble factors derived from MSCs was confirmed as a vital component to facilitate hepatocyte homeostasis[16-18]. Therefore, studies are needed to determine whether such a robust co-culture system could maintain an ideal liver support functions *in vitro* and avoid the cytotoxicity of liver failure serum.

The pool of 18 liver failure serum samples induced a higher detachment rate, lower viability and decreased liver support functions of mono-layered hepatocytes, which is consistent with the main findings described previously[9,10]. In addition, presence of the post-incubation effect of liver failure serum suggested that some of the metabolic functions such as CHE activity and Gln concentration were still maintained at a lower level in hepatocyte homo-culture compared with the control group. In contrast, the newly developed 3-D hepatocyte co-culture system could tolerate the cytotoxicity of the serum from ACLF patients and

have a similar performance to that incubated with healthy human serum *in vitro*. These results demonstrated that co-culture system displayed a better advantage over the pure liver cells in hepatocyte-based therapy for end-stage liver diseases. Moreover, hepatocytes co-cultured with MSCs achieved a higher proliferation capacity despite of the presence of the liver failure serum.

Recent studies have shown that VEGF, also known as the vascular permeability factor, plays an essential role in liver regeneration[32-35]. It has been shown that expression of VEGF in both hepatocytes and non-parenchymal cells are increased during liver regeneration induced by partial hepatectomy[33,35]. Furthermore, exogenous VEGF administration after partial hepatectomy facilitates the proliferative activity in the injured liver[32,34]. In contrast, neutralization of VEGF significantly inhibits the proliferative activity in the liver during regeneration after partial hepatectomy[32]. Therefore, VEGF is not only an angiogenic factor but also plays a role in the survival of liver. On the other hand, the serum VEGF levels were increased in the patients with acute hepatitis compared with controls and other types of liver disease such as chronic hepatitis, fulminant hepatitis and cirrhosis[36]. In addition, serum VEGF in survivors of fulminant hepatitis was significantly increased in the recovery phase compared with the levels on admission. In the liver cirrhosis patients, serum VEGF levels were significantly lower than those of the control group, suggesting that serum VEGF level may be associated with hepatocyte regeneration grade[36]. More recently, Namisaki and coworkers reported that VEGF treatment could significantly reduce the mortality rate of acute severe liver injury and on-going acute hepatic failure in rats[37]. Thus, VEGF levels in serum may represent a predictor of clinical outcome for patients with hepatic failure[38]. In the present study, we compared the expression levels of some key chemokines related to liver regeneration in the liver failure serum with those in the healthy human serum. Our results revealed that HGF and TNF-α were remarkably elevated in response to ACLF while EGF and VEGF levels were significantly decreased, which may partially account for the inhibitory effect of liver failure serum on hepatocyte mono-layered culture rather than stimulatory role. Since multipotent MSCs are a stable source of VEGF-producing cells *in vivo* and *in vitro*[39,40], have the ability to trans-differentiate into hepatocyte-like cells under certain circumstances[41], and are immunosuppressive by modulating the immune function of the major cell populations involved in alloantigen recognition and elimination[42], our co-culture system may be a promising candidate in future cell-based therapy.

In conclusion, 3-D spheroid culture system by co-culturing primary hepatocytes with bone marrow MSCs can tolerate the cytotoxicity of ACLF serum and has a better performance in maintaining the function of hepatocytes. Hence, primary hepatocytes co-cultured with MSCs may be suitable for the application in clinical practice of BAL and hepatocyte transplantation.

COMMENTS

Background

Bioartificial livers (BAL) and hepatocyte transplantation are the cell-based therapies which served as a bridge to transplantation or liver regeneration via providing temporary hepatic support. However, none of the cell-based therapy currently available can be used as a well defined and practical option in clinical settings. This is primarily due to the accumulation of a wide range of putative toxic substances within the circulation of hepatic failure patients.

Research frontiers

Serum from acute liver failure or fulminant hepatic failure (FHF) patients inhibited the growth, proliferation and metabolic functions of various hepatocytes. The authors reported a new-brand BAL configuration for the development of a three-dimensional (3-D) porcine hepatocyte culture system by co-culturing with bone marrow mesenchymal stem cells (MSCs) *in vitro*. So it is necessary to investigate whether 3-D hepatocyte culture system could resist the cytotoxicity of circulating inhibitory factors in the serum or plasma from liver failure patients.

Innovations and breakthroughs

Although most of the studies concerning the effects of patients' plasma or serum on liver cells use monolayer-cultured hepatocytes or transformed cell lines, and direct contact exists between the patient's serum, plasma, or blood and the exogenous hepatocytes in the current cell-based therapy, 3-D hepatocyte culture system has not been investigated. This study focused on the *in vitro* effects of sera from patients with viral hepatitis B-induced acute-on-chronic liver failure (ACLF) in China on the metabolic functions and proliferation of 3-D hepatocytes spheroids by co-culturing with bone marrow MSCs.

Applications

The study found that 3-D spheroid culture system by co-culturing primary hepatocytes with bone marrow MSCs can tolerate the cytotoxicity of ACLF serum and has a better performance in maintaining the function of hepatocytes. It could be applied in clinical practice of BAL and hepatocyte transplantation.

Terminology

Three-dimensional (3-D) hepatocyte culture system is a culture system of porcine hepatocytes and bone marrow MSCs in a co-culture manner *in vitro*. Co-culture of hepatocytes with non-parenchymal cells is beneficial for optimizing cell functions via heterotypic interactions.

Peer review

This is an interesting study aimed to investigate whether the function of hepatocytes co-cultured with bone marrow mesenchymal stem cells could be maintained in the presence of serum from patients with acute-on-chronic liver failure.

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S- Editor Sun H **L- Editor** MaJY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2407

World J Gastroenterol 2011 May 21; 17(19): 2407-2410
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Incidence of brain metastasis in patients with esophageal carcinoma

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Received: June 4, 2010 Revised: November 25, 2010
Accepted: December 2, 2010
Published online: May 21, 2011

Abstract

AIM: To determine the incidence of brain metastasis in a contemporary group of patients with carcinoma of the esophagus.

METHODS: Retrospective analysis of 53 patients with esophageal carcinoma who received radiotherapy as a component of treatment between 1998 and 2007, including patient and tumor characteristics, and subsequent diagnosis of brain metastasis. The association between the histological type of esophageal cancer and the incidence of brain metastasis was assessed using Fisher's exact test.

RESULTS: Forty-four of the fifty-three patients in this study had adenocarcinoma and nine had squamous cell carcinoma, ranging from stage ⅡA-ⅣB. Primary treatment was surgery with neoadjuvant chemoradiotherapy (trimodality therapy) in 19% of patients; chemoradiotherapy in 42%; and surgery and adjuvant radiotherapy in 7%. Twenty-five percent of patients in this study received palliative radiotherapy. The overall incidence of brain metastasis in this cohort was 13%. Adenocarcinoma was the primary tumor histology in all of the patients who developed brain metastasis, representing an incidence of 16% in this subgroup. No patients with squamous cell carcinoma received trimodality therapy. The association between histology and brain metastasis was not statistically significant.

CONCLUSION: The incidence of brain metastasis in this contemporary cohort of patients with esophageal carcinoma is higher than previously reported and was confined to those with adenocarcinoma.

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Key words: Brain metastasis; Esophageal carcinoma

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Smith RS, Miller RC. Incidence of brain metastasis in patients with esophageal carcinoma. *World J Gastroenterol* 2011; 17(19): 2407-2410 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2407.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2407

INTRODUCTION

The incidence of esophageal cancer has been increasing in the United States over the past thirty years[1], with an expected 16 470 cases and 14 530 deaths in 2009[2,3]. Underlying this increase is a steady rise in the incidence of adenocarcinoma histology, surpassing squamous cell between 1990 and the early 2000s as the most common histology in the United States[1,4-6].

Brain metastasis secondary to esophageal carcinoma

is considered to be a rare event, with a reported incidence of between 0.5% and 4.8%[4,7-13]. However, many of these studies included patients from the 1980s and 1990s when squamous cell was the predominant histology. In patients with non-small cell carcinoma of lung, the risk of brain metastasis is correlated with histological type, ranging from 40% to 50% in those with adenocarcinoma, *vs* 10% to 20% among those with squamous cell carcinomas[14-18]. Whether the incidence of brain metastasis in patients with esophageal carcinoma will increase as the predominant histology shifts from squamous cell to adenocarcinoma is unknown. Our recent clinical experience suggests that the incidence of brain metastasis in patients with esophageal carcinoma is higher than what has been reported. Therefore, a review of our database of patients with esophageal carcinoma at this community-based cancer center was undertaken to determine the incidence of brain metastasis in a contemporary group of patients with this disease. All of the patients received radiotherapy as a component of their treatment, but none had a diagnosis or suspicion of brain metastasis at the time of initial consultation.

MATERIALS AND METHODS

After obtaining Institutional Review Board approval, the medical records of all patients with a primary diagnosis of esophageal carcinoma between 1998 and 2007 treated in the Department of Radiotherapy at our center (Mayo Health System practice) were reviewed. Patient demographics, clinical characteristics, treatment modality, diagnosis of brain metastasis, and survival data were collected. All cases of brain metastases were diagnosed by radiographic imaging (CT or MRI scan). The association between the histology of esophageal cancer and the incidence of brain metastasis was assessed using the Fisher's exact test, with *P*-value of less than 0.05 considered to be statistically significant.

RESULTS

The clinical characteristics of fifty-three patients with a diagnosis of carcinoma of the esophagus are shown in Table 1. None of the patients had a diagnosis or suspicion of brain metastasis at the time of referral for radiotherapy evaluation. Forty-four patients had adenocarcinoma as the primary histology. Staging information was unavailable for three patients, and tumor grade was not available for twelve patients. Primary treatment modality is also shown in Table 1, with the majority (42%) receiving definitive chemoradiotherapy. No patients with squamous cell carcinoma underwent trimodality therapy. Thirteen patients with stage IVB disease received palliative radiation only. Forty-nine of the 53 patients have died. The median length of survival was 14 mo (range 3 to 56 mo) for squamous cell carcinoma patients and 13 mo (range 2 to 72 mo) for those with adenocarcinoma.

Seven (13%) of the fifty-three patients in this review

Table 1 Summary of patient characteristics

Characteristics	
No. of patients	53
Age (mean, yr)	68 (40-92)
Gender	44 male, 9 female
Length of follow-up (mean, mo)	16, (2-72)
Histology of primary tumor	
Adenocarcinoma	44
Squamous cell carcinoma	9
Grade of tumor	
1	2
2	13
3	19
4	7
Unknown	12
Stage	
ⅡA	5
ⅡB	2
Ⅲ	18
ⅣA	8
ⅣB	17
Unknown	3
Treatment	
Chemo-radiation/surgery	10
Chemo-radiation	22
Surgery/chemo-radiation	4
Surgery/radiation	4
Radiation (palliative)	13
Sites of metastases	
Brain	7
Liver	14
Lung	1
Chest	1
Spine	3
Other	8

Table 2 Characteristics of patients with brain metastasis

Characteristics	
No. of patients	7
Age (mean, yr)	66 (57-75)
Gender	6 male, 1 female
Histology of primary tumor	
Adenocarcinoma	7
Squamous cell carcinoma	0
Grade of tumor	
2	1
3	3
4	2
Unknown	1
Stage	
ⅡA	1
Ⅲ	2
ⅣA	2
ⅣB	2
Treatment	
Chemo-radiation/surgery	3
Chemo-radiation	2
Surgery/chemo-radiation	1
Surgery/radiation	1
Radiation (palliative)	0
Time between original diagnosis and brain metastasis (mean, mo)	10, (2-25)

were subsequently diagnosed with brain metastases, all by radiographic imaging (CT or MRI scan). Characteristics of patients diagnosed with brain metastasis are shown in Table 2. Adenocarcinoma was the histology of the primary tumor in all seven patients, representing an incidence of 16% for the adenocarcinoma subgroup. The association between the histological type and brain metastasis was not statistically significant ($P = 0.24$). In six of these seven patients, the diagnosis of brain metastasis occurred within 11 mo of initial diagnosis of esophageal cancer.

DISCUSSION

This review identified a higher incidence of brain metastasis (13%) in a contemporary series of patients with carcinoma of the esophagus than has been previously reported[4,7-13]. The incidence of brain metastasis in those studies ranged from 0.5% to 4.8%. Information on the incidence of brain metastases by histology type is limited. Three studies from Japan reported the incidence of brain metastasis in patients diagnosed with esophageal cancer in the 1980s to early 2000s as ranging from 1.4%-1.5%[7,8,11]. Squamous cell carcinoma was the predominant histological type of esophageal cancer in Japan over that time period[7,11,19]. One of the studies concluded that although squamous cell was the primary tumor histology in over 95% of patients in Japan, the patients with brain metastasis "frequently exhibited other histologic types"[11].

Several studies in the United States also reported low incidence of brain metastasis in patients with esophageal cancer[1,4-6,10,13]. In a large series of patients from The University of Texas M.D. Anderson Cancer Center diagnosed with primary carcinoma of the esophagus between 1993 and 2001, adenocarcinoma was the predominant histology (68% of cases versus 25% with squamous cell carcinoma). The overall incidence of brain metastasis was 1.7% and the authors concluded that histology did "not appear to be a risk factor for the development of brain metastasis"[4]. However, Gabrielsen *et al*[13] reported a 3.6% incidence of brain metastasis in a review of 334 patients with esophageal carcinoma who underwent esophagectomy and concluded that there was a trend ($P = 0.16$) toward higher incidence of brain metastasis in those with adenocarcinoma. Although our results revealed the incidence of brain metastasis to be confined to those with adenocarcinoma, we could not demonstrate that histology was a significant risk factor for subsequent diagnosis of brain metastasis because of the small sample size, especially in the group with squamous cell carcinoma.

Rice and colleagues reported a correlation between adjuvant therapy and incidence of brain metastasis in a large series of patients with esophageal carcinoma from 1985 to 2002 who underwent esophagectomy with or without adjuvant therapy[10]. The overall incidence of brain metastasis was 3.8%; however, the rate at five years post treatment was 2.5% with surgery alone, and as high as 18.4% in patients who underwent surgery with both preoperative and postoperative therapy. Interestingly, all cases of brain metastasis occurred in patients with adenocarcinoma, consistent with the pattern seen in our study. The incidence for those receiving multi-modality therapy was similar to the 16% observed for the adenocarcinoma group in our study. This finding deserves more investigation, including whether treatment type influenced the subsequent development of brain metastasis or if multi-modality therapy may have been a surrogate for advanced stage (although this was not supported in a matched-pair analysis in the above study) and/or adenocarcinoma histology.

In a population based study from the Mayo Clinic, the median survival of patients with adenocarcinoma of the esophagus improved from 8.5 to 11.7 mo between 1971 and 2000; however, this was not deemed to be a significant change[20] and is similar to the median length of survival of 13 mo in our study. Therefore, it is not clear that the higher rate of brain metastasis reported in this study can be explained by patients living significantly longer with modern therapy. The results observed in this study might reflect a difference in underlying tumor biology and metastatic potential for CNS involvement between the two primary histologies. This would be similar to the pattern seen in patients with non-small cell lung carcinoma, where the incidence of brain metastasis is at least two-fold higher in those with adenocarcinoma versus squamous cell carcinoma[14-18].

The relatively short interval between diagnosis of the primary tumor and development of brain metastases noted in our study (median 7 mo) is similar to the 5.6 mo reported by Weinberg *et al*[4]. Furthermore, in the study by Rice *et al*[10], twenty of the twenty-nine patients who developed brain metastasis did so within one year following esophagectomy. Based on these findings, close attention to neurological signs and symptoms, with consideration of brain imaging as clinically indicated, should be a component of initial and follow-up evaluations in patients with esophageal cancer, especially in those with adenocarcinoma.

Limitations of this study include the retrospective nature and the relatively small sample size, precluding the ability to demonstrate statistical correlation with histology or treatment modality. In addition, this review was limited to patients who received radiotherapy as a component of their treatment; therefore, it may not be representative of all patients with esophageal cancer. However, none of the patients in our study had a diagnosis or suspicion of brain metastasis at the time of referral for radiotherapy evaluation.

In conclusion, this study revealed a higher incidence of brain metastasis than previously reported in patients with esophageal cancer, and occurred exclusively in those with adenocarcinoma, possibly identifying a trend that may increase as the incidence of this particular histology continues to rise. However, other factors, including gender, stage, and treatment type, could not be thoroughly assessed. Further research, to include a larger sample size in a contemporary group of patients with esophageal carcinoma, is required to confirm the findings in this study and to better understand the possible association between histology, patient characteristics, and/or treatment modality and the development of brain metastasis.

ACKNOWLEDGMENTS

We thank Qing Chen, PhD for assistance with the statistical analysis and Michael Haddock, MD for his thoughtful critique.

COMMENTS

Background

This is an original study analyzing the incidence of brain metastasis in patients with esophageal carcinoma. The study assessed whether the incidence is higher in a contemporary group of patients than previously reported.

Research frontiers

Previous studies have shown very low incidence of brain metastasis in patients with esophageal carcinoma when the primary histological type was squamous cell carcinoma. The predominant histology has shifted to adenocarcinoma; therefore, this study addressed the question of whether the incidence of brain metastasis is higher than when squamous cell was the predominant histology. This would be similar to the pattern seen in patients with non-small cell lung carcinoma, where the incidence of brain metastasis is two- to five-fold higher in patients with adenocarcinoma *versus* squamous cell carcinoma.

Innovation and breakthroughs

The authors reported a higher incidence of brain metastasis than previously reported and all patients with brain metastasis had adenocarcinoma. In addition, there was a relatively short interval between the diagnosis of the primary tumor and development of brain metastasis, similar to data reported by other studies.

Applications

The findings of this study highlight the need for attention to neurological signs and symptoms with consideration of brain imaging as clinically indicated, in patients with esophageal cancer, especially those with adenocarcinoma.

Peer review

This is an original study concerning the incidence of brain metastasis in patients with esophageal carcinoma. The topic is interesting, the number of cases is not negligible; the study is well described and provides indication for further researches. The references are updated.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2411

World J Gastroenterol 2011 May 21; 17(19): 2411-2416
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Resected specimen evaluation, anorectal manometry, endoanal ultrasonography and clinical follow-up after STARR procedures

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Received: April 15, 2010 Revised: May 8, 2010
Accepted: May 15, 2010
Published online: May 21, 2011

Abstract

AIM: To investigate stapled transanal rectal resection (STARR) procedures as surgical techniques for obstructed defecation syndrome (ODS) by analyzing specimen evaluation, anorectal manometry, endoanal ultrasonography and clinical follow-up.

METHODS: From January to December 2007, we have treated 30 patients. Fifteen treated with double PPH-01 staplers and 15 treated using new CCS 30 contour. Resected specimen were measured with respect to average surface and volume. All patients have been evaluated at 24 mo with clinical examination, anorectal manometry and endoanal ultrasonography.

RESULTS: Average surface in the CCS 30 group was 54.5 cm^2 statistically different when compared to the STARR group (36.92 cm^2). The average volume in the CCS 30 group was 29.8 cc, while in the PPH-01 it was 23.8 cc and difference was statistically significant. The mean hospital stay in the CCS 30 group was 3.1 d, while in the PPH-01 group the median hospital stay was 3.4 d. As regards the long-term follow-up, an overall satisfactory rate of 83.3% (25/30) was achieved. Endoanal ultrasonography performed 1 year following surgery was considered normal in both of the studied groups. Mean resting pressure was higher than the preoperative value (67.2 mmHg in the STARR group and 65.7 mmHg in the CCS30 group *vs* 54.7 mmHg and 55.3 mmHg, respectively). Resting and squeezing pressures were lower in those patients not satisfied, but data are not statistically significant.

CONCLUSION: The STARR procedure with two PPH-01 is a safe surgical procedure to correct ODS. The new Contour CCS 30 could help to increase the amount of the resected tissue without differences in early complications, post-operative pain and in hospital stay compared to the STARR with two PPH-01 technique.

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Key words: Stapled transanal rectal resection; Contour CCS; Obstructed defecation

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Naldini G, Cerullo G, Menconi C, Martellucci J, Orlandi S, Romano N, Rossi M. Resected specimen evaluation, anorectal manometry, endoanal ultrasonography and clinical follow-up after STARR procedures. *World J Gastroenterol* 2011; 17(19): 2411-2416 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2411.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2411

INTRODUCTION

The prevalence of constipation in adults ranges from 2% to 27% in North America, particularly over 65 years old and with a female predominance[1]. There are two major types of constipation: secondary constipation and primary functional constipation, which can be divided into slow transit constipation, constipation-predominant irritable bowel syndrome, and obstructed defecation. Approximately half of constipated patients suffer from obstructed defecation[1]. Obstructed defecation syndrome (ODS) is characterized by a cohort of symptoms including incomplete evacuation with painful effort, unsuccessful attempts with long period spent in bathroom, bleeding after defecation, use of perineal support and/or odd posture. ODS can also depend on intussusception of the rectal wall, extending into the anal canal, usually defined as internal prolapse. This condition is even frequently associated with rectocele[2]. Many different surgical techniques have been described in literature to correct ODS with important limitations and different patterns of post-operative complications[3]. The stapled transanal rectal resection (STARR) procedure is a surgical technique introduced to treat ODS due to rectocele and rectal intussusceptions and it has been demonstrated to be safe and effective[4,5]. Recently, a new device for the STARR procedure called the Curved Cutter Stapler 30 mm (CCS30) was developed by ETHICON ENDO-SURGERY Inc.® to correct ODS. The theoretical advantages of CCS30 include the ability to resect a single larger specimen and that it is more symmetrical than the other technique, with the avoidance of lateral "dog-ears".

The purpose of our study was to compare the specimen features (average volume and surface) obtained using the traditional two staplers technique (STARR) with Contour CCS 30 (Transtar), and to evaluate the early and late postoperative outcome. Moreover, we evaluated if a different resection (larger and more symmetric) may be associated with a higher complication rate or with differences in clinical, manometric and sonographic results at the 24 mo follow-up.

MATERIALS AND METHODS

Population under study and pre-operative assessment

From January to December 2007, 30 patients (all female; mean age 46.4 years old) suffering from ODS were treated in our department, 15 with double PPH-01 stapler and 15 with Contour CCS 30. All patients underwent a preoperative assessment mainly based on clinical evaluation, proctoscopy, defecography, anorectal manometry, and endoanal ultrasonography. A particular effort was made to investigate patient's obstetric and gynaecologist history, as well as previous anal or abdominal surgery. A colonoscopy was performed when malignant or inflammatory disease was suspected. Longo OD and Wexner scores were preoperatively filled in with all patients.

Inclusion criteria

Patients selected for surgery were those with: (1) failure of medical therapy (1.5 L/d of water, low-fiber diet, lactulose 10 g/d) with persistence of at least three of the following symptoms: feeling of incomplete evacuation, painful effort, unsuccessful attempts with long periods spent in bathroom, defecation with use of perineal support and/or odd posture, digital assistance, evacuation obtained only with use of enemas; and (2) at least two of the following findings at defecography: rectoanal intussusception extending 10 mm into the anal canal, rectocele deeper than 3 cm on straining or entrapping barium contrast after defecation. The presence of hemorrhoids was not a contraindication to the operation[3].

Exclusion criteria

Patients with non-relaxing puborectalis muscle at defecography, with synchronous genital prolapse, or cystocele requiring associated transvaginal operations, fecal incontinence, mental disorders, or general contraindications to surgery were excluded[3]. Patients with pelvic floor dyssynergia confirmed by clinical and instrumental evaluation were treated with pelvic floor training.

All patients gave informed, written consent. In the first phase, patients were operated on with STARR procedure (double PPH-01) while Contour Transtar was used as incoming technique in a second instance so that a total of 30 patients were enrolled in the present study, equally distributed according to the two techniques described (15 STARR with double PPH-01 and 15 Transtar with CCS 30). All surgical procedures were carried out in the lithotomy position by a single senior surgeon (GN). Preoperative enema and antibiotic prophylaxis with intravenous Metronidazole 500 mg were performed.

Spinal or general anaesthesia were both carried out and a particular effort was made to the muscle curarization in order to avoid, with a constant muscular relaxation, a sudden sphincter stretching during surgery.

Surgical techniques and specimen measurement

PPH-STARR was performed using two PPH 01-stapling devices (ETHICON Endosurgery, Cincinnati, USA) as described elsewhere[3]. In addition, 2/0 vicryl sutures were used to oversew the staple line in order to reduce postoperative bleeding (Figure 1). For the Contour Transtar-procedure, a CCS-30 stapler kit (ETHICON Endosurgery, Cincinnati, USA) was used. Briefly, the procedure starts with gentle dilatation of the anus and insertion of the circular anal dilator (CAD), which is then fixed to the perineum with 4 sutures. The internal prolapse is verified by insertion and withdrawal of a gauze swab. Starting at the 2 o'clock position, a 2/0 Vicryl traction suture is placed at the apex of the prolapse. A further 4 traction sutures are then placed around the circumference of the prolapse. A final suture to mark the site and depth of the first stapler firing is placed at the 3 o'clock position. The Contour Transtar device is introduced into the rectum

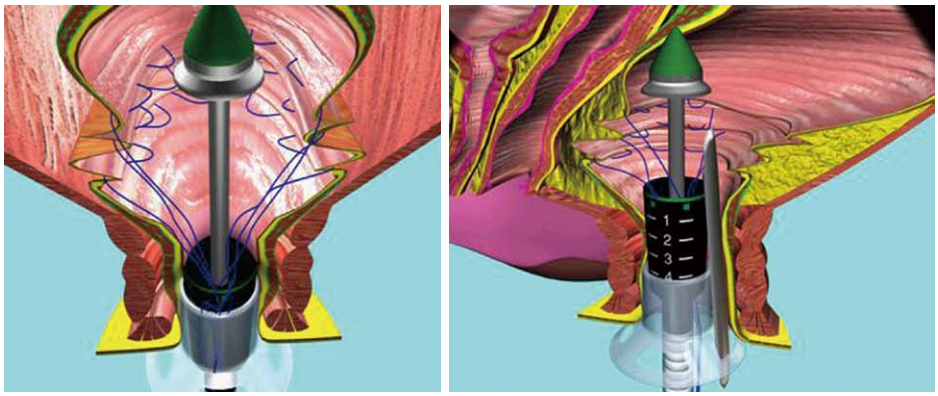

Figure 1 Stapled transanal rectal resection with double PPH-01.

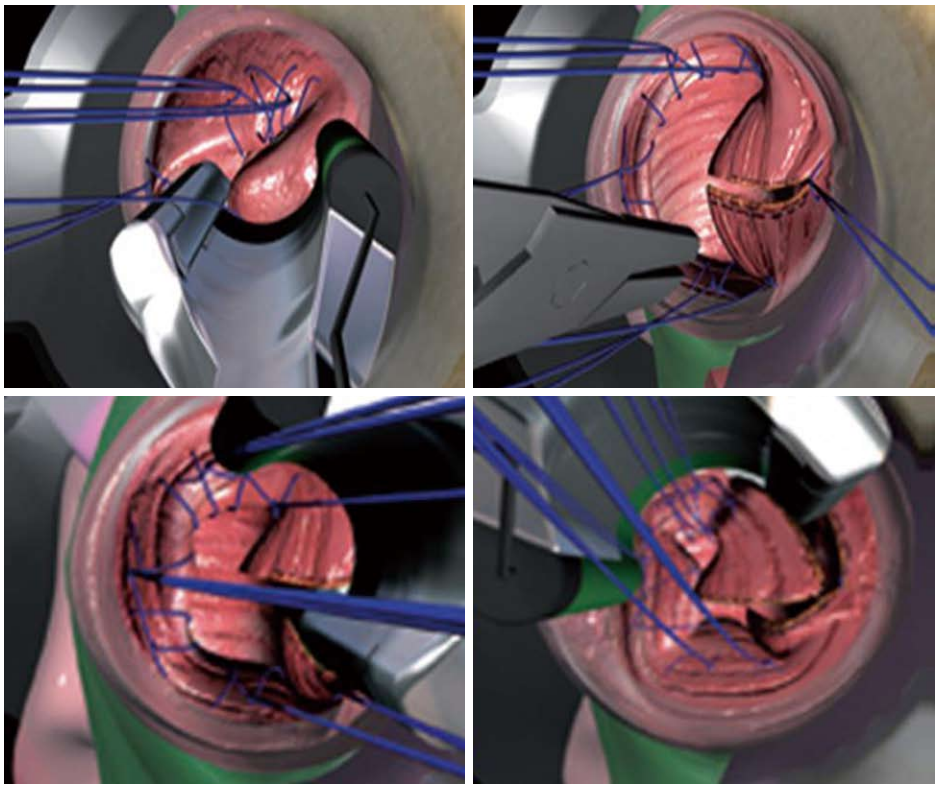

Figure 2 Stapled transanal rectal resection with contour CCS 30.

and the prolapse at the 3 o'clock position and pulled into the jaws using the previously placed traction suture and marking suture. The stapler is closed and, after checking the vagina for inadvertent incorporation of the vaginal mucosa, the first stapler firing is performed to produce a radial cut into the prolapse. After changing the stapler cartridge, the device is re-introduced into the rectum and sequential circumferential resection of the prolapse is performed using 4 to 5 separate stapler firings to complete the resection. In order to secure haemostasis, single 2/0 vicryl stitches were used to under-run the staple line. Finally an easy-flow-drainage is placed in the anus as an indicator of bleeding (Figure 2).

With concern to the specimen, it was opened and extended so that surface and volume could be measured. The volume was measured using a controlled volume jar and obtained by variations in the volume of the jar.

Post-operative management and follow-up

Patients were treated with a standard protocol for pain control with intramuscular Ketorolac 30 mg and intravenous paracetamol 10 mg as a rescue dose. The post operative pain was evaluated with the Visual Analogical Scale (VAS) at the first and second day after the surgical procedure (twice a day).

All patients were prospectively evaluated after 7 d and 1 mo from the discharge. As regards long term follow-up, patients were asked to be clinically evaluated at 12 mo following surgery and all agreed. Moreover, they were all contacted and evaluated again 24 mo after surgery. Clini-

Table 1 Specimen and clinical data of the two groups under study

	STARR with double PPH-1 (15)	STARR with CCS 30 (15)
Specimen (mean)		
Surface (cm^2)	36.9	54.5[1]
Volume (cc)	23.8	29.8[1]
VAS postoperative score (mean)		
1st day	2.55	1.3
2nd day	0.95	1.1
Post-operative morbidity	None	1 (3.3%)[2]
Hospital stay (mean)	3.4	3.1

[1]Comparison is statistically significant ($P < 0.05$); [2]One case of acute bleeding. Stapled transanal rectal resection (STARR) with PPH-1 *vs* STARR with CCS 30. VAS: Visual Analogical Scale.

Table 2 Clinical and manometric results after 1 year of follow-up (30 patients)

	STARR with double PPH-1 (15)	STARR with CCS 30 (15)
Longo score		
Preoperative	19.07	20.05
Postoperative	5.06[1]	6.04[1]
Postoperative pressures (mean)		
Resting	67.2	65.7
Squeezing	118.2	120.9
Compliance (%)		
Normal	4 (26.6)	4 (26.6)
Reduced	7 (50)	6 (40)
Increased	4 (26.6)	5 (33.3)

[1]Comparison is statistically significant ($P < 0.05$). STARR: Stapled transanal rectal resection.

cal examination and Longo OD/Wexner questionnaires were achieved as well as endoanal ultrasonography (Bruel and Kjaer 10 MHz 3-D rotating probe) and anorectal manometry.

Statistical analysis

All variables including demographic, clinical, operative and manometric findings were recorded and statistically analysed. Analyses were performed using the Statistical Package for the Social Sciences, SPSS 17.0 for Windows, XP, SPSS Inc., Chicago IL. Log-rank test was used to assess the difference between the groups. A *P* value less than 0.05 was considered significant.

RESULTS

The two groups under study (15 patients operated on with double PPH-01 and 15 patients with CCS 30) were homogeneous with respect to the demographic (mean age) and clinical data (obstetric/gynaecologist and general history; clinical examination). With regards to the resected specimen, average surface in the CCS 30 group was 54.5 cm^2 statistically different when compared to the STARR group (36.92 cm^2). The average volume in the CCS 30 group was 29.8 cc, while in the PPH-01 it was 23.8 cc and difference was statistically significant as well (Table 1). With concern to post-operative pain during the first and second postoperative day, we found that in the CCS 30 group the VAS was 1.3 (range 0-4) and 1.1 (range 0-3) respectively, while in the group treated with two PPH-01 technique (STARR) the VAS was 2.55 (range 0-4) and 0.95 (range 0-3, 5). One month following surgery patients did not complain of pain anymore. The mean hospital stay was 3.1 d (range 2-5 d) in the CCS 30 group and 3.4 d (range 3-6 d) in the PPH-01 group (Table 1).

Only one complication, acute bleeding, occurred in the CCS 30 group (3.3%), promptly treated with surgery in order to control the bleeding. We have not recorded other perioperative complications such as rectal abscess, post-operative hematomas, tenesmus, or rectal strictures.

As regards the long-term follow-up, endoanal ultrasonography performed 1 year following surgery was considered normal in both of the studied groups. Above all, neither major damage nor sonographically demonstrable sphincter fragmentations were noticed in the endoanal exam performed at the follow-up. Urgency was complained by 5 patients (3 in the double PPH group and 2 in the Transtar one) and incontinence by 3 patients (2 in the double PPH group and 1 in the Transtar one), both resolved in some measure during the follow-up period with an overall satisfactory rate of 83.3% (25/30). In the study groups, the postoperative Longo and Wexner scores for ODS showed an improvement that was statistically significant with respect to the preoperative value.

Findings of anorectal manometry at the 1 year follow-up are showed in Table 2. In both groups of patients, the mean resting pressure is higher than the preoperative value (67.2 mmHg in the STARR group and 65.7 mmHg in the CCS30 group *vs* 54.7 mmHg and 55.3 mmHg respectively). Resting and squeezing pressures are lower in those patients not satisfied, but data are not statistically significant. Even if data are not statistically significant, postoperative rectal compliance seems to be lower in the STARR group than in the CCS group (Table 2).

DISCUSSION

Treatment of ODS is a widely debate topic, and the first consideration concerning the right indications of surgical procedure is to correct the ODS[6,7]. Defecography shows a rectocele or rectal intussusception in 81 and 35 percent of asymptomatic female respectively[8], therefore the presence of the rectocele and rectal intussusception are not an indication for surgery. In fact, only symptomatic patients with rectocele and rectal intussusception are suitable for a surgical treatment. For this reason, and according to the literature, we recommend a strict and careful selection of patients[3]. Moreover, we also believe that patients with pelvic floor dyssynergia, clearly demonstrated by clinical and instrumental evaluation, represent a particular population so that pelvic floor training should be considered the main therapeutic choice with respect to surgery.

The STARR procedure with two PPH-01 is a safe, well tolerated surgical procedure effectively restoring the anatomy and the function of the anorectum in patients with ODS due to rectocele and rectal intussusception, with a low rate of complication and a short hospital stay[9,10]. In the literature it is not clearly established which surgical technique is the most effective for the treatment of ODS[3]. In fact, no randomized trial has yet clearly demonstrated the best approach[3]. Moreover, in the era of healthcare cost management, it could be useful to underline that official prices of double PPH-01 and CCS 30 techniques are €800 and €1, 789 respectively. The new CCS 30 Contour has the advantage of a new semicircular head allowing the resection under direct vision into the anal canal. Moreover, it allows a more regulated specimen than using two PPH-01 and our data confirm this assessment in agreement with the international literature, as the amount of rectal resection can be easily regulated with regards to the depth of the rectal intussusception. Analyzing our data, we found a statistically significant difference in surface and volume between the two PPH-01 and CCS 30 Contour specimens. In accordance to the concept of a more regulated resection with CCS 30, the average surface and volume have turned out greater in the STARR group with this new device. Therefore, STARR with the CCS 30 Contour is a procedure that allows removal of a larger, more regular and more symmetrical specimen and, as a consequence, in a perfect cylinder compared with the 2 irregular specimens obtained by STARR with two PPH-01.

This data could provide an explanation for the reduced rectal compliance in the STARR group compared to the CCS30 group. In fact, the resection performed with the double PPH technique could result in an hourglass shape of the rectum, with a stricture at staple line level. On the contrary, the resection obtained with CCS30, performed with four or more firings, could results in a larger and softer staple line.

Although in the literature correlations between the amount of the prolapse removed and the functional improvement as well as between the functional failure and the insufficient removal of the prolapsed have not been demonstrated yet, we believe that Contour CCS 30 might increase the functional results of the STARR procedure even if our data and the short follow-up cannot support this theory yet.

Analyzing our data there are not significant differences between the CCS 30 Contour and the STARR with two PPH-01 groups concerning the hospital stay, postoperative VAS evaluation and, above all, we have not recorded any differences between the two groups concerning major early complications[11]. The literature reports 5% of post-operative bleeding following STARR with two PPH-01[6]. Although the average surface and volume of the resected specimen in STARR with CCS 30 group were larger compared to STARR with double PPH-01 group, in our experience complication rate is not increased. Actually the literature reports only one case of acute complications after STARR with CCS 30 contour; retroperitoneal and mediastinal emphysema treated with medical therapy[12].

In our study, incidences of fecal incontinence and urgency in both groups confirm the results of the international literature, but no significant alteration was found with regards to endoanal ultrasonography and anorectal manometry performed at the follow-up. In a recent study Renzi *et al*[13] report 2.9% of post-operative bleeding after STARR with CCS30 contour and our data confirmed these rates. Most authors report incontinence to flatus (IF) and urge to defecate (UD) that tends to resolve in few weeks[2]. Arroyo in a recent series of 104 patients, treated for ODS with a double PPH-01 STARR, reports an incidence of IF and UD at 1 mo of 22.1% and 26.9%, respectively[6]. In a published series of 90 patients, Boccasanta reports an IF incidence of 8.9% and 17.8% of UD 1 mo following surgery[2].

Some authors[3] tried to explain the incontinence advocating a sphincter or mucosal injury or an excessive anal dilation, but our findings with postoperative endoanal ultrasonography did not show any sphincter damage following surgery. According to Pechlivanides, IF and UD could be due to rectal wall edema and reduced rectal compliance[9]. Actually, in our experience, anorectal manometry at 1 year following surgery revealed a decreased rectal compliance, particularly in the STARR group with two PPH. On the other hand, an increased resting pressure is a common finding in these patients and might be a consequence of the lower rectal compliance as a compensatory mechanism. Our results are based on a short outcome and a small non-randomized population, so these theories should be further investigated on the basis of a longer follow-up.

In conclusion, in our experience STARR with Contour CCS 30 is a safe and feasible technique allowing the excision of a major amount of tissue without any increasing of the early complication rate or sphincter injuries, as demonstrated by the endoanal ultrasonography. Moreover, resected tissue after a Contour CCS 30 procedure is clearly more symmetric and larger in dimension, without any difference in post-operative pain and hospital stay compared to the STARR with two PPH-01 technique. On the other hand, in the current literature[14] a correlation between the amount of the prolapse removed and the functional improvement in patients with ODS has not been reported yet and so far it has not been demonstrated to have any correlation between functional failure and insufficient removal of the prolapse. Further studies should investigate longer clinical outcomes and allow us to evaluate if a larger rectal resection results in a functional improvement emphasizing the real advantages of CCS 30 with respect to STARR.

COMMENTS

Background

Obstructed defecation syndrome is a common disease, particularly in the female population, treated with two different stapled transanal rectal resection (STARR)

procedures.

Innovations and breakthroughs

Both STARR procedures are worldwide performed. All specimen data, manometric and sonographic evaluation should be considered with a clinical follow-up as well in the evaluation of these surgical procedures.

Peer review

The authors ought to be congratulated for a very well conducted study and for the large series of STARR procedures performed.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2417

World J Gastroenterol 2011 May 21; 17(19): 2417-2423
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

p53 antibodies, metallothioneins, and oxidative stress markers in chronic ulcerative colitis with dysplasia

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Received: January 10, 2011 Revised: February 6, 2011
Accepted: February 13, 2011
Published online: May 21, 2011

Abstract

AIM: To investigate the role of p53 antibodies (p53Abs), metallothioneins (MTs) and oxidative stress markers in the early detection of dysplasia in chronic ulcerative colitis (UC).

METHODS: The study included 30 UC patients, 15 without dysplasia (group Ⅱ) and 15 with dysplasia (group Ⅲ), in addition to 15 healthy volunteers (group Ⅰ, control subjects). The enzyme-linked immunosorbent assay technique was used to measure serum p53Abs and MTs, while advanced oxidation protein products (AOPPs), and reduced glutathione (GSH) levels were measured by spectrophotometric method in all subjects.

RESULTS: In group Ⅱ and group Ⅲ compared to group Ⅰ, there were significant increases in serum levels of AOPPs (145.94 ± 29.86 μmol/L and 192.21 ± 46.71 μmol/L *vs* 128.95 ± 3.06 μmol/L, $P < 0.002$ and $P < 0.001$, respectively), MTs (8.18 ± 0.35 μg/mL and 9.20 ± 0.58 μg/mL *vs* 6.12 ± 0.25 μg/mL, $P < 0.05$ and $P < 0.05$, respectively), and p53Abs (20.19 ± 3.20 U/mL and 34.66 ± 1.34 U/mL *vs* 9.42 ± 1.64 U/mL, $P < 0.001$ and $P < 0.001$, respectively). There were significantly higher levels of AOPPs ($P < 0.05$) and p53Abs ($P < 0.001$) in UC patients with dysplasia compared to those without dysplasia, while MTs showed no significant difference between the 2 groups ($P > 0.096$). In contrast, GSH levels showed a significant decrease in both patients' groups (1.87 ± 0.02 μmol/mL and 1.37 ± 0.09 μmol/mL *vs* 2.49 ± 0.10 μmol/mL, $P < 0.05$ and $P < 0.05$ in groups Ⅱ and Ⅲ, respectively) compared with group Ⅰ, and the levels were significantly lower in group Ⅲ than group Ⅱ ($P < 0.05$). There was a positive correlation between AOPPs and both MTs ($r = 0.678$, $P < 0.001$) and p53Abs ($r = 0.547$, $P < 0.001$), and also between p53Abs and MTs ($r = 0.739$, $P < 0.001$). There was a negative correlation between AOPPs and GSH ($r = -0.385$, $P < 0.001$), and also between GSH and both MTs ($r = -0.662$, $P < 0.001$) and p53Abs ($r = -0.923$, $P < 0.001$).

CONCLUSION: Oxidative stress and oxidative cellular damage play an important role in the pathogenesis of chronic UC and the associated carcinogenetic process. p53Abs levels could help in early detection of dysplasia in these conditions.

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Key words: Ulcerative colitis; Advanced oxidation protein products; Reduced glutathione; Metallothionein

Peer reviewer: Jay Pravda, MD, Inflammatory Disease Research Center, Gainesville, Florida, 32614-2181, United States

Hamouda HE, Zakaria SS, Ismail SA, Khedr MA, Mayah WW. p53 antibodies, metallothioneins, and oxidative stress markers in chronic ulcerative colitis with dysplasia. *World J Gastroenterol* 2011; 17(19): 2417-2423 Available from: URL: http://www.wjg-

net.com/1007-9327/full/v17/i19/2417.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2417

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease limited to the colon and affecting only mucosa and submucosa except in the most severe cases. Although the exact pathogenesis of UC remains unknown, various hypotheses have been put forward, and a number of factors are associated with its occurrence[1,2]. The development of dysplasia and cancer is one the most concerning complications of longstanding UC[3].

Both genetic and environmental factors contribute to the pathogenesis of colorectal cancer in UC. Oxidative stress also seems to be involved in the pathogenesis of UC because the inflammatory cells, neutrophils, and macrophages produce large amounts of reactive oxygen species (ROS)[2]. Oxidative stress in inflamed tissue can pave the way for malignant tumors, and it is a major pathogenetic factor in the well-established correlation between inflammatory diseases and cancer[4]. As it is difficult to detect UC-associated dysplasia endoscopically, the development of new diagnostic modalities at an early or precancerous stage is crucial to improve the prognosis of UC-associated neoplasia[5,6].

Advanced oxidation protein products (AOPPs) are new protein markers of oxidative stress with pro-inflammatory properties, which accumulate in many pathological conditions[7,8]. AOPPs are formed mainly as a consequence of the action of chlorinated compounds, leading to the formation of dityrosine residues and protein cross-linking[9,10]. Being the products of oxidative imbalance themselves, AOPPs further participate in the potentiation and perpetuation of both oxidative stress and inflammation[11].

Glutathione (GSH) and its related enzymes are essential enzymatic defense systems in the colonic mucosa that have many important functions, such as maintaining the reduced state of proteins and protecting the cells against ROS, drugs or heavy metal ions[12,13].

Metallothioneins (MTs) are a superfamily of small proteins that are present in virtually every living organism and have highly a conserved number and position of cysteine residues, enabling them to incorporate monovalent and divalent metal atoms and to reduce reactive oxygen and nitrogen species[14]. MTs are known to participate in fundamental cellular processes such as cell proliferation and apoptosis[15].

Altered genes may not only lead to a functional change that contributes to the appearance of a malignant phenotype, but may also generate molecules that will induce humoral or cell-mediated specific immune responses[16]. p53 is the most striking tumor suppressor gene. Mutations of the p53 gene are the most frequently reported somatic gene alterations in human cancer, leading to accumulation of p53 gene products in tumor cells that can initiate an immune response with generation of circulating anti-p53 antibodies (p53Abs)[17,18]. The earlier observation of p53Abs in sera of patients with lung, liver, colon, and breast cancer[17,19] not only raised the question of the relationship between p53 gene mutation, p53 accumulation, and the anti-p53 humoral response, but also opened the way to the development of new markers for cancer diagnosis[16].

The aim of this study was to investigate the role of p53Abs, MTs and some oxidative stress markers in the early detection of dysplasia in chronic UC patients.

MATERIALS AND METHODS

The study included 45 subjects in 3 groups. Group I (control group) comprised 15 healthy volunteers (8 male, 7 female), aged 40.3 ± 14.6 years. Group II comprised 15 UC patients (9 male, 6 female), aged 52.9 ± 17.8 years without colonic dysplasia. Group III comprised 15 UC patients (7 male, 8 female), aged 65.5 ± 11.2 years with mild to moderate degrees of dysplasia.

Patients were selected from the inpatients and outpatients of the Tropical Medicine Department of Tanta University Hospital, Egypt. Written consent was obtained from every investigated subject. Disease duration was calculated from the date of diagnosis of UC, taken from the medical records.

Patients with autoimmune disease, any malignant tumor, and/or chronic liver diseases and those with concurrent infections, previous surgery, chemotherapy or who had undergone colorectal surgery and those with nutritional support were excluded from the study. The diagnosis of UC was based upon a clinical history of diarrhea and/or rectal bleeding for 6 wk or more, typical radiological and endoscopic findings, and characteristic microscopic changes on biopsy specimens.

Colonic biopsy samples

All patients underwent colonic biopsy sampling during surveillance colonoscopy, which was performed according to established American Gastroenterological Association guidelines for UC surveillance colonoscopy[20]. An aliquot of the colon sample was immediately fixed in formalin for the histopathological evaluation which was performed by the pathologist in a blinded fashion throughout the study. Dysplasia was diagnosed in line with classification proposed by the Inflammatory Bowel Disease Dysplasia Morphology Study Group[21]. At the time of the endoscopic procedure, blood samples were taken from each subject under complete aseptic conditions, and the sera obtained by centrifugation were stored at -80℃ until the time of use.

Measurements

All groups were subjected to the following: (1) A thorough medical history-taking, full clinical examination and

routine laboratory investigations including complete blood picture and serum albumin, and lipid profile including serum total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol; (2) Estimation of serum AOPP level: determination of AOPPs based on spectrophotometric detection according to Witko-Sarsat *et al*[9] (1998). Briefly, 200 μL of serum (diluted 1:5 with phosphate-buffered saline (PBS), 200 μL of chloramin T (0-100 μmol/L) for calibration and 200 μL of PBS as blank were applied on a microtiter plate; 10 μL of 1.16 mol/L potassium iodide and 20 μL of acetic acid were added to each well and absorbance at 340 nm was measured immediately. The concentration of AOPPs was expressed as μmol/L chloramine T equivalents units; (3) Spectrophotometric determination of serum GSH level: the method is based on the reduction of 5.5'-dithiobis-(2-nitrobenzoic acid) by GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance was measured at 405 nm using a commercial kit (Biodiagnostic, Egypt)[22]; (4) Estimation of serum metallothionein level: MTs in the serum were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Ray Biotech, Inc) after acid (1 mol/L HCl) and heat (100℃, 10 min) treatment to eliminate the co-existent antibody-reactive protein according to Cousins[23] (1991); and (5) Assay of serum p53Abs: p53Abs were detected by a commercially available ELISA kit (Quantkine) kit supplied by Clinilab according to Kirsch *et al*[24] (1998). Cut-off absorbance value was calculated according to the manufacturer's instructions (cut-off = 26.3). Therefore, we judged samples to be positive for serum when the p53Abs level was higher than 26.3 U/mL.

Statistical analysis

Statistical analysis was performed using SPSS for Windows 10.0. One way analysis of variance was used to compare groups and Tukey's test was used to determine the significance between 2 groups. The Pearson correlation coefficient (*r*) was used to identify a correlation between different parameters.

RESULTS

The demographics and biochemical characteristics in all studied groups are shown in Table 1.

Serum AOPP levels were significantly increased in UC groups Ⅱ and Ⅲ compared with the control group ($P < 0.002$ and $P < 0.001$, respectively), with significantly higher levels in patients with dysplasia than in those without dysplasia ($P < 0.001$). GSH serum levels were significantly reduced in groups Ⅱ and Ⅲ compared with controls ($P < 0.05$ and $P < 0.05$, respectively), with significantly lower levels in patients with dysplasia than in those without dysplasia ($P < 0.05$). MT serum levels were significantly increased in groups Ⅱ and Ⅲ compared with controls ($P < 0.05$ and $P < 0.05$, respectively), with no significant difference between the UC groups ($P > 0.096$). p53Ab serum levels were significantly increased in groups Ⅱ and Ⅲ compared with controls ($P < 0.001$ and $P < 0.001$, respectively) with significantly higher levels in patients with dysplasia than in those without dysplasia ($P < 0.001$, Table 2, Figures 1 and 2).

Correlation studies in UC patients (Table 3) showed a positive correlation between AOPPs and both MTs ($r = 0.678$, $P < 0.001$) and p53Abs ($r = 0.547$, $P < 0.001$), and also between p53Abs and MTs ($r = 0.739$, $P < 0.001$). On the other hand, there was a negative correlation between AOPPs and GSH ($r = -0.385$, $P < 0.001$), and also between GSH and both MTs ($r = -0.662$, $P < 0.001$) and p53Abs ($r = -0.923$, $P < 0.001$).

Using a cut-off value (≥ 26 U/mL), we demonstrated that p53Abs were present in 40.0% of UC patients with dysplasia and in 13.3% without dysplasia.

Figures 3 and 4 show photomicrographs of the mucosa in UC without and with dysplasia.

Table 1 Demographic and biochemical characteristics in all studied groups (mean ± SD)

	Group Ⅰ (*n* = 15)	Group Ⅱ (*n* = 15)	Group Ⅲ (*n* = 15)
Age (yr)	40.3 ± 14.6	52.9 ± 17.8	65.5 ± 11.2
Sex (male/female)	8/7	9/6	7/8
Disease duration (yr)	------	6 ± 1.1	7 ± 2.1
TG (mg/dL)	90.3 ± 14.5	92.0 ± 10.8	96.5 ± 10.8
TC (mg/dL)	160.5 ± 13.76	167.6 ± 15.3	170.5 ± 13.8
LDL-C (mg/dL)	124 ± 16.46	142.33 ± 24.41	142.66 ± 24.33
HDL-C (mg/dL)	54.50 ± 11.81	44.06 ± 9.12	41.60 ± 10.85
Hb (g/dL)	13.1 ± 1	11.78 ± 1.2	11.3 ± 0.9
WBC (/mm^3)	5180 ± 967	5630 ± 1252	5033.3 ± 976
PLT (10^4/mm^3)	34.8 ± 5	36 ± 4.9	38 ±4.2
Albumin (g/dL)	4.1 ± 0.23	4.08 ± 0.35	2.9 ± 0.29

Group Ⅰ: Control group; Group Ⅱ: Ulcerative colitis without dysplasia; Group Ⅲ: Ulcerative colitis with dysplasia; TG: Triglyceride; TC: Total cholesterol; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelet count. $P > 0.05$ are presented as not significant.

Table 2 Statistical comparison between all studied parameters in all studied groups (mean ± SD)

	Group Ⅰ (*n* = 15)	Group Ⅱ (*n* = 15)	Group Ⅲ (*n* = 15)	*P*
AOPP (μmol/L)	128.95 ± 3.06	145.94 ± 29.86	192.21 ± 46.71	< 0.002[1,a] < 0.001[1,b] < 0.001[1,c]
GSH (μmol/L)	2.49 ± 0.10	1.87 ± 0.02	1.37 ± 0.09	< 0.05[1,a] < 0.05[1,b] < 0.05[1,c]
MT (μg/mL)	6.12 ± 0.25	8.18 ± 0.35	9.20 ± 0.58	< 0.05[1,a] < 0.05[1,b] > 0.096[c]
p53Abs (U/mL)	9.42 ± 1.64	20.19 ± 3.20	34.66 ± 1.34	< 0.001[1,a] < 0.001[1,b] < 0.001[1,c]

[1]Significant; [a]Group Ⅱ *vs* group Ⅰ; [b]Group Ⅲ *vs* group Ⅰ; [c]Group Ⅲ *vs* group Ⅱ. AOPP: Advanced oxidation protein product; GSH: Glutathione; MT: Metallothionein; p53Abs: p53 antibodies.

Table 3 Correlation matrix between all studied parameters

	r	*P*
AOPP (μmol/L) and GSH (μmol/mL)	-0.385	0.001[1]
AOPP (μmol/L) and MT (μg/mL)	0.678	0.001[1]
AOPP (μmol/L) and p53Abs (U/mL)	0.547	0.001[1]
GSH (μmol/L) and MTs (μg/mL)	-0.662	0.001[1]
GSH (μmol/L) and p53Abs (U/mL)	-0.923	0.001[1]
p53Abs (U/mL) and MT (μg/mL)	0.739	0.001[1]

[1]Significant. AOPP: Advanced oxidation protein product; GSH: Glutathione; MT: Metallothionein; p53Abs: p53 antibodies.

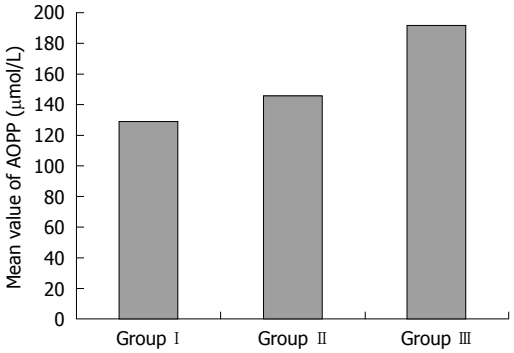

Figure 1 Comparison of advanced oxidation protein product levels in groups Ⅰ, Ⅱ and Ⅲ.

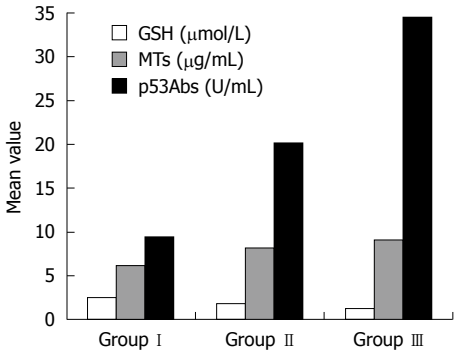

Figure 2 Comparison of glutathione, metallothioneins, and p53 antibodies levels in all studied groups. GSH: Glutathione; MTs: Metallothioneins; p53Abs: p53 antibodies.

DISCUSSION

Chronic UC is associated with an increased risk of developing colorectal cancer. The risk of developing cancer, or its precursor lesion, dysplasia, increases exponentially with the duration of the disease[6]. For early detection of UC-associated colorectal cancer, surveillance colonoscopy is recommended in UC patients at high risk. However, poor acceptability by patients reduces its effectiveness. In addition, it is difficult to detect UC-associated dysplasia endoscopically; therefore, a suitable marker for selecting patients at high risk is needed[4]. Accordingly, this study aimed to investigate the value of AOPPs, GSH, MTs and p53Abs levels for the early detection of dysplasia in chronic UC patients.

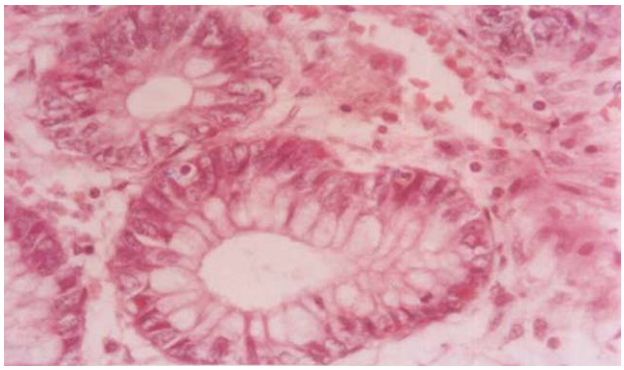

Figure 3 Photomicrograph of ulcerative colitis mucosa without dysplasia showing acute inflammatory cells (stromal) and multiple apoptotic bodies (400 ×). Hematoxylin and eosin stain.

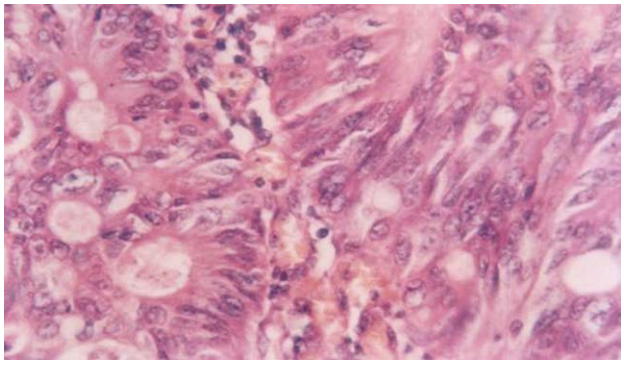

Figure 4 Photomicrograph of ulcerative colitis with dysplasia showing hyperchromatic nuclei shrunken cells, cytoplasmic organelles and inclusions (400 ×). Hematoxylin and eosin stain.

The activities of phagocytic leukocytes are greatly increased in the colons of UC patients resulting in enhanced generation of pro-oxidant molecules that have been known to play an important role in the initiation and promotion of multi-step carcinogenesis through specific gene alterations, genetic instability and aberrant methylation[25-27]. Indices of oxidative damage are widely used as markers of oxidative stress. AOPPs are new protein markers of oxidative stress with pro-inflammatory properties, accumulated in many pathological conditions[11,13]. In the present study, serum AOPP levels were significantly increased in UC patients, with significantly higher levels in UC patients with dysplasia than in those without dysplasia. Our results are in agreement with other previous studies which showed an increased formation of AOPPs in inflammatory bowel disease[28,29], and those which showed that colorectal cancer is associated with oxidative stress with increased AOPP levels[30-32].

The epithelium of the colon contains multiple antioxidant systems, including antioxidant enzymes and low-molecular-weight antioxidant molecules such as GSH[33]. GSH and enzymes associated with it play crucial role in cell defense against ROS, which are implicated in various types of cancer[34,35]. In the present study, the significant decrease in GSH level in UC patients with significantly lower levels in patients with dysplasia are in accordance

with previous studies, which showed depletion of the GSH system in UC patients, leading to increased susceptibility towards toxic or carcinogenic compounds[13]. The significant decrease in GSH level in UC patients may result from an increased turnover of GSH to prevent oxidative damage in these patients. The lower levels of GSH in patients with dysplasia may still favor an overproduction of free radicals which in turn may induce damage to the cell membrane and cellular molecules (DNA, RNA) leading to neoplasia[34,35].

The thiol-rich protein MT plays a major role in detoxification of toxic metals and in protection against oxidative damage. In normal tissues, MT expression is usually undetectable, except in certain types of cells such as myoepithelial, renal and thyroid epithelial cells. It has been observed that the large bowel epithelial cells express MT in up to 40% of patients with UC, and a correlation has been found between the degree of MT expression and severity of inflammation[36].

Our study showed a significant increase in MT levels in both groups of UC patients, but a non significant difference between them. This finding is in agreement with the studies of Brüwer *et al*[36] and Waeytens *et al*[37], who found an increased expression of MT in epithelial cells of the intestinal mucosa in inflammatory bowel disease patients. The increased MT concentration in UC patients suggests induction of MT synthesis in response to the potential harmful effects of ROS and nitrogen intermediates produced during the inflammatory response. The generated ROS may activate MT expression through multiple pathways, including directly by stimulating an antioxidant response element and specific metal response elements in the promoter region as well as indirectly by events associated with second-messenger protein kinase pathways[17,18].

Several lines of evidence indicated that many types of tumors have high concentrations of MT and this may play a role in various carcinogenic processes. Also high levels of MT expression have been reported in initiation, progression, and metastasis of cancer[14].

MT overexpression may represent an important early step in the development of colorectal carcinoma in UC patients and those with low grade dysplasia[38]. It has been hypothesized that mutation-induced metallothionein overexpression may interfere with the function of zinc finger DNA binding transcription factors involved in the control of the expression of a wide range of genes regulating cell proliferation and apoptosis, such as p53, and conferring a growth advantage on the mutated cells[39].

The p53 gene mutations seem to be an early event in cancerous change in UC patients[25]. These mutations lead to inactivation of the p53 protein, and cellular accumulation in the nucleus which can initiate an immune response with generation of circulating p53Abs. These p53Abs reflect a humoral response that occurs early in tumor development. Previous studies reported high diagnostic sensitivity and specificity of p53Abs in patients with various types of cancers[40].

In the present study serum p53Abs showed significantly increased levels in both groups of UC patients, with significantly higher levels in patients with dysplasia. The widely accepted hypothesis for increased serum p53Abs is as follows; a point mutation of the p53 gene is associated with overexpression of p53 protein. Overexpressed mutant p53 is considered a nonself protein, triggering the immunocompetent cells and secretion of p53Abs[40]. The type and location of p53 gene mutations may impact on the generation of p53Abs. Most of these mutations lead to the synthesis of a stable protein which accumulates in tumor cells and may be important for the development of the immune response[40]. It has also been suggested that mutations in exons 5 and 6 (coding for mutant protein that binds to heat shock protein 70) are immunogenic, while mutations in exons 7 and 8 are not related to the generation of p53Abs[41]. Our results are in line with the study of Cioffi *et al*[15], 2004 who suggested that assessment of serum p53Abs is an indirect marker for p53 gene mutations, and the abnormally high p53 protein levels could be considered to have a potential for use as a complementary test to improve surveillance program performance in UC patients. Normally p53 comes into action when DNA is damaged and arrests the cell cycle so as to allow repair. In the case of failure of repair, p53 directs the cell to apoptosis; therefore, p53 mutation leads to uncontrolled proliferation of cells[42].

Using a cut-off value (≥ 26 U/mL), we demonstrated that p53Abs were present in 40.0% of UC patients with dysplasia and in 13.3% without dysplasia. Further investigations on a large scale are needed to better define the sensitivity, specificity, positive and negative predictive values of p53 alterations in patients with UC with and without dysplasia.

In conclusion, oxidative stress and oxidative cellular damage play an important role in the pathogenesis of chronic UC and the associated carcinogenetic process. p53Ab levels could help in early detection of dysplasia in UC patients.

COMMENTS

Background

Patients with longstanding ulcerative colitis (UC) have an increased risk of developing dysplasia and colorectal cancer. This risk appears to be related to the cumulative effect of chronic inflammation and correlates directly with the extent and duration of disease. Earlier colonoscopy screening and surveillance for detecting mucosal dysplasia is important to select colorectal cancer-prone individuals for prophylactic colectomy but, to date, this has not been translated into a positive survival benefit. p53 antibodies (p53Abs), metallothioneins (MTs) and some oxidative stress markers may aid in the early detection of dysplasia in chronic UC patients and could be used as complementary tests to improve surveillance program.

Research frontiers

Despite several acknowledged limitations, periodic surveillance colonoscopy continues to be used to diagnose dysplasia and colorectal cancer complications of longstanding UC. Investigating the role of p53Abs, MTs and some oxidative stress markers may open the way to the development of new cancer diagnosis.

Innovations and breakthroughs

The present study revealed that the decreased serum protective antioxidant glutathione (GSH) level associated with an increased production of oxygen free

radicals results in protein oxidation and hence advanced oxidation protein products (AOPPs) with subsequent increase of MTs leading to p53 instability and the development of high levels of p53Abs in longstanding UC, that may precede and accompany dysplasia. These data are in line with previous studies and call for wider research on the associations in UC.

Applications

AOPPs, GSH and MTs are associated with dysplastic changes in chronic UC. p53Ab levels could be used as a complementary test in a surveillance program. Further investigations on large scale populations are needed to compare their sensitivity, specificity, positive and negative predictive values, in addition to, alterations of the p53 tumor suppressor gene, which occurs early in patients with UC and may precede dysplasia.

Terminology

p53: the *p53* gene is the tumor suppressor gene whose abnormalities are the most frequently reported gene alterations in human cancer. MTs: a superfamily of small proteins with a highly conserved number of cysteine residues and they reduce reactive oxygen and nitrogen species. GSH: essential enzymatic defense system in the colonic mucosa. AOPPs are protein markers of oxidative stress with pro-inflammatory properties.

Peer review

This is an excellent study which is based on the early detection of serological biomarkers in order to predict the risk of developing colorectal cancer in individuals with UC.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2424

World J Gastroenterol 2011 May 21; 17(19): 2424-2430
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Monoclonal immunoscintigraphy for detection of metastasis and recurrence of colorectal cancer

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Supported by A Grant of the Ministry of Science of the Republic of Serbia, No.175018

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Received: October 6, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: May 21, 2011

Abstract

AIM: To assess the clinical role of monoclonal immunoscintigraphy for the detection of metastasis and recurrence of colorectal cancer.

METHODS: Monoclonal immunoscintigraphy was performed in patients operated on for colorectal adenocarcinoma suspected of local recurrence and metastatic disease. The results were compared with conventional diagnostics.

RESULTS: Immunoscintigraphic investigation was done in 53 patients. Tumor recurrence occurred in 38 patients, and was confirmed by other diagnostic modalities in 35. In 15 patients, immunoscintigraphic findings were negative, and confirmed in 14 with other diagnostic methods. Comparative analysis confirmed good correlation of immunoscintigraphic findings and the results of conventional diagnostics and the level of tumor marker carcinoembryonic antigen. Statistical analysis of parameters of radiopharmaceutical groups imacis, indimacis and oncoscint presented homogenous characteristics all of three radiopharmaceuticals. The analysis of immunoscintigraphic target focus was clearly improved using tomography.

CONCLUSION: Immunoscintigraphy is highly specific and has a good predictive value in local recurrence of colorectal cancer.

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Key words: Immunoscintigraphy; Monoclonal antibodies; Colorectal carcinoma; Tumor metastasis; Tumor recurrence

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URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2424.htm
DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2424

INTRODUCTION

Radiolabeled monoclonal antibodies (mAbs) against tumor-associated antigens enable imaging of primary tumors of the gastrointestinal system, and their metastases and/or recurrence, with high sensitivity and specificity. Whole body immunoscintigraphy and/or single photon emission computed tomography (SPECT) are accessible imaging methods focusing on specific type of tumors. Nuclear medicine imaging enables determination of the pathophysiological and biochemical parameters of the viable tumor tissue, including metabolic changes as well as the presence of specific proteins/receptors on the surface of the tumor cells. Positron emission tomography (PET) is the best method for imaging metabolic changes based on increased rate of tumor glycolysis and/or protein metabolism. Dual modality hybrid imaging techniques PET/CT enable precise morphological and anatomical localization.

Radio-immunoguided surgery has been introduced as a method of more accurate detection of tumor extension and enables radical resection. Radioimmunotherapy with mAbs as postoperative adjuvant treatment is currently been investigated[1].

The aim of the present study was to evaluate the clinical reliability of immunoscintigraphy for detection of metastasis and recurrence of colorectal carcinoma, using three different radiopharmaceuticals.

MATERIALS AND METHODS

Methodology

Imacis 1 contains a cocktail of (111 MBq ^{131}I) mAb 19-9 F (ab')$_2$ and mAb anti CEA F (ab')$_2$. It is labeled with ^{131}I. Its half-life of 8 d and β-minus emission leads to significant radiation exposure of the patient. In addition, its high energy (364 KeV) makes it less than optimal for imaging, necessitating special collimation for contemporary gamma cameras. The two other radiopharmaceuticals used in this study were labeled with ^{111}In. Indimacis 19-9 contains 19-9 F(ab')$_2$/DTPA fragments of mAbs. It is a pure γ emitting isotope with a physical half life of 67 h, an abundance of photon emissions at 173 and 247 keV, while Oncoscint CR 103 is an immunoconjugate produced by site-specific modification of the mAb B72.3, which is a murine immunoglobulin that is able to recognize high molecular weight glycoprotein (TAG-72) expressed by a majority of adenocarcinomas[2-6].

Imacis 1 was administered by slow injection for approximately 30 min. Potassium iodide (600 mg/d) was administered orally for 10 d (starting 24 h before injection) to block the uptake of free iodine into the thyroid gland. Imaging was carried out after 96-120 h. Planar images (6 min/image or at least 200 000 counts over the whole field of view) including anterior and posterior projections of the thorax, abdomen and pelvis, were taken using large field-of-view cameras, fitted with parallel hole high energy collimators. Indimacis 19-9 at a dose of 185 MBq was administered by slow infusion of 100 mL 0.9% sodium chloride over 30 min. Anterior and posterior spot views of the abdomen, pelvis and/or chest (500 000 counts/view) were obtained 24 h and 48 h following the infusion. SPECT of abdominal and pelvic regions, including 360° rotating orbit, sampling every 6° with an 40-s acquisition per stop, using a 128 × 128 or 64 × 640 word matrix was carried out. Reconstruction was performed using Butterworth filter, order 6-10. Oncoscint CR 103 at a dose of 185-200 MBq was administered by slow injection for approximately 5 min, following the same acquisition protocol as above. To achieve more precise localization of the pathological lesions, as well as to increase target-to-background ratio, the dual isotope acquisition and subsequent subtraction of the obtained images were carried out. Thus, images of the vascular system (^{99m}Tc-red blood cells), liver and spleen (^{99m}Tc-sulfur colloid) or kidney (^{99m}Tc-DTPA) are obtained and used for subtraction. Scintigraphy was performed with a ROTA/Orbiter scintillation camera and Micro Delta computer.

Patients

The selection of patients was based upon complete diagnostic records [anamnestic data, physical examination, blood analysis, ultrasonography, contrast radiography, rectoscopy/colonoscopy, CT, magnetic resonance imaging (MRI), tumor marker assay] and clinical follow-up of at least 6 mo. The investigation was performed whenever there was a rise in serum levels of tumor markers [carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 19-9)], and metastasis or recurrence could not be located according to clinical, radiological (chest X-rays), sonographic or endoscopic findings.

In all the patients, tumor marker (CEA and CA 19-9) blood levels were estimated every month in the same laboratory. Blood samples for tumor marker estimation were taken from the cubital vein of the patients and stored at -20℃ until analysis. Physiological values of CEA were considered up to 7 U/L, while for CA 19-9, they were up to 33 U/mL. Fifteen patients were treated with Imacis 1, 18 with Indimacis 19-9, and 20 with Oncoscint CR 103.

Statistical analysis

The study data were analyzed in program R version 2.8.1. Tables and graphs were created in Microsoft Office Excel 2007. For statistical analysis of inter-rater agreement of samples with normal distribution, the following graphs were used: Normal Q-Q plot, histogram, and Kolmogorov-Smirnov's and Shapiro-Wilk's tests. In order to test the differences between parameters based on their nature, we used Kruskal-Wallis's test, exact Wilcoxon's rank sum test and Fisher's exact test. The value of $\alpha = 0.05$ was accepted as statistically significant. In case of multiple testing of the same set of data, Bonferoni's correction was used ($\alpha_1 = 0.05/\beta = 0.0167$). For the inter-rater agreement of significant parameters, Cohen's κ coefficient test was used.

Table 1 General parameters in patients examined by all three radiopharmaceuticals

Parameter	Imacis 1	Indimacis 19-9	Oncoscint CR 20	Test
No. of patients	15	18	20	-
CEA (μg/L)				Kruskal-Wallis
Mean (SD)	11.6 (12.2)	8.6 (6.5)	41.2 (60.4)	$\chi^2 = 5.71$
Median (rang)	8.9 (1.2-40)	5.9 (3.2-21)	14 (1.3-234)	$P = 0.0577$
NA	2/15 (13.3%)	8/18 (44.4%)	1/20 (5.0%)	
CA 19-9 (U/mL)				Kruskal Wallis
Mean (SD)	27.6 (10.4)	16.2 (4.5)	49.3 (56.5)	$\chi^2 = 10.71$
Median (rang)	22.0 (15-42)	14.6 (11.2-27)	24.5 (14-183)	$P = 0.0047$
NA	6/15 (40.0%)	8/18 (44.4%)	6/20 (30.0%)	
US				Fisher's eact
0	9 (60.0%)	12 (66.7%)	16 (80.0%)	$P = 0.5838$
1	5 (33.3%)	6 (33.3%)	4 (20.0%)	
NA	1 (6.7%)	-	-	
CT				
0	8 (53.3%)	6 (33.3%)	8 (40.0%)	
1	5 (33.3%)	5 (27.8%)	3 (15.0%)	Fisher's exact
2	-	5 (27.8%)	6 (30.0%)	$P = 0.1787$
3	-	1 (5.6%)	2 (10.0%)	
4	-	-	1 (5.0%)	
NA	2 (13.3%)	1 (5.6%)	-	
MR				
0	2 (13.3%)	-	4 (20.0%)	
1	-	-	3 (15.0%)	Fisher's exact
2	-	-	1 (5%)	$P = 0.6$
NA	13 (86.67%)		12 (60.0%)	
Colonoscopy				
0	2 (13.3%)	9 (50.0%)	5 (25.0%)	
1	1 (6.67%)	3 (15.0%)	3 (15.0%)	Fisher's exact
2	3 (20.0%)	2 (11.1%)	2 (10.0%)	$P = 0.55942$
NA	9 (60.0%)	4 (22.2%)	10 (50.0%)	
Rectoscopy				
0	5 (33.3%)	8 (44.4%)	5 (25.0%)	
1	4 (26.7%)	1 (5.6%)	2 (10.0%)	Fisher's exact
2	4 (26.7%)	1 (5.6%)	1 (5.0%)	$P = 0.42891$
NA	2 (13.3%)	8 (44.4%)	12 (60.0%)	
Immunoscintgraphy				
0	7 (46.7%)	5 (27.8%)	4 (20.0%)	
1	2 (13.3%)	5 (27.8%)	3 (15.0%)	Fisher's exact
2	5 (33.3%)	6 (33.3%)	5 (25.0%)	$P = 0.31134$
3	1 (6.7%)	2 (11.1%)	7 (35.0%)	
NA				
SPECT				
0	-	2 (11.1%)	3 (15.0%)	
1	-	6 (33.0%)	1 (5.0%)	
2	-	6 (33.0%)	3 (15.0%)	Fisher's exact
3	-	2 (11.1%)	7 (35.0%)	$P = 0.06137$
NA		2 (11.1%)	6 (30.0%)	

Type 0- no disease; 1- liver metastases; 2- recurrence; in colonoscopy and rectoscopy: 1- recurrence; 2-stricture and polyposis; 3- liver metastases and recurrence; 4- peritoneal carcinosis. CEA: Carcinoembryonic antigen; NA: Not Analyzed; US: Ultrasound; CT: Computed tomography; MR: Magnetic resonance; SPECT: Single photon emission computed tomography. Oncoscint CR 20: Indium In 111 satumomab pendetide; CA 19-9: Carbohydrate antigen 19-9.

RESULTS

Analysis of general parameters in three radiopharmaceutical groups-homogeneity

Data from various patients were statistically analyzed using Fisher's exact test in a study group consisting of 53 patients investigated with three radiopharmaceuticals. Analyzed parameters were: age, sex, surgical treatment, pathological verification and diagnostic examination findings. These analyses showed the homogeneity between the three different radiopharmaceuticals (Table 1).

Tumor marker CEA

With Kruskal-Wallis's test, levels of tumor markers CEA and CA 19-9 were analyzed in 53 patients. CA 19-9 level in the Indimacis 19-9 group was lower than in the other two groups, but it was still elevated. CEA level was elevated in all patients, but was significantly lower in those without pathological findings, and elevated in those with metastatic disease and/or recurrence (Table 2).

Complementary diagnostics

The findings of the complementary diagnostic methods

Table 2 Median carcinoembryonic antigen in types of immunoscintigraphy

Type of disease	*n*	CEA median
0	14	5
1	8	16.25
2	10	9.45
3	9	10
4	1	40

Type 0-no disease; 1-liver metastases; 2-recurrence; 3-liver metastases and recurrence; 4-peritoneal carcinosis. CEA: Carcinoembryonic antigen.

Table 3 Comparison of Cohen's κ coefficient for immunoscintigraphy and other diagnostic methods

Method	*n*	κ
US	52	0.157
CT	50	0.384
MRI	10	0.667
Colonoscopy	31	0.469
Rectoscopy	30	0.655

US: Ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging.

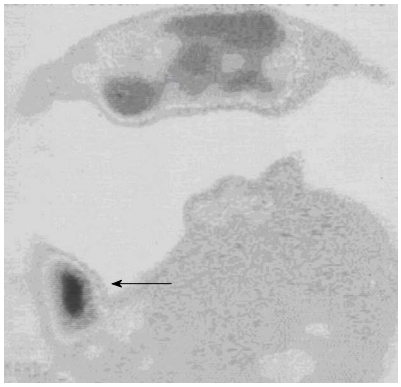

Figure 1 Immunoscintigraphy, planar, anterior view. Accumulation of radiopharmaceutical (IMACIS 1) in metastatic tumor of the right lower part of the liver (arrow).

and immunoscintigraphy were analyzed with Cohen's κ coefficient for the statistical analysis of inter-rater agreement: ultrasonography, slight; rectoscopy, substantial; colonoscopy, moderate; CT, fair; and MRI, substantial (Table 3). Whole body immunoscintigraphy was superior in correlation with complementary diagnostic methods for the detection of pelvic and extrahepatic metastases. Tumor recurrence occurred in 38 patients, and was confirmed by other diagnostic modalities in 35. In three patients, immunoscintigraphic findings were false positive, because hepatic metastases were not confirmed by other imaging modalities. This can be explained by the local inflammation after liver surgery. Thus, sensitivity of the method was 97%, specificity 82%, positive predictive value 92%, negative predictive value 93%, and accuracy 92% (Figures 1-3).

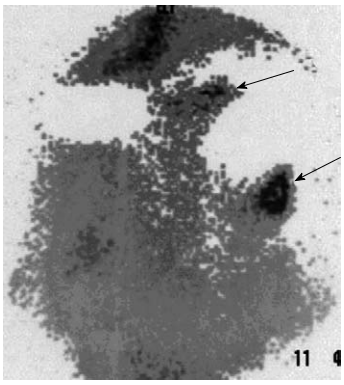

Figure 2 Immunoscintigraphy, planar, posterior view. Accumulation of radiopharmaceutical (INDIMACIS 19-9) in liver metastases (arrows).

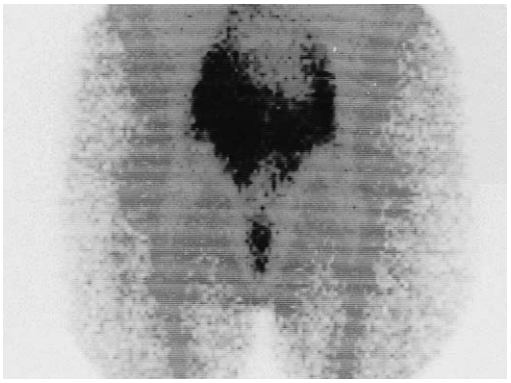

Figure 3 Immunoscintigraphy, planar, anterior view. Accumulation of radiopharmaceutical (Oncoscint) in peritoneal carcinosis.

Target background ratio

In 28 patients with positive immunoscintigraphic findings (both Indimacis 19-9 and Oncoscint), target/background (tg/bg) ratio was analyzed. Higher tg/bg ratio was found in metastatic foci with Indimacis 19-9 (Table 4). Further analysis of SPECT tg/bg ratio and planar tg/bg ratio points out the advantages of SPECT acquisition in immunoscintigraphic detection of metastases (exact Wilcoxon's rank sum test: planar images W = 169, $P = 0.0005$ and SPECT images W = 174.5, $P = 0.0001$).

DISCUSSION

The analyses showed homogeneity between the groups for the three different radiopharmaceuticals. CEA and CA 19-9 were analyzed in 53 patients. The CA 19-9 level was lower in the Indimacis 19-9 group compared with the other two groups, but it was still elevated. Tumor marker CEA was elevated in all patients, but significantly lower in those without pathological findings, and elevated in those with metastatic disease and/or recurrence. Thus, we can conclude that both parameters are valuable for evaluation and follow-up of disease.

The findings of the complementary diagnostic methods and immunoscintigraphy were analyzed and whole body immunoscintigraphy was superior in correlation

with complementary diagnostic methods for the detection of pelvic and extrahepatic metastases.

Tumor recurrence occurred in 38 patients, and was confirmed by other diagnostic modalities in 35 (Figures 1-3). In three patients, immunoscintigraphic findings were false positive due to local inflammation after liver surgery. In 15 patients, findings were negative, which were confirmed in 14 patients using other diagnostic methods, and one patient had a false-negative result, which was a small lesion in the rectal lumen (1 cm) that was confirmed by rectoscopy. Thus, sensitivity of the method was 97%, specificity 82%, positive predictive value 92%, negative predictive value 93%, and accuracy 92%.

A higher tg/bg ratio was found for metastatic foci with Indimacis 19-9. Further analysis pointed out the advantages of SPECT acquisition for immunoscintigraphic detection of metastases.

In most of the investigated cases, immunoscintigraphy was complementary to other imaging methods and significantly influenced the patient management. The most appropriate applications of this method should be the detection of recurrence, assessment of viability, as well as follow-up of disease progression and regression after therapy. Its diagnostic role is complementary to the radiological methods, which show limitations such as viability assessment after surgery, radio- and chemotherapy (CT, MRI), as well as when contrast radiography and colonoscopy cannot be performed (patients with colostomas and strictures), or when recurrence has an extraluminal position. However, other morphological methods (CT, ultrasongraphy, MRI) are superior for detection of liver metastases, while immunoscintigraphy is more sensitive and specific for the discovery of recurrences of colorectal carcinoma.

With regard to the false-negative findings for small intraluminal tumors, we conclude that for intraluminal tumors, endoscopic methods such as rectoscopy are the methods of choice. Apart from localization, the disadvantage of immunoscintigraphy is low spatial resolution of gamma cameras, which can lead to overlooking of small lesions (1 cm). These disadvantages can be overcome by using new generation gamma cameras with increased resolution as well as fusion images with CT and MRI, and especially hybrid systems. Thus, the recent introduction of a hybrid imaging device that contains a low-dose CT system and a gamma camera (SPECT/CT) on a single gantry has enabled the sequential acquisition of the two imaging modalities, with subsequent merging of data into a composite image display. These hybrid studies have led to a revolution in the field of imaging, with highly accurate localization of tumor sites, assessment of invasion into surrounding tissues, and characterization of their functional status[7-9]. In the absence of SPECT/CT systems, tomography (SPECT) for a better distinction of the tumor is highly recommended. Further improvement in the detection of small tumor recurrences with immunoscintigraphy, using radioimmunoguided surgery (RIGS) and intraoperative detection of tumor deposits using special gamma probe systems, after i.v. application of radiopharmaceuticals, is discussed later.

Table 4 Target/background ratio (mean and SD) in planar and single photon emission computed tomography immunoscintigraphic foci

	Planar	SPECT	*n*
Indimacis 19-9 mean (SD)			
Recurrence	1.2	3.5	1/13
Metastasis	2.03 (0.44)	3.04 (0.8)	13/13
Oncoscint mean (SD)			
Recurrence	1.6 (0.24)	2.15 (0.29)	4/5
Metastasis	1.45 (0.26)	1.84 (0.28)	15/15

SPECT: Single photon emission computed tomography.

For accurate diagnosis, it is necessary to estimate tg/bg ratio, especially for the detection of liver metastases (subtraction method is highly recommended), and non-specific uptake of the radiopharmaceuticals in organs due to metabolism and excretion (liver and kidneys), and tissues mainly due to local inflammation. In our patients, false-positive findings can be attributed to the accumulation of radiopharmaceuticals in inflammation. We must emphasize that in all three false-positive patients, tg/bg ratio was on the lower edge of values for positive findings, which means lower than in tumor tissue, but the difference was not obvious and it was not easy to make a clear cut-off. All three patients underwent liver surgery during 6 mo before our investigation, which caused local inflammation with increased accumulation of radiopharmaceuticals. This means that, to prevent false-positive findings, the time of investigation should be longer after surgery, or repeated after 1 or 2 mo, with the expectation of obtaining lower values in non-specific accumulation and higher values in the case of tumor tissue, with obligatory quantification, i.e. estimation of tg/bg ratio[10,11]. Although the antibodies are tumor specific, there is a certain non-specific accumulation in tissues, due to increased vascularization and local inflammation because specific radioimmunotherapy has never been employed widely[12]. Also, as in previously described methods recommended for detection of false-negative cases, apart from physiological methods (immunoscintigraphy with SPECT), additional morphological investigation is recommended, such as hybrid SPECT/CT imaging, in order to distinguish accumulation in the unchanged, inflamed tumor tissue from newly developed tumor formation. Furthermore, even tumor marker/antigen (CEA, CA19-9) levels can be moderately increased due to local inflammation, which results in binding with specific radiolabeled antibodies[13,14]. This also confirms the importance of follow-up in unclear cases. However, even with the above-mentioned limitations, accuracy of the method is very high.

The results from the literature mainly correspond to ours. Thus, some authors have confirmed the significance of the method for detection of recurrence, but have not confirmed its validity for detection of liver metastases[15,16], whereas many[17-19] have emphasized the significance of tomography. However, on the contrary, some investigations[20-22] have found immunoscintigraphy inferior to other imaging methods, especially for the detection of lymph node metastases and for planning adequate surgical

approaches for recurrent colorectal carcinoma.

Our previous results, as well as those of other authors[23-29] have pointed out the particular application of these antibodies for disease staging, and detection of local recurrence and extra-hepatic metastases in colorectal carcinoma, and that they have an important role in the therapeutic decision making process. The clinical value of PET and immunoscintigraphy with ^{131}I or ^{111}In anti-CEA mAb for diagnosis of recurrent colorectal cancer has been confirmed by Ito *et al*[30], who have concluded that PET/CT reflects more accurately the biological character of tumors, but cannot provide the specificity of immunoscintigraphy that enables us to distinguish patients for antibody-based therapy. The superior value of PET with fluorodeoxyglucose for detection of distant metastases (liver, bone, and lung) and lymph node involvement has been estimated in comparison to ^{99m}Tc-labeled anti-CEA Fab for detection of recurrence of colorectal carcinoma[31]. Immunoscintigraphy is superior for detection of local recurrent colorectal cancer, whereas PET is better for detection of distal metastases[32].

RIGS[33] enables localization of small tumor deposits. Roveda *et al*[34] have performed immunoscintigraphy with ^{131}I or ^{111}In anti-CEA and 19.9 mAb using a gamma probe, and have found it particularly useful for endoscopic study of the pelvis after anterior resection, which is difficult to achieve by other diagnostic procedures. Both immunoscintigraphy and RIGS enable a more accurate diagnosis according to Hladic *et al*[35]. Florio *et al*[36] have found positive intraoperative gamma probe detection, although negative for immunoscintigraphy. RIGS applied in primary colorectal cancer enables the detection of occult lymph node metastases[37].

In summary, imaging methods (CT, US, MRI) have an advantage for detection of liver metastases, whereas immunoscinitgraphy is more specific for the assessment of recurrence of abdominal tumors. Thus, immunoscintigraphy should be applied in patients with suspected local recurrence and inconclusive results of routine diagnostic workup.

COMMENTS

Background

Considering that some tumors produce characteristic antigens, scintigraphy with monoclonal antibodies to these antigens seems to be a very promising method for detection. The aim of this study was to evaluate the clinical reliability of immunoscintigraphy for the detection of metastasis and recurrence of colorectal carcinoma, using three different radiopharmaceuticals.

Research frontiers

The results demonstrate that immunoscintigraphy is an accurate method for the detection of cancer recurrence. Together with single photon emission computed tomography (SPECT)/CT and radioimmunoguided surgery, it could have potential for selection of patients for immunotherapy or, in the future, radioimmunotherapy.

Innovations and breakthroughs

Imaging methods (CT, ultrasonography, magnetic resonance imaging) have advantages for detection of liver metastases, whereas immunoscintigraphy is more specific for the assessment of recurrence of abdominal tumors.

Applications

Immunoscintigraphy should be used in patients with suspected local recurrence and inconclusive results from routine diagnostic workup.

Terminology

Monoclonal immunoscintigraphy is scintigraphy with radiolabeled monoclonal antibodies on tumor markers/antigens.

Peer review

The research article by Artiko and his team deals with the usefulness of immnunoscintigraphy for the detection of metastases and the recurrence of colorectal cancer. The results indicate that immunoscintigraphy is reliable and has a specific advantage for the detection of tumor recurrence, and can also be useful for suspected local recurrence. There was a good correlation between immunoscintigraphic evaluation and the results of conventional diagnostic methods.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2431

World J Gastroenterol 2011 May 21; 17(19): 2431-2436
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Long-term outcome and efficacy of endoscopic hemorrhoid ligation for symptomatic internal hemorrhoids

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Received: January 4, 2011 Revised: March 1, 2011
Accepted: March 8, 2011
Published online: May 21, 2011

Abstract

AIM: To assess the long-term outcome of endoscopic hemorrhoid ligation (EHL) for the treatment of symptomatic internal hemorrhoids.

METHODS: A total of 759 consecutive patients (415 males and 344 females) were enrolled. Clinical presentations were rectal bleeding (593 patients) and mucosal prolapse (166 patients). All patients received EHL at outpatient clinics. Hemorrhoid severity was classified by Goligher's grading. The mean follow-up period was 55.4 mo (range, 45-92 mo).

RESULTS: The number of band ligations averaged 2.35 in the first session for bleeding and 2.69 for prolapsed patients. Bleeding was controlled in 587 (98.0%) patients, while prolapse was reduced in 137 (82.5%) patients. After treatment, 93 patients experienced anal pain and 48 patients had mild bleeding. Patient subjective satisfaction was 93.6%. Repeat treatment or surgery was performed if symptoms were not relieved in the first session. In the bleeding group, the recurrence rate was 3.7% (22 patients) at 1 year, and 6.6% and 13.0% at 2 and 5 years. In the prolapsed group, the recurrence rate was 3.0%, 9.6% and 16.9% at 1, 2 and 5 years, respectively.

CONCLUSION: EHL is an easy and well-tolerated procedure for the treatment of symptomatic internal hemorrhoids, with good long-term results.

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Key words: Bleeding; Endoscopy; Hemorrhoid; Ligation; Prolapsed

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Su MY, Chiu CT, Lin WP, Hsu CM, Chen PC. Long-term outcome and efficacy of endoscopic hemorrhoid ligation for symptomatic internal hemorrhoids. *World J Gastroenterol* 2011; 17(19): 2431-2436 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2431.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2431

INTRODUCTION

Hemorrhoids are the most prevalent anorectal disorder among adults, and over 90% of patients undergoing sigmoidoscopy or colonoscopy are found to have hemorrhoids of varying degrees. Hemorrhoids are defined as internal or external based on whether they are located above or below the dentate line. Internal hemorrhoids can be classified into 4 grades using the Goligher system[1]: Grade 1, hemorrhoids with bleeding; Grade 2, hemorrhoids with bleeding and protrusion, with spontaneous reduction; Grade 3, hemorrhoids with bleeding and protrusion that require manual reduction; and Grade 4, prolapsed hemorrhoids that cannot be replaced. Nonoperative management is considered for

patients with symptoms (anal bleeding or rectal prolapse) and grades 1, 2, and 3 internal hemorrhoids. Treatments include local injection therapy, anal divulsion, elastic band ligation, cryotherapy, infrared and laser photocoagulation, direct application of electrical current, and bipolar coagulation[2,3]. Based on the results of a meta-analysis, MacRae and McLeod[4] concluded that rubber band ligation should be recommended for Grades 1 to 3 internal hemorrhoids, and that patients treated by this method were less likely to require additional therapies than those treated with local injection therapy or infrared coagulation.

Since first introduced in the United States in 1951, rubber band ligation has become the mainstay of treatment for bleeding and prolapsing internal hemorrhoids, and is now a well-established, safe, and effective technique[5-7]. It has been shown to be substantially better than medication alone in terms of outcome, and is not associated with significant morbidity[8]. Conventional band ligation is performed with rigid anoscopic devices with limited maneuverability and a narrow field of view, and no ability to document treatment photographically[4]. These drawbacks can be overcome by using a video-endoscopic system that provides a detailed image of the operative field as well as photographic capability[9]. Our previous studies showed a good result of endoscopic hemorrhoid ligation (EHL) in patients with symptomatic internal hemorrhoids at initial therapy and one year after treatment[10,11]. The aim of this study was to further assess the long-term outcome and efficacy of EHL for treatment of symptomatic internal hemorrhoids.

MATERIALS AND METHODS

From November 2000 to October 2004, 759 consecutive patients with symptomatic internal hemorrhoids were treated with EHL and enrolled in this study prospectively. All the procedures were performed by the same endoscopist who has more than 10 years of endoscopic surgery experience. Before EHL, the endoscopist needs to identify the dentate line, and then EHL is easy to perform without learning curve. The whole procedure takes about 2-3 min, and compared to the traditional technique by anosocpe, EHL offers good view and also recordable for the trainee to learn the procedure.

There are 415 male and 344 female patients with a mean age of 54.2 years (range, 18-92 years). Rectal bleeding or prolapse was the major patient complaints. Sixty-eight patients were cirrhotic and 36 patients were uremia.

All patients underwent flexible sigmoidoscopy after fleet enema 1pc or colonoscopy after standard colonic preparation with polyethylene glycol 2000 mL or sodium phosphate 90 mL in splitting doses to exclude other causes of rectal bleeding. Patients were excluded if polyps or evidence of malignancy was found at colonoscopy. All patients gave informed consent for the ligation procedure. Patients were not asked to discontinue the use of aspirin or other non-steroid anti-inflammatory drugs (NSAID) before the procedures. The contraindications of EHL were the same as traditional band ligation, such as thrombosed hemorrhoids, anal fistula or peri-anal abscess.

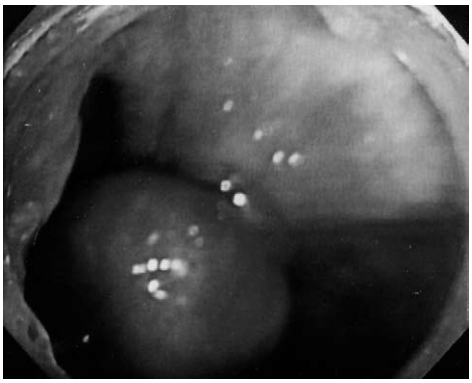

Figure 1 Endoscopic view of internal hemorrhoid, 2-5 mm above the dentate line, ligated with a rubber band.

After the initial endoscopic examination, patients were treated if Grade 2 or greater internal hemorrhoids were present. As with esophageal variceal ligation, a transparent plastic endoscopic ligation cap (Sumitomo Co., Tokyo, Japan) was attached to the top of a diagnostic upper gastrointestinal (GI) endoscope (GIF-XQ230; Olympus Optical Co, Ltd, Tokyo, Japan). The dentate line then was identified, and ligation was performed 2-5 mm above the dentate line (Figure 1). The hemorrhoid was suctioned into the cap with the tip of the endoscope in the anal canal, and a single elastic band was released. If further ligation was required, another rubber band was placed on the cap. All ligations were performed in an outpatient setting without any premedications such as sedatives or analgesics.

Safety data were recorded and all adverse events were documented. After first treatment session, patients were asked to complete a questionnaire to evaluate the subjective satisfaction, which was classified as excellent, good, fair or poor. Patients were seen 1 wk after the procedure and then monthly and sigmoidoscopy after fleet enema 1pc was performed at all visits. In all cases, hemorrhoid severity and recurrent symptoms were assessed yearly after the ligation session. Failure of treatment was defined as persisted symptoms beginning 1 mo after ligation. Recurrent bleeding was defined as anal bleeding with two separate bowel movements or massive bleeding that required further treatment after one month of ligation. Recurrent prolapse was defined as recurrent prolapse symptoms that were troublesome to patient lasting more than 2 mo after initial successful treatment and requiring further treatment.

Student *t* test was used for analysis and *P* value less than 0.05 defined as statistically significant.

RESULTS

All patients were treated with one session initially, and were followed regularly. The mean follow-up period was 55.4 mo (range, 45-92 mo). Overall patient satisfaction was 93.6% after the first treatment session.

Rectal bleeding was the chief complaint of 593 patients: 146 had anemia (hemoglobin < 12 g/dL) due to

Figure 2 Pre-treatment showed grade 4 hemorrhoids.

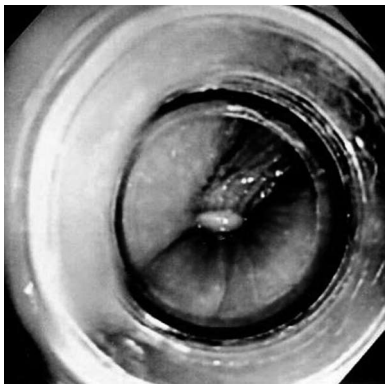

Figure 3 After ligation, grade 4 hemorrhoids showed spontaneous reduction.

Table 1 Severity of hemorrhoids in the prolapsed group before and after the first endoscopic ligation session

	Before treatment	After treatment
Goligher grade	Ⅳ 23	Ⅳ 7
		Ⅲ 16
	Ⅲ 130	Ⅲ 22
		Ⅱ 93
		Ⅰ 15
	Ⅱ 13	Ⅰ 13

hemorrhoid bleeding; 273 experienced intermittent dripping of blood from the anal area; and 174 noted blood intermittently on toilet tissue. Rectal bleeding was controlled in 587 patients (98.0%). The average number of band ligations performed in the first session for rectal bleeding was 2.35. There were 6 patients in whom symptoms were not controlled in the first session, and received a second treatment session. Bleeding in these patients was controlled after second session, thus, all of the patients had their symptoms controlled. The recurrence rate was 3.7% at 1 year (22 patients), 6.6% at 2 years, and 13.0% at 5 years.

Rectal prolapse requiring manual reduction was the major complaint of 166 of the 759 patients, 131 of whom also had intermittent mild rectal bleeding. The severity of hemorrhoid prolapse was classified by Goligher's grading. Most patients (82.5%, 137/166) had their hemorrhoids reduced by at least one grade in the first session (Table 1). Figures 2 and 3 showed improvement of pre- and post-treatment of ligation for Grade 4 hemorrhoids. The average number of band ligations performed in the first session was 2.69. For failed control of symptoms in the first session, 4 patients received further surgical hemorrhoidectomy due to thrombosed hemorrhoids and perianal abscess, while 21 patients received the second session of whom 17 had their symptom controlled. Three patients received a third EHL session and 2 patients showed improvement while the other received a hemorrhoidectomy for poor response to EHL. Five patients in the prolapsed group were lost to follow-up. The recurrence rate at 1 year was 3.0% (5 patients), at 2 years was 9.6%, and at 5 years was 16.9% (Figure 4).

The symptom control rate was higher in the bleeding group than in the prolapsed group (98.0% *vs* 82.5%, respectively; $P = 0.043$). A total of 93 patients (12.3%) experienced mild anal pain or tenesmus sensation 1-3 days after treatment, which was relieved by oral mefenamic acid. There were 48 patients (6.3%) who had mild bleeding 1-14 d after ligation (mild bleeding means some blood noted in tissue papers), and most of them were treated by injection of 1-3 mL diluted solution of epinephrine (1:100 000) in divided doses directly into the wound. Two cirrhotic patients had experienced massive bleeding due to post-ligation ulcers (Figure 5) and required blood transfusion. Bleeding was controlled with local injection of epinephrine. No EHL-related mortality was observed.

The patients' subjective satisfaction was 93.6% (excellent or good response) after the first treatment session. The mean follow-up period was 55.4 mo (range, 45-92 mo). The percentage of symptom free patients during follow-up period is shown in Figure 6.

DISCUSSION

Rubber band ligation has been used to treat internal hemorrhoids since Blaisdale introduced a ligation device in 1951. This device is used via an anoscope to grasp hemorrhoid tissue with small prongs and an elastic band is applied. The hemorrhoid and its redundant mucosal tissues become thrombosed and slough off, usually within 5-7 d. One notable advantage of band ligation is the production of submucosal scarring that prevents subsequent development of new hemorrhoid tissue. Rubber band ligation is technically simple and can be used in the outpatient setting without local anesthesia. The reported success rate varies between 69% and 97%, depending on the degree of internal hemorrhoids, the ligation technique, and the duration of follow-up[8]. Serious complications, such as life-threatening massive bleeding[12,13] and sepsis are extremely rare, but should not be discounted[14,15]. Dickey and Garrett[16] found that hemorrhoid banding using video-endoscopic anoscopy and a single-handed ligator compared favorably with traditional hemorrhoid banding by anoscopy. This video-endoscopic technique may be preferred in the office setting.

There are other several non-operative methods for management of internal hemorrhoids, such as sclerothera-

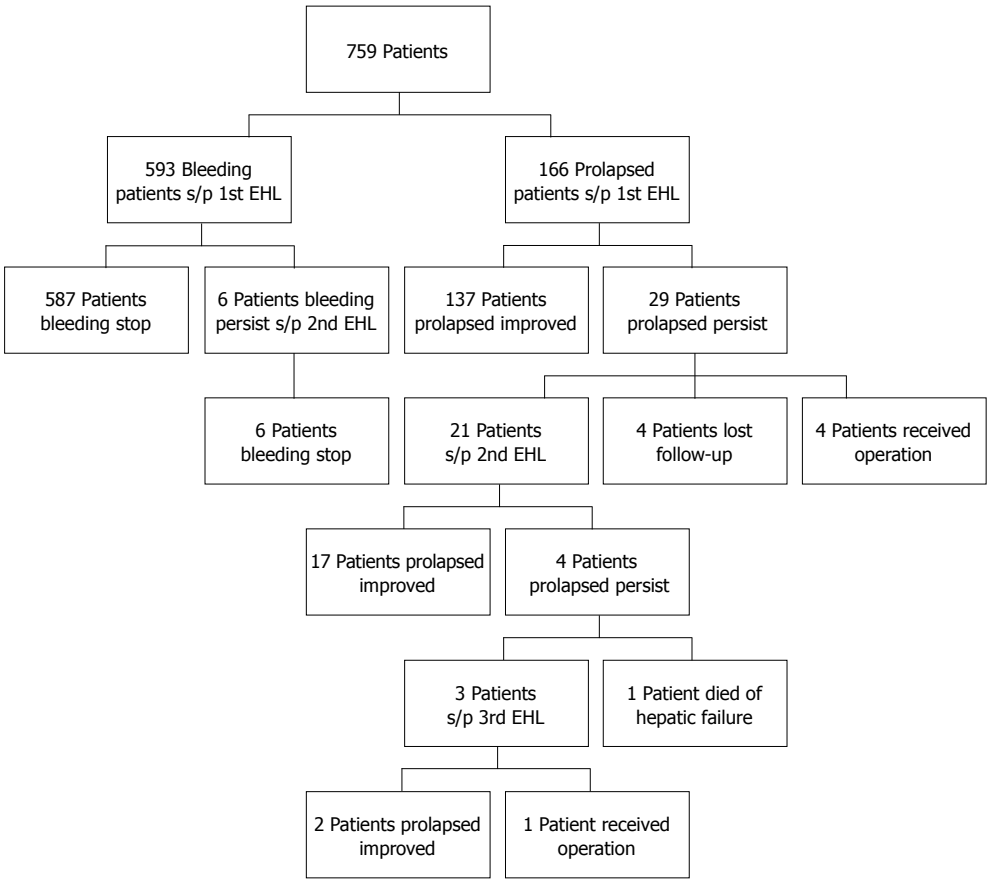

Figure 4 Outcomes of the 759 patients undergoing endoscopic hemorrhoid ligation. EHL: Endoscopic hemorrhoid ligation.

Figure 5 Ulcer formation after ligation.

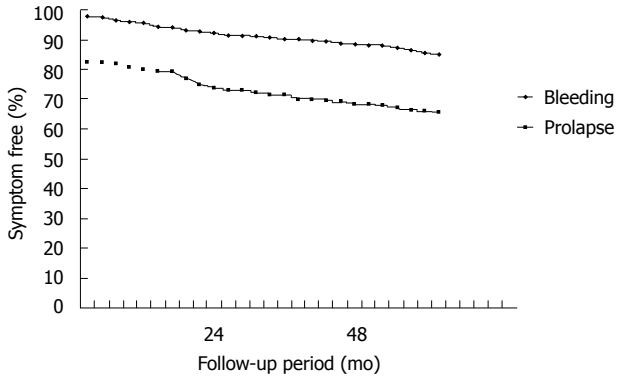

Figure 6 Five-year follow-up for the symptom-free percentage of bleeding and prolapsed patients.

py, cryotherapy, direct-current electrotherapy and infrared photocoagulation. MacRae et al performed a meta-analysis covering 23 studies that compared rubber band ligation, sclerotherapy, hemorrhoidectomy, infrared photocoagulation, and manual dilation of the anus for patients with Grade 1-3 hemorrhoids[17]. They found that rubber band ligation was more effective than sclerotherapy, less likely to require additional therapy than either infrared photocoagulation or sclerotherapy, and more likely to cause pain.

Stiegmann and Goff[18] first proposed elastic band ligation for the treatment of esophageal and gastric varices using a device attached to the tip of a video-endoscope to deploy the bands. Endoscopic band ligation of esophageal varices now is preferred to sclerotherapy because of equivalent efficacy, ease of use, and relatively fewer complications[19,20]. The application of the same device and technique to eradicate internal hemorrhoids is a logical extension of this established procedure. Trowers *et al*[9] reported preliminary experience with endoscopic hemorrhoid ligation in 1997 in which 95% of internal hemorrhoids were reduced by more than one grade after treatment. Berkelhammer and Moosvi[21] used retroflexed endoscopic band ligation to treat bleeding internal hemorrhoids. Excellent results were achieved in 80% of patients with Grade 2 hemorrhoids. In

addition, the results with treatment of patients with Grade 2 hemorrhoids were more likely to be excellent compared with those for patients with Grade 3 hemorrhoids.

There are two different endoscopic hemorrhoid ligation devices, a smaller one that is attached to gastroscope just as variceal ligators, and a larger one with a greater diameter cap used with a colonoscope. Our previous study showed that these two devices provided similar good results for the treatment of symptomatic internal hemorrhoids[10]. A later study in which the variceal ligation device was used with a gastroscope showed good initial and one-year results with more than 90% of hemorrhoids reduced by at least one grade[11].

In this study, we used the gastroscope for hemorrhoid ligation and found that 82.5% (137/166) of patients had their hemorrhoids reduced by at least one grade in the first session, with 93.6% subjective satisfaction. In addition, more than 80% of patients had sustained results. The symptom control rate was better in bleeding group other than prolapsed group. Bleeding hemorrhoids were easily identified by endoscopic view, while the prolapsed loosen hemorrhoid tissue might need more bands ligated to induce adequate submucosal fibrosis which would make the prolapsed tissue fixed in rectum. Only minor complications occurred such as mild anal bleeding and pain, and only 5 patients required further surgical interventions. Two cirrhotic patients had experienced massive bleeding due to post-ligation ulcers but the bleeding was controlled with local injection of epinephrine solution. Because cirrhotic or uremia patients were risky for bleeding tendency, these patients were at a higher risk for operation, EHL provided an alternative therapy for hemorrhoids of these patients and the risk was low compared with operation. The greatest advantage of EHL is that it can be performed repeatedly if needed. Patients who failed in symptom control after their first EHL session treatment received further treatment sessions, and most patients had good results. After successful treatment, the one-year recurrent rate was 3.7% in bleeding group and 3.0% in prolapsed group and the five-year recurrent rate was 13.0% in bleeding group and 16.9% in prolapsed group.

In conclusion, endoscopic hemorrhoid ligation is an important progress in the treatment of symptomatic internal hemorrhoids. Endoscopic hemorrhoid ligation is simple, safe, and effective. Multiple bands can be applied in one session, and further bands can be applied in subsequent sessions if a single session fails to completely eradicate the internal hemorrhoids. The treatment success rate is high, and the long-term recurrence rate is low.

COMMENTS

Background

Previous studies showed a good result of endoscopic hemorrhoid ligation (EHL) in patients with symptomatic internal hemorrhoids at initial therapy and one year after treatment.

Research frontiers

The aim of this study was to further assess the long-term outcome and efficacy of EHL for treatment of symptomatic internal hemorrhoids.

Innovations and breakthroughs

Endoscopic hemorrhoid ligation is an important advance in the treatment of symptomatic internal hemorrhoids. Endoscopic hemorrhoid ligation is simple, safe, and effective. Multiple bands can be applied in one session, and further bands can be applied in subsequent sessions if a single session fails to completely eradicate the internal hemorrhoids. The treatment success rate is high, and the long-term recurrence rate is low.

Applications

EHL is an easy and well-tolerated procedure for the treatment of symptomatic internal hemorrhoids, with good long-term results.

Terminology

EHL means endoscopic hemorrhoidal ligation, which is a new device of rubber band ligation for the treatment of symptomatic internal hemorrhoids.

Peer review

This is a well written and comprehensive report characterizing a substantive cohort in endoscopic hemorrhoidal ligation for the treatment of symptomatic internal hemorrhoids.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2437

World J Gastroenterol 2011 May 21; 17(19): 2437-2445
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Effects of appendectomy and oral tolerance on dextran sulfate sodium colitis

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Received: August 4, 2010 Revised: October 15, 2010
Accepted: October 22, 2010
Published online: May 21, 2011

Abstract

AIM: To evaluate the concomitant effects of appendectomy and oral tolerance on colitis.

METHODS: Delayed-type hypersensitivity (DTH) was investigated at a 7-d interval after ovalbumin (OVA) administration and immunization under normal and colitis conditions in appendectomized or sham-operated mice. Pathological scores for the colon were graded after ingestion of colon-extracted protein (CEP) and induction of dextran sulfate sodium (DSS) colitis in appendectomized or sham-operated mice. Thereafter, Th1 and Th2 in Peyer's patches and spleen lymphocytes were detected in CEP-treated and bovine serum albumin (BSA)-treated control mice.

RESULTS: In appendectomized mice, DTH was not inhibited at day 7 after OVA administration and at the initial phase of DSS colitis, whereas it was inhibited at day 14 and day 21. However, in sham-operated mice, it was inhibited during the whole procedure and the onset of DSS colitis. The protective role of CEP against DSS colitis was present in sham-operated mice, with predominant improvement of colonic pathological changes, while vanished in the appendectomized mice. A shift from Th1 to Th2 in Peyer's patches resulted from a decrease of Th1 cells with the ingestion of CEP. Compared with BSA in the sham-operated group, no predominant changes were observed in the appendectomized mice.

CONCLUSION: Appendectomy interferes with the protective role of CEP in DSS colitis via a shift from Th2 to Th1 during oral tolerance induction.

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Key words: Appendicectomy; Oral tolerance; Dextrin sulfate sodium; Murine colitis; Th1-Th2 balance

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Yue M, Shen Z, Yu CH, Ye H, Ye YF, Li YM. Effects of appendectomy and oral tolerance on dextran sulfate sodium colitis. *World J Gastroenterol* 2011; 17(19): 2437-2445 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2437.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2437

INTRODUCTION

Ulcerative colitis (UC) is one of the major inflammatory bowel diseases (IBDs), and is a chronic inflammatory disorder of the intestine. Multiple factors cause the initiation and pathogenesis of this disorder, including genetic polymorphisms, environmental factors, and immune responses[1]. Studies on mucosal immunological disturbance help greatly understand the course of UC, and establish better therapeutic strategies.

Oral tolerance is one of the major features in gut immunology, and refers to the active non-response to dietary antigens and commensal enteric bacteria or substances administered orally[2,3]. IBD results from environmental trigging agents that act on suspected hosts to cause uncontrolled immune reactions in the gut, and is considered to be a consequence of the breakdown of normal mucosal tolerance[4]. Oral tolerance has been confirmed to be a therapy for a variety of autoimmune diseases including IBD[5].

As a part of the intestine, the appendix has long been considered as a deteriorated organ, until epidemiological research revealed that patients who have had an appendectomy have a lower incidence of UC than those with an intact appendix[6]. Furthermore, appendectomy shows a protective effect on colitis in TCR-$\alpha^{-/-}$ mice and trinitrobenzene sulfonic acid (TNBS)-induced murine colitis[7-9]. These results suggest that appendectomy is a protective factor against UC[10].

Although appendectomy and oral tolerance have been shown to have a potential role in intestine immunology, the combined effect of appendectomy and oral tolerance in the normal gut immune environment and in colitis is still unclear. In this study, the combined effect of appendectomy and oral tolerance was evaluated in mice.

MATERIALS AND METHODS

Mice

Male BALB/c mice, 4-5 wk old, were purchased from Vital River (Beijing, China). Mice were kept under special pathogen-free conditions with free access to food and water, unless required by the experimental protocol. Every six mouse was allocated to the treatment or control group. The study protocol was approved by the Ethics Committee of Zhejiang University School of Medicine.

Reagents

Ovalbumin (OVA; chicken egg albumin, grade V), complete Freund's adjuvant (CFA), bovine serum albumin (BSA), phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St Louis, MO, USA). Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (USA). Antibodies including CD3ε, CD4, CD8α, CD69, interferon (IFN)-γ and interleukin (IL)-4, either conjugated with PE, FITC, PE-cy5 or APC, or their corresponding controls in flow cytometry were purchased from Becton Dickinson Biosciences (USA). RPMI 1640 was purchased from Invitrogen (USA) and Percoll from Amersham Bioscience (USA).

Surgical procedure

BALB/c mice were anesthetized with ketamine hydrochloride under sterile conditions. For all surgical interventions, mice underwent a standardized procedure, starting with a midline laparotomy, followed by mobilization and exteriorization of the appendix. In mice that were subjected to appendectomy, division of the appendix was performed between two ligatures that were placed proximal to the border of the cecum and appendix. The cecal stump was irrigated with saline. In all groups, the abdominal wall was closed in two layers, using a running suture technique. Mice were monitored during the immediate recovery phase after anesthesia, and were assessed daily throughout the recovery period of 1 wk.

Oral tolerance induction

Intragastric administration of 250 μg OVA or colon-extracted protein (CEP) for five times (both were controlled by BSA) was applied in each group, followed by subcutaneous injection of 200 μg OVA in 200 μL 50% CFA.

Delayed-type hypersensitivity and evaluation

For delayed-type hypersensitivity (DTH) test, all mice were injected subcutaneously in the right rear footpad with 25 μL physiological saline that contained 100 μg OVA. After 24 h, the increase in footpad thickness was measured using a helicocaliper, and controlled by the normal left footpad that was treated with the same volume of PBS[11].

Colitis induction and evaluation

DSS colitis was induced by feeding 5% (w/v) DSS diluted in water for 7 d, during which, disease activity index was calculated daily according to body weight, stool consistency, and the occult blood test[12]. Mice were all sacrificed 7 d after treatment with DSS for pathological examination of the descending colon and flow cytometric detection.

Microscopic examination of the colon was performed in a blinded fashion on formalin-fixed tissue sections that were stained with hematoxylin and eosin, as previously described[13]. The sections were classified as 0-3 to evaluate the amount and depth of inflammation, and 0-4 to evaluate the amount of crypt damage or regeneration. These pathological changes were also quantified as the percentage of involvement by the disease process: 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; and 4, 76%-100%. Each section was then scored for each feature separately by establishing the product of the grade for that feature and the percentage involvement (0-12 for inflammation and extent, and 0-16 for regeneration and crypt damage).

CEP preparation

CEP was prepared as described previously[14]. The colon was sampled at 7 d after DSS treatment, minced on ice, and centrifuged for 20 min at 15000 × *g*. Protein concentration was measured after supernatant collection and filtration for sterilization.

Experimental design and procedure

The experiment procedure is shown schematically in Figure 1. The combined effects of appendectomy and OVA-induced oral tolerance were tested under conditions of normal gut immune status and DSS colitis. Oral

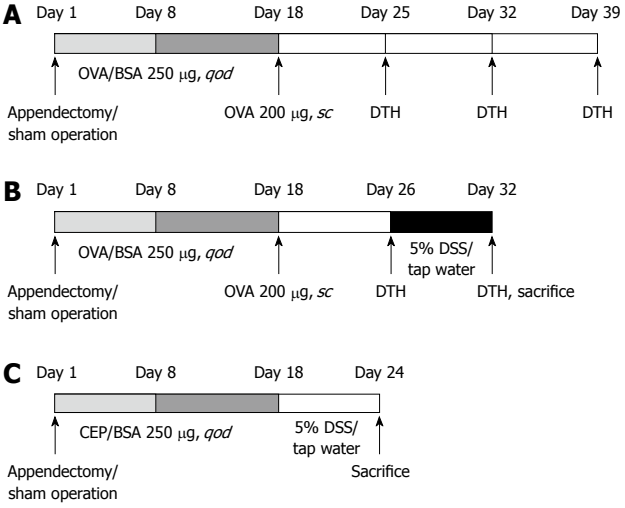

Figure 1 Experimental design and procedure. OVA: Ovalbumin; BSA: Bovine serum albumin; DTH: Delayed-type hypersensitivity; DSS: Dextran sulfate sodium; *qod*: Every other day; *sc*: Subcutaneous injection; CEP: Colonextracted.protein

tolerance was produced in mice by repeated intragastric administration of OVA at day 7 after appendectomy or sham operation, followed by 200 g OVA injected subcutaneously on day 18. DTH assessment was performed on days 25, 32 and 39 (7, 14 and 21 d after OVA immunization). To observe the effect of appendectomy on OVA oral tolerance in colitis, on day 26, a group of mice was challenged with 5% DSS, and DTH assessment was performed at the beginning and end points.

To observe the combined effect of appendectomy and CEP on protection of colitis, another group of mice was challenged with 5% DSS on day 18, and pathological assessment and lymphocyte analysis were performed at the end points.

Cell preparation and cytokine analysis

For lymphocyte isolation of SPL and PPL[15], spleen or Peyer's patch was removed, minced, passed through a nylon strainer, and counted after staining with trypan blue. Th1 and Th2 cells were separated by IFN-γ and IL-4 staining. Lymphocytes were cultured at 37℃ for 6 h in medium that contained ionomycin (500 ng/mL), PMA (5 ng/mL) and GolgiStop.

Statistical analysis

All data are presented as mean ± SD. Paired *t* test was used throughout the experiment, except that the statistical significance of the pathological differences was evaluated by non-parametric Wilcoxon rank sum test. $P < 0.05$ was defined as statistical significance.

RESULTS

Appendectomy postponed oral tolerance response

To assess the establishment of oral tolerance and verify the role of appendectomy, both the appendectomized and the sham-operated mice were injected subcutaneously with 200 g OVA. DTH was scored by measuring the thickness of the footpad at days 7, 14 and 21 after OVA injection (Figure 2).

Compared with the BSA-treated mice, the footpad thickness in the OVA-treated mice increased less significantly at days 14 and 21 in the appendectomized and sham-operated mice. However, on day 7, there was no difference in footpad thickness between the OVA- and BSA-treated appendectomized mice, but there was an obvious difference in the sham-operated mice. This suggests that appendectomy postponed the oral tolerance response.

Appendectomy suppressed oral tolerance in DSS-induced colitis

To evaluate whether appendectomy had an impact on oral tolerance in DSS-induced colitis, appendectomized and sham-operated mice were treated with 5% DSS at day 26 after OVA or BSA ingestion and OVA injection. The immune response was assessed by DTH at days 1 and 7 after DSS treatment (Figure 3).

In the appendectomized mice, DTH assessment showed no difference between OVA and BSA ingestion. In the group of sham-operated mice, although the OVA-ingested mice showed slighter immunoresponse than that of BSA-ingested mice on the first day, we could not observe the difference of immunoresponse between OVA- and BSA-ingested mice group. This was consistent with previous results and suggested that appendectomy did not enhance the oral tolerance protection in colitis. Furthermore, OVA treatment showed no protective effect against colitis both in the appendectomized and the sham-operated mice after 7 d, and the index of footpad thickness showed no significant difference. Taken together, these results suggested that OVA-induced oral tolerance was only present in sham-operated mice, and appendectomy suppressed oral tolerance in DSS-induced colitis.

Appendectomy eliminated the protective role of CEP against DSS colitis

It is possible that the non-protection of OVA-induced oral tolerance to DSS colitis resulted from the fact that OVA is not a specific oral tolerance antigen to DSS colitis. Therefore, we replaced OVA with CEP in the appendectomized and the sham-operated mice on day 8. After repeated intragastric administration of CEP or BSA, for five times on day 10, the mice were challenged with 5% DSS. Murine DSS colitis was characterized by destruction of the basic structure of colonic villi and almost total cast of epithelium, with the disappearance of crypts and massive infiltration of lymphocytes. In the appendectomized mice, CEP- and BSA-treated groups showed similar histological features, with massive lymphocytes infiltration, loss of goblet cells, crypt damage, mucosal ulceration, and accompanying submucosal edema (Figures 4 and 5). In the sham-operated mice, the CEP-treated group showed less amelioration of colonic inflammation compared with the BSA-treated control group. Pathological score showed that, in sham-operated mice, ingestion of CEP resulted in mild pathological changes in the descending colon, with a significantly lower

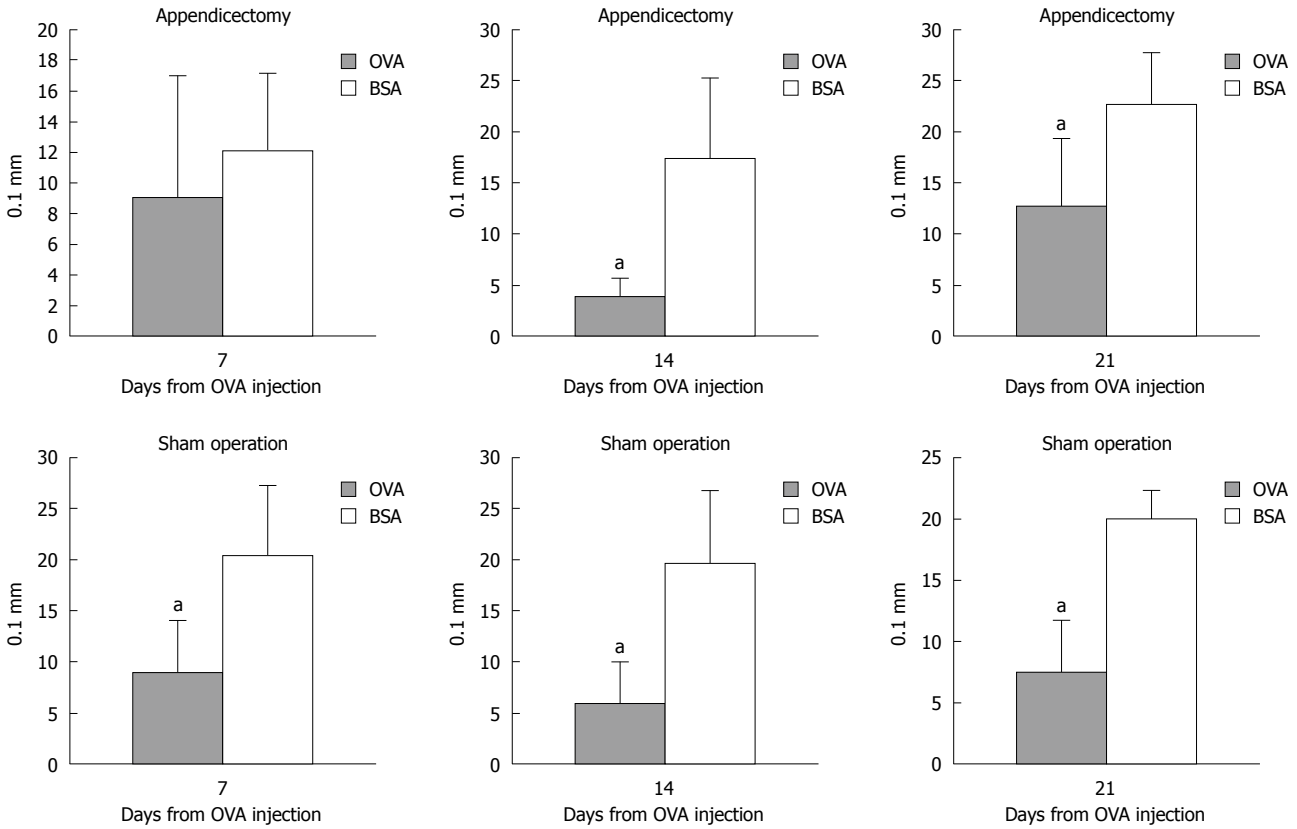

Figure 2 Delayed-type hypersensitivity was scored at days 7, 14 and 21 after ovalbumin injection in appendectomized and sham-operated mice. $^{a}P < 0.05$ *vs* bovine serum albumin (BSA) control mice in appendectomized or sham-operated groups. OVA: Ovalbumin.

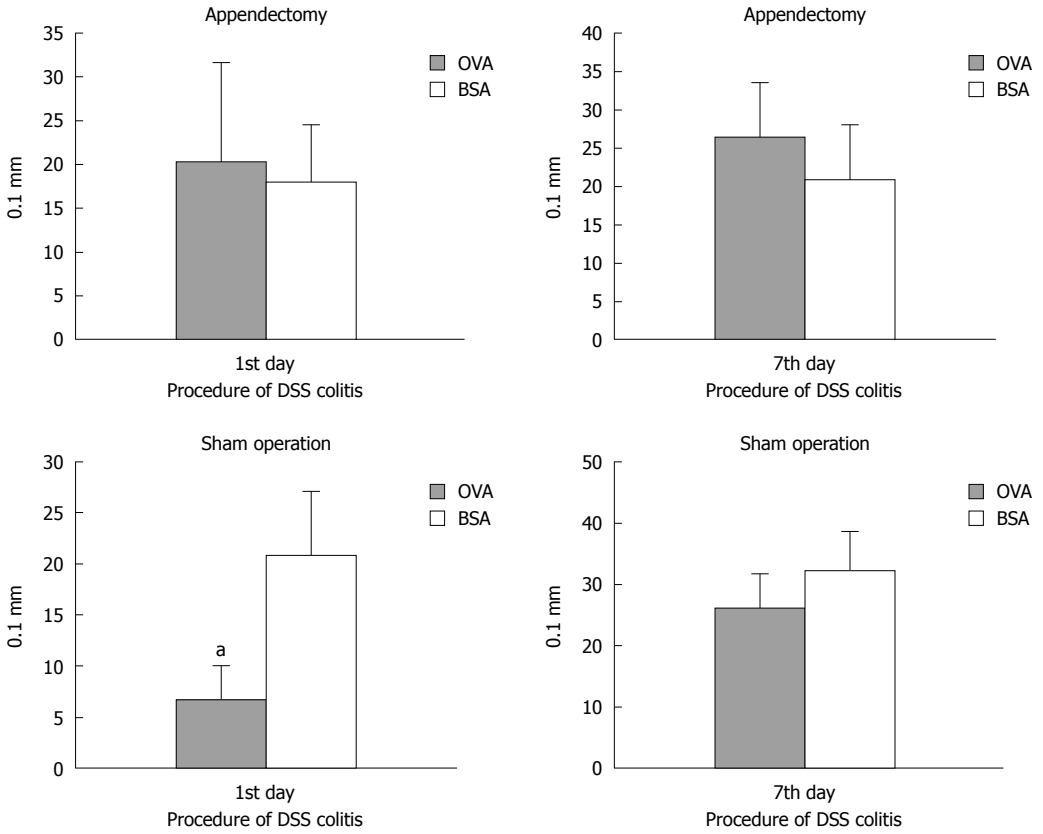

Figure 3 Delayed-type hypersensitivity on days 1 and 7 with 5% dextran sulfate sodium treatment after ovalbumin- or bovine serum albumin-ingestion and ovalbumin-injection in appendectomized and sham-operated mice. $^{a}P < 0.05$ *vs* bovine serum albumin (BSA) control mice in sham-operated group. DSS: Dextran sulfate sodium; OVA: Ovalbumin.

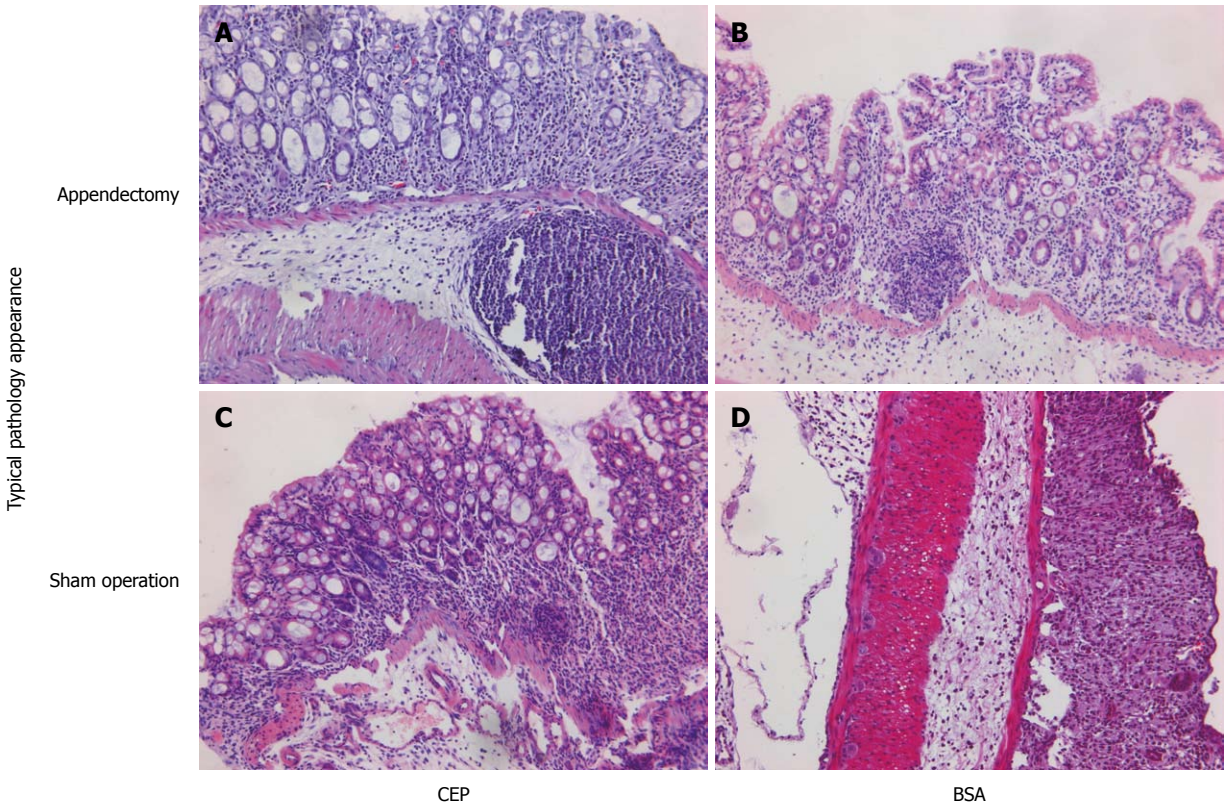

Figure 4 Histological findings in colon-extracted protein- or bovine serum albumin-treated mice in appendectomized (A and B) and sham-operated (C and D) groups. In the appendectomy group, there were similar histological features, massive lymphocyte infiltration, loss of goblet cells, crypt damage, mucosal ulceration, and accompanying submucosal edema, whereas in sham-operated mice, there was less amelioration of colonic inflammation. CEP: Colon-extracted protein; BSA: Bovine serum albumin.

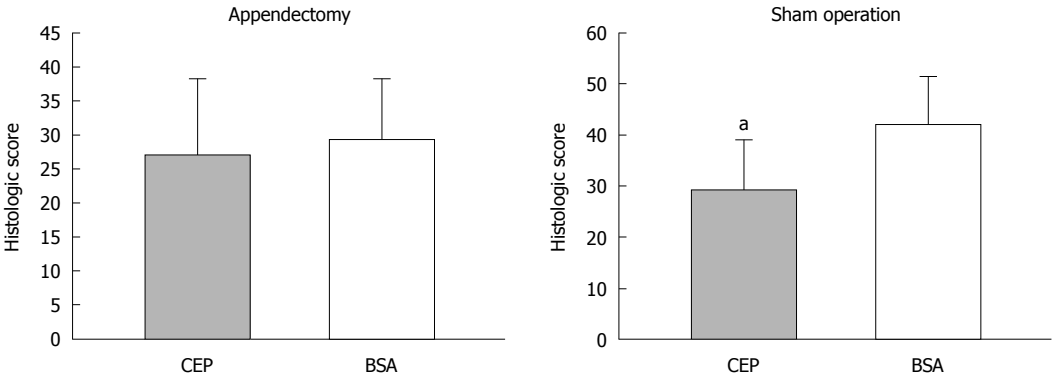

Figure 5 Blinded histological assessment of colitis at day 7 after treatment with 5% dextran sulfate sodium in appendectomized and sham-operated mice with colon-extracted protein or bovine serum albumin ingestion, based on the validated scoring system. [a]*P* < 0.05 *vs* bovine serum albumin (BSA)-treated mice in sham-operated group. CEP: Colon-extracted protein.

pathological score compared with the BSA controls. However, in the appendectomized mice, no significant difference was observed between the CEP- and BSA-treated groups. Our results suggested that appendectomy eliminated the protective role of CEP against DSS colitis.

Inhibition of the protective role of CEP against DSS colitis by appendectomy was associated with Th1/Th2 in PPL

We investigated the possible role of Th1 and Th2 in the difference of CEP protection against DSS colitis between the appendectomized the sham-operated mice (Figures 6 and 7). Th1 and Th2 of PPL and SPL were isolated at the end of colitis and stained with antibodies to IFN-γ and IL-4. The ratio of Th1/Th2 in SPL showed no significant difference between appendectomized and sham-operated mice. However, in PPL, Th1 decreased predominantly after ingestion of CEP in the sham-operated mice compared with BSA control mice, which resulted in the same trend change in Th1/Th2 ratio. Meanwhile, this situation was not observed in Th2 cells. With regard to the appendectomized mice, the number of Th1 and Th2 and their ratio did not change significantly.

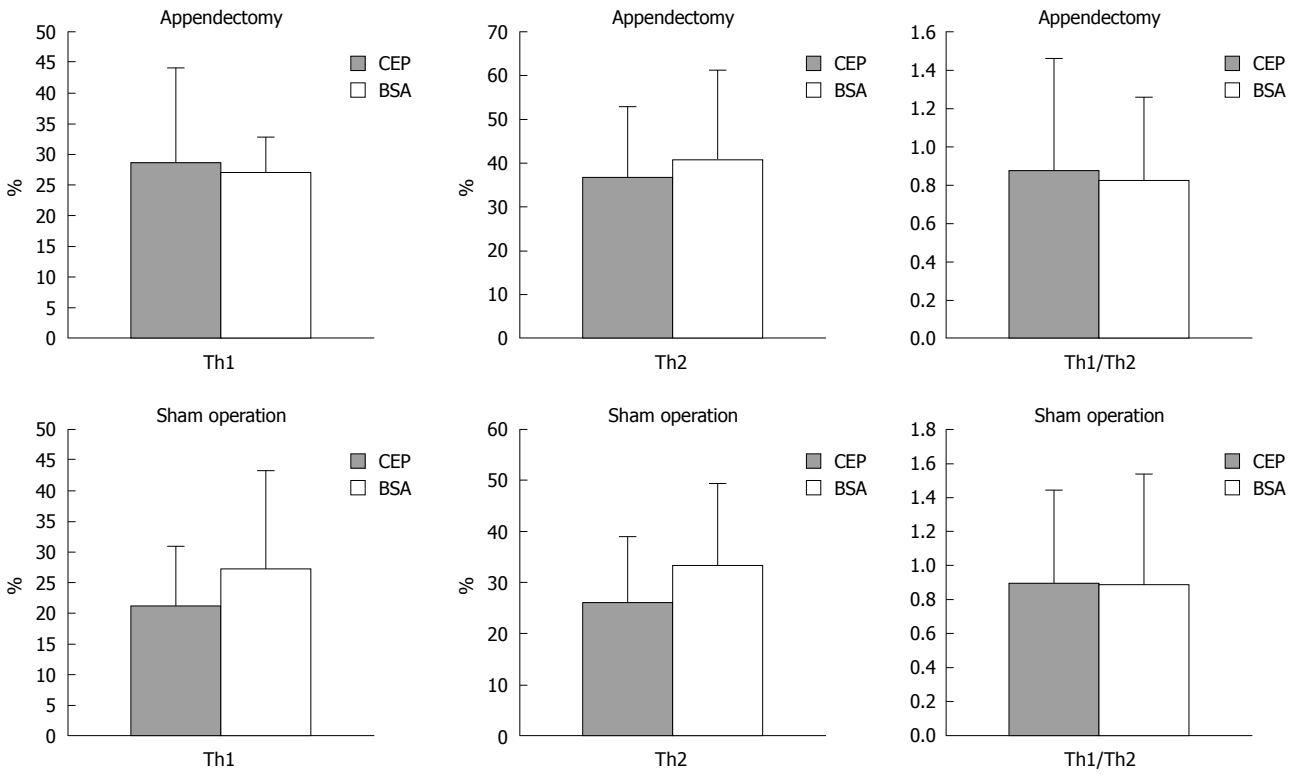

Figure 6 Th1, Th2 and the ratio of Th1/Th2 in spleen lymphocytes at day 7 after treatment with 5% dextran sulfate sodium in appendectomized and sham-operated mice with colon-extracted protein or bovine serum albumin ingestion. CEP: Colon-extracted protein; BSA: Bovine serum albumin.

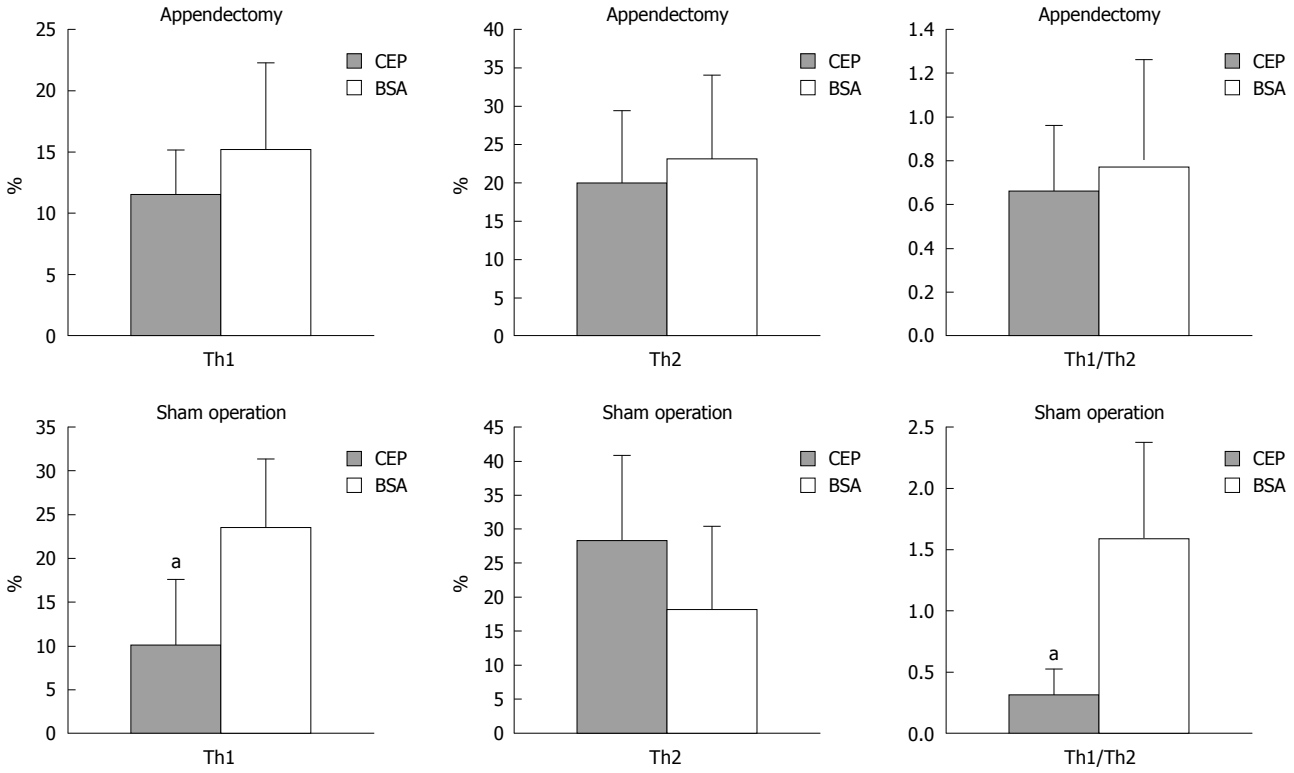

Figure 7 Th1, Th2 and the ratio of Th1/Th2 in Peyer's patch lymphocytes at day 7 after treatment with 5% dextran sulfate sodium in appendectomized and sham-operated mice with colon-extracted protein or bovine serum albumin ingestion. $^{a}P < 0.05$ *vs* bovine serum albumin (BSA)-treated mice in the sham-operated group. CEP: Colon-extracted protein.

DISCUSSION

UC is a chronic relapsing inflammatory disorder of the large intestine. Results from experimental and clinical studies have indicated that there are multifactors in initiation and pathogenesis of this disorder, involving interactions among genetic, environmental, and immune factors[16]. Studies on mucosal immunological disturbance help greatly to understand the course of UC and might be useful to establish better therapeutic strategies. Among the immunotherapeutic options for UC, there are two phenomena that differ from other anti-cytokine strategies. One is the relationship between appendectomy and UC, and the other is the application of oral tolerance.

Appendicular lymphoid tissue, together with Peyer's patches, intestinal intraepithelial and lamina propria lymphocytes, and mesenteric lymph nodes, has been considered as one component of the gut-associated lymphatic tissue (GALT). Several studies have suggested an inverse association between appendectomy and UC[17,18]. UC with prior appendectomy is associated with a milder disease, as defined by the lesser requirement for immuno-suppression or colectomy for severe colitis[19]. A meta-analysis has reported a strong and consistent inverse association between appendectomy and the development of UC[20]. Further support for the involvement of the appendix in IBD is provided by the observation that appendectomy prevents the development of spontaneous experimental colitis in T-cell receptor α mutant mice[7]. However, things seem to be more complicated in humans. Histopathological findings that show the skip lesion of inflammation in the appendix in UC patients suggest that inflammation at one site in the large intestine of predisposed individuals might trigger UC at distant sites. Okazaki *et al*[21] have reported a case with a positive response of UC to appendectomy, with improved colonoscopic and microscopic findings. Several studies have shown inadequate and obscure effects on UC, including a cohort study in Denmark that has proved that appendectomy has no beneficial effect on admission rates in patients with UC[22]. If we disregard those studies with poor or inadequately described methods, the evidence still supports an inverse association between appendectomy, particularly at a young age, and later risk of UC[17]. All the problems might come from the fact that the specific immunological changes after appendectomy remain poorly defined.

Oral tolerance is one of the main features in GALT. It can be described as a state of specific immunological unresponsiveness to antigens that are capable of inducing a protective or injurious immune response under other circumstances. Neurath *et al*[23] have reported that induction of oral tolerance could be beneficial to mural TNBS colitis, *via* the mediation of transforming growth factor β, suppressor lymphocytes, or suppression of humoral immunity[24,25]. Kraus *et al*[26] have confirmed a defect in oral tolerance in patients with IBD, with keyhole limpet hemocyanin, which implies that disturbance of gut tolerance is present in IBD. Good results have been obtained from a double-blind clinical trial for the treatment of Crohn's disease by oral administration of Alequel, a mixture of autologous CEP, although the patients included might not be sufficient to achieve statistically significant results, but they still showed a good tendency[27].

Thus, appendectomy and oral tolerance were both involved in the disturbance of gut immunity in IBD. Their relationship and corresponding mechanism has not been clearly defined. Our study assessed the contribution of the appendix during oral tolerance induction by OVA in the normal or inflammatory gut environment. Our results clearly demonstrated that appendectomy postponed the occurrence of oral tolerance under normal and inflammatory conditions. At the same time, the protective role of CEP against mural DSS colitis could be inhibited when appendectomy was performed.

Concerning the effect of appendectomy on the traditional oral tolerance model induced by OVA feeding, our data from the normal gut immune status are in agreement with those from DSS colitis. The appendiceal lymphoid tissue is predominantly composed of CD4+ T-helper cells and B lymphocytes. Although the exact function of the appendix is undetermined, it is potentially involved in antigen sampling from the gut lumen and induces mucosal immunological responses as a part of the GALT, together with Peyer's patches. Therefore, it is possible that the suppressive effect on oral tolerance is transient in normal mice, as indicated by the fact that DTH reactions were re-inhibited after 2-3 wk in the appendectomized mice. At the onset of DSS colitis, the inhibition of oral tolerance is still predominant, with no significant inhibition of DTH reaction in the appendectomized mice. No difference was noticed at the end of colitis in IBD, because no inhibition of DTH with the defect in oral tolerance was observed[26]. Clearly, the appendix is involved in oral tolerance, at least at the early inductive stage, but to maintain oral tolerance, it seems not to be as important as Peyer's patches, which have a similar histological composition and may have compensative effects.

For the clinical application of oral tolerance in IBD, the effectiveness of oral administration of CEP has been demonstrated in a series of rodent experiments[14,28,29]. TNBS colitis, which features Th1 oversensitivity, is the main model involved in the proof of protection from oral tolerance. The fact that it functions well in DSS colitis implies that oral tolerance could be an innovative strategy for immunotherapy of IBD. While as the appendix may be involved, the protective role of oral tolerance could be indistinctive in the appendectomized mice which shows the possibility of limited application in patients with appendix removed. Therefore, if oral tolerance is to work effectively, whether the appendix remains intact should be confirmed, as well as the dose to ensure a better response. Although we demonstrated the correlation between appendectomy and oral tolerance in UC, the mechanisms that mediate the protection afforded by appendectomy or oral tolerance, and their relationship, are still under investigation.

Mucosal immune dysfunction has been frequently proposed as a key factor in the development of IBD. Studies in animals or humans with IBD imply an abnor-

mal intestinal mucosal barrier function, overproduction of Th1 or Th2 cytokines, and uncontrolled activation of $CD4^+$ T cells by components of colonic bacteria as contributing factors to the pathogenesis of UC[30]. The Th1/Th2 paradigm has been shown to be crucial for oral tolerance to maintain the immune homeostasis in the gut, and at the same time, its disequilibrium correlates with occurrence of IBD[31,32]. Another strong proof of the participation of the Th1/Th2 paradigm in IBD is a series of studies on the effect of worm infection, which can induce a predominant Th2 immune response. Epidemiological research has demonstrated a high prevalence rate of IBD and low worm infection rate in developed countries, while the opposite results have been obtained in developing countries[33]. Worm infection can even be therapeutic for colitis in IL-10 knockout mice or TNBS colitis with a deviation in the Th1/Th2 balance[34,35]. The question remains whether Th1/Th2 is involved in the effect of the appendix on oral tolerance induced by CEP administration in DSS colitis.

A variety of experimental models, including the DSS model, have been developed to investigate mechanisms involved in the pathogenesis of IBD[36]. Oral administration of DSS to mice can cause acute or chronic colitis with similar histopathology as human UC, and has proven to be valuable for the assessment of cause and effect relationships between specific treatment and colitis. However, the specific pathogenic mechanisms that underlie DSS colitis remain elusive. DSS-mediated colonic injury is thought to result from mucosal damage, followed by the upregulation of Th1 and Th2 cytokines, as well as other pro-inflammatory mediators[37]. We investigated the Th1/Th2 ratio and the secretion of IFN-γ and IL-4 from SPL and PPL in appendectomized or control mice after CEP ingestion and DSS colitis. The deviation from Th1 to Th2 disappeared, together with improvement of colonic pathology in PPL caused by CEP induction in the appendectomized mice, whereas in sham-operated mice, there was a predominant Th1 to Th2 deviation that resulted from Th1 inhibition caused by CEP ingestion compared with BSA. This implies a crucial role for the appendix in the development of mucosal oral tolerance, and this could be via the deviation of Th1/Th2 in PPL. With regard to the complexity of the immune network in GALT, our results suggest that the appendix is involved in the initial phase of the immune response, which is complied with the histologic composition. The continued changes remain to be further investigated.

In summary, the appendix gives us an approach to investigate gastrointestinal immunology. We studied the effect of appendectomy on CEP-induced tolerance to DSS colitis and its relationship with the Th1/Th2 paradigm. Our results imply that the appendix plays a role in maintenance of oral tolerance induced by CEP in DSS colitis, and this function could be related to the deviation from Th1 to Th2 in PPL. These results provide a new insight into the pathogenesis of IBD, and perhaps more efficient use of appendectomy or oral tolerance as a therapeutic tool.

ACKNOWLEDGMENTS

We thank Professor Yu-Lan Liu, Professor Shan Wang and the Surgical Oncology Laboratory of Peking University, China for their technical support.

COMMENTS

Background

Oral tolerance refers to the active non-response to dietary antigens and commensal enteric bacteria or substances administered orally. Inflammatory bowel disease (IBD) results from environmental trigging agents acting on suspected hosts to cause uncontrolled immune reactions in the gut. Oral tolerance has been validated to be therapeutic in a variety of autoimmune diseases including IBD. Appendectomy showed a protective effect against colitis. However, the combined effect of appendectomy and oral tolerance in the normal gut immune environment and colitis is still unclear.

Research frontiers

Studies on mucosal immunology disturbance help greatly to understand the course of ulcerative colitis, and may be useful to establish better therapeutic strategies.

Innovations and breakthroughs

The combined effect of appendectomy and oral tolerance was evaluated by challenge in dextran sulfate sodium (DSS) colitis with oral tolerance induction, with or without appendectomy.

Applications

The result is novel and offers great potential for understanding the role of the appendix in the maintenance of immune homeostasis during oral tolerance in the gut. Appendectomy interferes with the protective role of colonic extracted protein against DSS colitis, *via* a shift from Th2 to Th1 during oral tolerance induction. The appendix might play a role in the maintenance of immune homeostasis during oral tolerance in the gut.

Terminology

Oral tolerance: oral tolerance is one of the major features in gut immunology, and refers to the active non-response to dietary antigens and commensal enteric bacteria or substances administered orally. It has been confirmed to be a therapy for a variety of autoimmune diseases including IBD.

Peer review

This study investigated the effect of appendectomy on oral tolerance protection against DSS-induced murine colitis. The results are novel and offer great potential for understanding the role of the appendix in the maintenance of immune homeostasis during oral tolerance in the gut. The study was performed in a detailed and convincing manner. The results are interesting.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2446

World J Gastroenterol 2011 May 21; 17(19): 2446-2449
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

A case of steroid-dependent myeloid granulocytic sarcoma masquerading as Crohn's disease

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Received: December 17, 2010 Revised: February 8, 2011
Accepted: February 15, 2011
Published online: May 21, 2011

Abstract

Small bowel tumors and Crohn's disease are common causes of small bowel obstruction. Early stage neoplasms can easily be mistaken for Crohn's disease. Therefore, thorough work-ups including imaging studies and endoscopic evaluation with biopsies are critical for accurate diagnosis. Here we report a case of an otherwise healthy female with progressive onset of multiple, recurrent obstructive symptoms secondary to terminal ileal narrowing who was referred for management of steroid-dependent Crohn's disease. After thorough evaluation, the diagnosis was revised to myeloid granulocytic sarcoma involving the terminal ileum. In this case, a delay in diagnosis can be detrimental for prognosis, as myeloid granulocytic sarcoma is highly predictive of underlying acute myeloid leukemia and needs urgent referral for chemotherapy and/or resection.

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Key words: Crohn's disease; Myeloid sarcoma; Ileum; Steroids

Peer reviewer: Sharad Karandikar, Consultant General and Colorectal Surgeon, Department of General Surgery, Birmingham Heartlands Hospital, Birmingham, B95SS, United Kingdom

Kwan LY, Targan SR, Shih DQ. A case of steroid-dependent myeloid granulocytic sarcoma masquerading as Crohn's disease. *World J Gastroenterol* 2011; 17(19): 2446-2449 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2446.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2446

INTRODUCTION

Small bowel tumors are uncommon and represent 1%-2% of all gastrointestinal (GI) neoplasms[1-3]. The most common locations are in order: (1) ileum; (2) duodenum; and (3) jejunum. Two large population studies reviewing small intestinal cancers reported the most common histologic types and respective subtypes were: (1) neuroendocrine (39%)/carcinoid (90%); (2) carcinoma (31%)/adenocarcinoma (69%); (3) lymphoma (18%)/large B cell (50%); and (4) sarcoma (10%)/GI stromal tumor (57%)[4,5]. Myeloid sarcoma of the small intestine is unique and rare, with only 3 of 10945 cases reported in a recent SEER (Surveillance, Epidemiology and End Results) population study[5]. Patients often present with abdominal pain, obstruction, GI bleeding, or an abdominal mass. For patients with underlying Crohn's disease, these symptoms can often mimic an active flare. Here we report a case of a steroid-dependent patient who was referred to our tertiary disease center. After endoscopy and workup, a rare case of myeloid granulocytic sarcoma of the ileum was diagnosed in an otherwise healthy, non-leukemic patient.

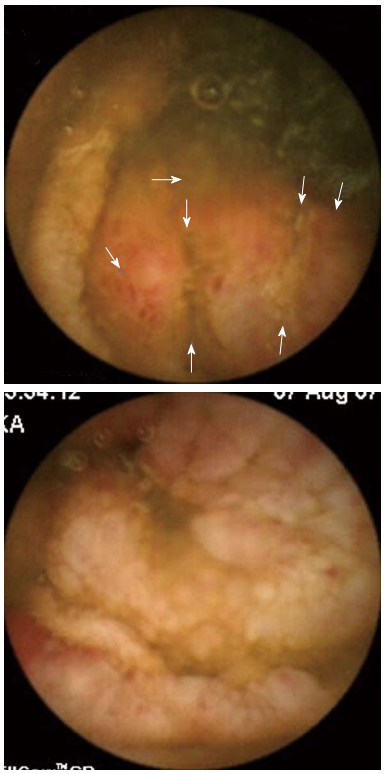

Figure 1 Capsule images of ulcerated, lumpy mucosa from the distal jejunum to proximal ileum.

CASE REPORT

A 39-year-old female with no significant past medical history, presented with a 5-mo history of nausea, vomiting, non-bloody water diarrhea, and right lower quadrant crampy abdominal pain, resulting in multiple, recurrent small bowel obstructions. Her initial hospitalization and diagnostic workup gave a preliminary diagnosis of Crohn's disease based on computerized tomography (CT) findings of a thick walled terminal ileum. She responded immediately to bowel rest and steroid therapy, and was then discharged home on a 6-wk taper regimen, and started on mesalamine (Pentasa) 4 g/d. However, she had recurrent obstructive symptoms when weaned below 10 mg prednisone daily resulting in another Emergency Department visit and a repeat course of oral prednisone. In the subsequent 2 mo, she was hospitalized twice more for recurrent small bowel obstruction, each time after attempts to wean off oral steroid therapy. She remained steroid-dependent despite mesalamine (Pentasa) 4 g/d and a trial of budesonide 9 mg/d. Previous NSAID use was limited to one regular strength ibuprofen per day for menstrual cramps. She associated symptoms of nausea, vomiting, bloating, and oral ulcers with increased stress.

Laboratory tests were unremarkable including inflammatory markers (erythrocyte sedimentation rate 9, C-reactive protein < 0.07) and inflammatory bowel disease (IBD)-7 serology panel, ASCA (anti-*S. cerevisiae* antibody) IgA < 12 EU/mL, ASCA IgG < 12 EU/mL, anti-OMPC (outer membrane protein C of *E. Coli*) IgA 3.9 EU/mL, anti-CBir1 3.8, and pANCA (perinuclear anti-neutrophil cytoplasmic antibody) < 12.1. Initial colonoscopy and ileoscopy were unremarkable with normal biopsies. Wireless capsule enteroscopy showed inflammation in the proximal ileum.

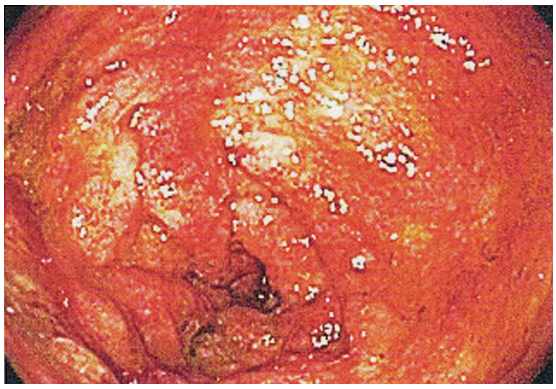

Figure 2 Colonoscopy image of erythematous, ulcerated, lumpy mucosa of the terminal ileum.

The patient was referred to our IBD center for management of steroid-dependent Crohn's disease. We reviewed her wireless capsule study and noted additional findings that included ulcerated lumpy mucosa from the distal jejunum to proximal ileum (Figure 1), suggestive of neoplastic changes. Colonoscopy using a pediatric colonoscope showed normal colonic mucosa and ileocecal valve, but there was erythematous, ulcerated, lumpy mucosa for at least 20 cm into the terminal ileum (Figure 2). We could not advance the pediatric colonoscope further due to ileal narrowing. Ileal biopsies showed an extramedullary myeloid cell tumor (granulocytic sarcoma) with immunologic characterization consistent with myeloid lineage (myeloperoxidase positive) with co-expression of CD34, lysozyme, CD15 and CD68. Biopsies were negative for B-cell markers (CD20, CD79a, PAX-5) and T-cell markers (CD2, CD3, CD4, CD5, CD7, and CD8) (Figure 3). Other work-ups included positron emission tomography/CT that showed increased activity of the thickened bowel in the mid-anterior right quadrant, normal bone marrow aspirate, normal peripheral blood smear and flow cytometry, and fluorescent *in situ* hybridization (FISH) cytogenetics were negative for inv(16) and t(8;21) gene fusion. She underwent standard chemotherapy for acute myeloid leukemia (AML) followed by resection of the ileum and is currently disease free for 2 years.

DISCUSSION

Clinical presentation of a small bowel neoplasm can be nonspecific and often mimics active Crohn's disease. Here we demonstrate a case of a presumed Crohn's flare concealing an underlying small bowel malignancy. We also report a rare case of a sporadic isolated extramedullary myeloid cell tumor of the terminal ileum.

Myeloid sarcoma occurs in about 2 per 1 000 000 adults and 0.7 per 1 000 000 children[6]. Myeloid granulocytic sarcoma can present as an isolated tumor (67%) or as multiple lesions involving a variety of anatomic locations: skin, bone/spine, lymph nodes[7,8]. The GI tract was reported to

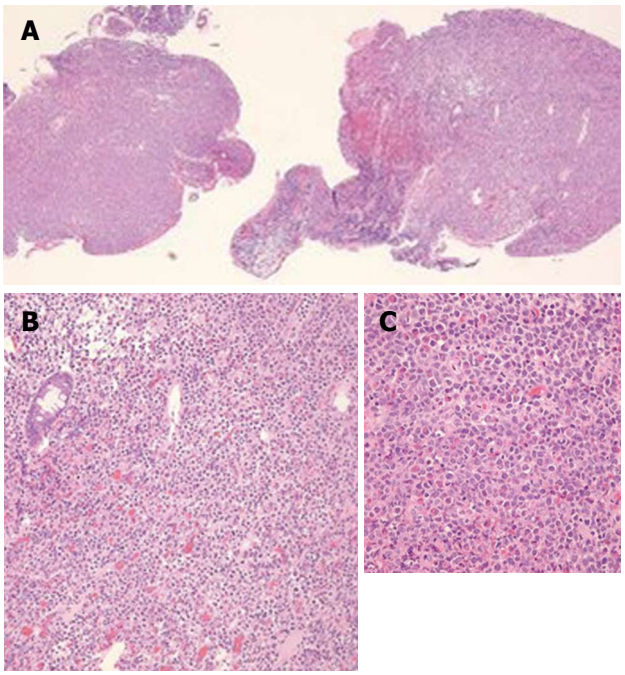

Figure 3 Biopsies from the terminal ileum showing extramedullary myeloid cell tumor (granulocytic sarcoma). A: 40 x magnification; B: 200 x magnification; C: 400 x magnification.

be involved in 4 of 61 (7%) cases[9] with the small intestine being the most frequent site (10%-11%)[7,9]. In a series of 20 cases of granulocytic sarcoma of the small intestine, 17 were localized; 11 of 17 involved the ileum, 12 were in non-leukemic patients, and one occurred during a myeloid leukemia blast crisis. Eight of 12 aleukemic cases progressed to AML within a mean of 10.8 mo[8]. Another report found 90% of aleukemic patients with granulocytic sarcoma progressed to AML within 10.5-11 mo[10].

Isolated myeloid infiltrative small bowel tumors are often mistaken for non-Hodgkin lymphoma or diffuse large B cell lymphoma[11]. Other considerations which must be ruled out include lymphoproliferative disorders and poorly differentiated carcinoma in adults, and melanoma, neuroblastoma, rhabdosarcoma, Ewing sarcoma, and medulloblastoma, in children[12]. Thus, accurate diagnosis of granulocytic sarcoma by histochemical and immunoperoxidase staining, cytogenetic, FISH, or flow cytometry is critical[8].

Cytogenetic abnormalities often reported in aleukemic cases of isolated GI tract granulocytic sarcoma are inversion of p16 and/or t(8; 21) fusion gene[8]. Here we report a case of isolated granulocytic sarcoma of the ileum in an aleukemic patient without inv(16) or t(8; 21) and with no clinical manifestations of a leukemic disorder at diagnosis.

New diagnosis of granulocytic sarcoma in a non-leukemic patient often predicts onset of AML and a potential blast crisis within 1 year. In a case series, 4 of 7 sporadic intestinal granulocytic sarcoma developed AML within an average of 8 mo (range, 4-21 mo)[13]. Another report found a median time of 9 mo for isolated granulocytic sarcoma to develop AML with median survival of 22 mo[14].

The prognosis of isolated myeloid sarcoma is variable but is better if diagnosed early[8,15,16]. The prognosis is poor once there is infiltration of the GI tract with leukemic cells[17]. Systemic chemotherapy, especially AML type induction chemotherapy can delay the time to develop AML and prolong the survival period[7,9,14,18,19]. Yamauchi *et al*[7] showed a longer non-leukemic period after diagnosis of granulocytic sarcoma (median 12 mo) in patients treated with systemic chemotherapy (median 3-6 mo). In a series of 74 cases of primary granulocytic sarcoma without transformation to AML within 1 mo of diagnosis, 58% who received chemotherapy were disease-free for up to 11 mo, and 19% for up to 2 years compared with 5% who did not receive chemotherapy[8]. Surgical intervention or radiotherapy has not been proven to influence survival but may be indicated for symptomatic complications of tumors such as obstruction or perforation[14]. Nevertheless, a management plan with the goal of complete eradication and remission of leukemia and its respective potential needs to be made on an individualized basis.

In summary, intestinal granulocytic sarcoma is rare but should be part of the differential diagnosis for any sporadic small bowel mass of unknown origin, especially in the non-leukemic patient. An early and accurate diagnosis predicts the best outcome and survival. Granulocytic sarcoma is often a precursor to a diagnosis of AML needing urgent systemic chemotherapy and/or any combination of surgical resection, radiotherapy, or stem cell transplantation. Incomplete workup and presumptive diagnosis of Crohn's disease for the initial manifestation of an ileal narrowing or obstruction can easily delay the diagnosis of malignant neoplasms which require urgent intervention and treatment.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2450

World J Gastroenterol 2011 May 21; 17(19): 2450-2453
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Optimized management of advanced hepatocellular carcinoma: Four long-lasting responses to sorafenib

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Received: September 2, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: May 21, 2011

Abstract

The therapeutic options for hepatocellular carcinoma (HCC) have been so far rather inadequate. Sorafenib has shown an overall survival benefit and has become the new standard of care for advanced HCC. Nevertheless, in clinical practice, some patients are discontinuing this drug because of side effects, and misinterpretation of radiographic response may contribute to this. We highlight the importance of prolonged sorafenib administration, even at reduced dose, and of qualitative and careful radiographic evaluation. We observed two partial and two complete responses, one histologically confirmed, with progression-free survival ranging from 12 to 62 mo. Three of the responses were achieved following substantial dose reductions, and a gradual change in lesion density preceded or paralleled tumor shrinkage, as seen by computed tomography. This report supports the feasibility of dose adjustments to allow prolonged administration of sorafenib, and highlights the need for new imaging criteria for a more appropriate characterization of response in HCC.

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Key words: Hepatocellular carcinoma; Sorafenib; Drug toxicity; Response criteria; Decision making

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Abbadessa G, Rimassa L, Pressiani T, Carrillo-Infante C, Cucchi E, Santoro A. Optimized management of advanced hepatocellular carcinoma: Four long-lasting responses to sorafenib. *World J Gastroenterol* 2011; 17(19): 2450-2453 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2450.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2450

INTRODUCTION

Therapeutic options for advanced hepatocellular carcinoma (HCC) have been so far inadequate[1-5]. Recent progress in molecular biology has allowed identification of new therapeutic targets, including vascular endothelial growth factor (VEGF), which is overexpressed and related to progression-free survival (PFS) and overall survival (OS) in HCC[6,7]. Sorafenib, an oral multi-kinase inhibitor, blocks tumor cell proliferation and angiogenesis by

targeting RAF, VEGF receptors, platelet-derived growth factor receptor β, c-KIT and FLT3 signaling pathways[8].

Efficacy and safety of sorafenib have been demonstrated by phase Ⅱ/Ⅲ trials, which have proven that 400 mg bid sorafenib significantly prolongs OS, reduces the risk of death by 31%, and increases the time to radiological progression[9-11]. Consequently, sorafenib has become the standard of care for patients with advanced HCC.

We present four cases of unresectable, systemic-treatment-naïve HCC, enrolled in phase Ⅱ (HOPE) and Ⅲ (SHARP) trials at the Department of Medical Oncology and Hematology of the Istituto Clinico Humanitas in Rozzano (Milan, Italy), who obtained long-lasting objective responses. All patients were diagnosed by histology and computed tomography (CT) or magnetic resonance imaging (MRI). Their peculiar responses and the personalized management of side effects provide suggestions to optimize the use of sorafenib in the management of HCC patients.

CASE REPORT

Patient 1

In December 2002, a 61-year-old caucasian man was examined by MRI, which showed a single 110 mm × 140 mm hepatic lesion and thrombosis of the main and right branches of the portal and superior mesenteric veins. The patient was Child-Pugh class A, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0, with α fetoprotein (AFP) of 10 ng/mL. Sorafenib treatment was started in January 2003. In February 2003, grade 2 diarrhea was controlled with loperamide. In April 2003, the lesion measured 70 mm × 66 mm; grade 3 diarrhea appeared and resolved in July 2003, after a 3-d pause from sorafenib and daily loperamide. In December 2003, persistent grade 2/3 diarrhea reappeared, and resolved after a 50% reduction in sorafenib dose. In January 2004, treatment was discontinued due to progressive disease: a new lesion appeared at the edge of the previous one. From March 2003 to January 2004, AFP values progressively increased from 20 to 218 ng/mL. The patient achieved a 70% objective response according to World Health Organization (WHO) criteria, and 12 mo PFS. The patient died in June 2005.

Patient 2

A 63-year-old caucasian man was diagnosed with hepatitis B and C virus-related cirrhosis in 1983, and with HCC in November 2002. In February 2003, at baseline, a 20 mm × 25 mm lesion in hepatic segment Ⅳ and a non-clearly definable lesion in segment Ⅱ were visible. Gynecomastia, moderate thrombosis and erythema were managed, since March 2003, with periodical treatment pauses. In September 2003, sorafenib dose was reduced by 50% (400 mg/d) due to persistent grade 2 diarrhea. A minor response was detected in May 2004, and the lesion in segment Ⅳ reduced to 15 mm × 15 mm (55%) in July 2004; the lesion in segment Ⅱ lost density and became undetectable on subsequent scans. In August 2005, after 30 mo on therapy, HCC disease progression was observed as an increase in the number of tumor lesions and an increase in the diameter of existing lesions. We observed progression in the liver and not at other sites. Thereafter, the patient was lost to follow-up and died in April 2008.

Patient 3

A 70-year-old caucasian man with hepatitis C virus (HCV)-related cirrhosis, with a good PS, was diagnosed in July 2002 with vascular-invading HCC. In January 2003, at baseline, a single hepatic lesion measured 60 mm × 50 mm and AFP was 15 ng/mL. In June 2003, a 50% reduction in sorafenib dose was required due to paresthesia and cramps in the hands and feet. From July to November 2003, the lesion gradually reached 35 mm × 35 mm. At that time, the area originally covered by the lesion ranged from a denser portion, which surrounded the persistent tumor, to a less dense area, towards the normal liver. In October 2004, the lesion was barely visible. In October 2005, a complete response was achieved. AFP slightly decreased throughout the study, stabilizing at 12 ng/mL. In January 2008, after PFS of 60 mo, a new 10-mm lesion was detected in segment Ⅷ, outside the area previously involved. The patient was treated with two chemoembolizations and he is in complete response as of July 2010.

Patient 4

A 69-year-old caucasian man who had HCV-related cirrhosis since 1987 was diagnosed with HCC in May 2005. At baseline, in June 2005, the patient presented with three hepatic lesions, thrombosis of the portal vein main branch, Child-Pugh class A and ECOG PS 0. After 10 d on sorafenib, treatment was stopped for 1 mo due to grade 3 hand-foot skin reaction, and restarted at 50% of the dose. In September 2005, a partial response was observed, and the density of the only remaining lesion was reduced. In October 2005, the treatment was paused for 9 d due to grade 3 hand-foot skin reaction. At resolution, treatment was restarted at a dose of 400 mg every other day. In May 2007, the lesion was radiographically unchanged, was biopsied and proven disease free. As of July 2010, 62 mo from enrollment, the patient, still on the reduced dose regimen, maintains a complete response (Figure 1).

DISCUSSION

Sorafenib is the only effective systemic therapy for the treatment of HCC, but side effects lead to treatment discontinuation in some patients. Nowadays, HCC is treated by hepatologists or oncologists. The former may be less accustomed to the typical side effects of anticancer drugs, and the latter may not be keen to manage problems related to underlying liver cirrhosis. This report proves the importance of a multidisciplinary approach in the management of advanced HCC patients.

The described cases highlight how, in case of sorafenib-related side effects, reductions and pauses in the admin-

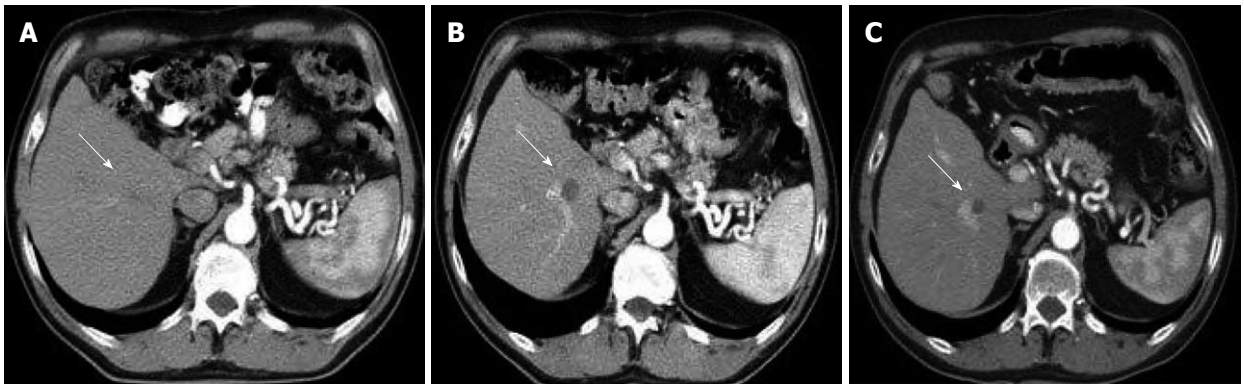

Figure 1 Patient 4 - arterial phase computed tomography scans. A: Pre-sorafenib, the 25-mm lesion in segment Ⅷ was biopsied, and measured > 100 HU. B: Partial response, tumor necrosis and intra-lesional HU of 40-70 after 4 mo on sorafenib. C: After 40 mo on sorafenib, the necrotic area measured < 40 HU intra-lesionally, and was disease-free at histological examination. The arrows show the liver lesion.

istered dose can allow long-term treatments. Efficacy of conventional cytotoxic agents is strictly related to the administered dose. With new targeted agents, length of treatment, rather than dose intensity, may be fundamental for tumor control. The winning strategy may lie in managing side effects, and tailoring the anticancer regimen to the characteristics of the patients, rather than discontinuing treatment at the appearance of signs of intolerance. Unfortunately, data on drug blood levels that are needed to achieve and maintain target inhibition are inadequate. The multi-target nature of sorafenib is one additional challenge, which means that various factors play a role in the activity of this agent[6-8].

In three of the four reported cases, objective responses were achieved following substantial dose reductions. In patient 2, partial response was observed at month 16 of the study (7 mo at full dose and 9 at half dose). In patient 3, sorafenib dose was reduced by 50% during the month 6 on treatment; an objective response was seen in month 8 of therapy, and a complete response was achieved after a total of 34 mo on study. In patient 4, sorafenib dose was reduced by 50% after just 10 d of therapy, and 20 d later, a partial response was observed. One month later, dose was further reduced to 25%, and complete response was documented after 24 mo on study. The lesson appears clear: the recommended sorafenib dose is 400 mg bid; when needed, dose reductions, by limiting side effects, offer a better quality of life and can allow long-term administration and achievement/maintenance of tumor control.

The radiological features of responding lesions are another issue worthy of attention. When assessing the efficacy of targeted therapies by imaging, a gradual change in tumor density and blood flow may be observed before tumor shrinkage. However, the uncommon radiological patterns can lead to late recognition of responses, or worse, to misleading evaluations. Indeed, in two of the reported cases (patients 3 and 4), lesions seemed unchanged and, at first sight, had been considered active. On the contrary, these lesions were responding and being substituted by residual scars (in patient 4, this was confirmed by liver biopsy). This issue deserves serious consideration and calls for new, more appropriate methods of appraisal. With targeted therapies, traditional methods of quantitative evaluation, such as WHO criteria or Response Evaluation Criteria in Solid Tumors, may not be optimal, and the need for qualitative standardized measurements becomes more pressing[9,12-14]. Although positron emission tomography is not reliable for evaluation of HCC, Hounsfield unit (HU) density scale for CT scan imaging, and signal patterns for MRI can be used to measure tumor necrosis. Combination of these methods with dimensional measurements allows more precise characterization of sorafenib responses in this disease[13,14]. In our experience, a lesion density reduction to 40 HU on CT scan can be considered indicative of tumor necrosis.

An additional mean of response evaluation may be provided by analysis of blood samples. At present, there is no agreement on specific biomarkers of response to sorafenib. We analyzed routine laboratory tests and did not identify any correlation in our patients, however, we did not search for alternative signals such as markers of inflammation and oxidative stress. The recommendations that derive from the reported cases are to tailor dose and schedule, to administer the drug until progressive disease is observed, and to evaluate critically dimensional and density changes in tumor lesions. Anticancer drugs have evolved in recent years, and so should the way physicians view tumor treatment strategies. Although this calls for more personalized treatment plans, agreement on standardized and more appropriate assessment techniques will allow more conscious decision making in the treatment of advanced HCC patients[13,14].

ACKNOWLEDGMENTS

The Authors thank Pasquala Ragucci for her valuable collaboration.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i19.2454

World J Gastroenterol 2011 May 21; 17(19): 2454
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Severe alcoholic hepatitis: Glucocorticoid saves lives and transplantation is promising

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Received: November 23, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: May 21, 2011

Abstract

Glucocorticosteroids have been used as the only treatment for a long time which significantly reduced the mortality of the patients with severe alcoholic hepatitis. The efficacy of transplantation has been recently addressed in a pilot study. The result seems promising but needs larger multicenter trials.

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Key words: Alcoholic hepatitis; Glucocorticoids; Transplantation

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Braillon A. Severe alcoholic hepatitis: Glucocorticoid saves lives and transplantation is promising. *World J Gastroenterol* 2011; 17(19): 2454 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i19/2454.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i19.2454

TO THE EDITOR

Amini and Runyon conclude that "the routine use of glucocorticoids for severe alcoholic hepatitis (SAH) poses significant risk with equivocal benefit"[1]. They refer to a 2008 Cochrane review but did not cite the authors' results that "Glucocorticosteroids significantly reduced the mortality of the patients with Maddrey's score of at least 32 or hepatic encephalopathy and with low-bias risk in a group of trials".

They also did not cite a recent review in a major journal which concluded that "Five patients need to be treated with corticosteroids to prevent one death"[2]. A more recent meta-analysis of individual patient data (221 allocated to corticosteroids *vs* 197 controls) confirms that corticosteroids significantly improve the 28-d survival in patients with SAH[3].

The conclusion "Histologic diagnosis of alcoholic hepatitis rules out the possibility of liver transplantation" is exaggerated according to the current literature. A teenager who develops liver failure after a deliberate overdose of paracetamol, or after contracting hepatitis B through irresponsible behaviour, has open access to liver transplantation[4]. Alcoholic patients must not be discriminated: after the transplantation, appropriate support measures must be taken with the alcohol services in the patient's locality. The efficacy of transplantation has been addressed in a study of 18 patients with SAH. Non-responders to steroids were identified by a Lille score: the 6-mo survival was 83% (compared with 44% in case-matched control) and none of the patients relapsed in the first year[5]. The result seems promising but needs larger multicenter trials.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 May 21; 17(19): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 May 21; 17(19): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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

March 21-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, Venise, 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 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 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 J Gastroenterol 2011 May 21; 17(19): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Instructions to authors

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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 systemically 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 consensuses 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

ISSN and EISSN

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 Directory of Open Access Journals. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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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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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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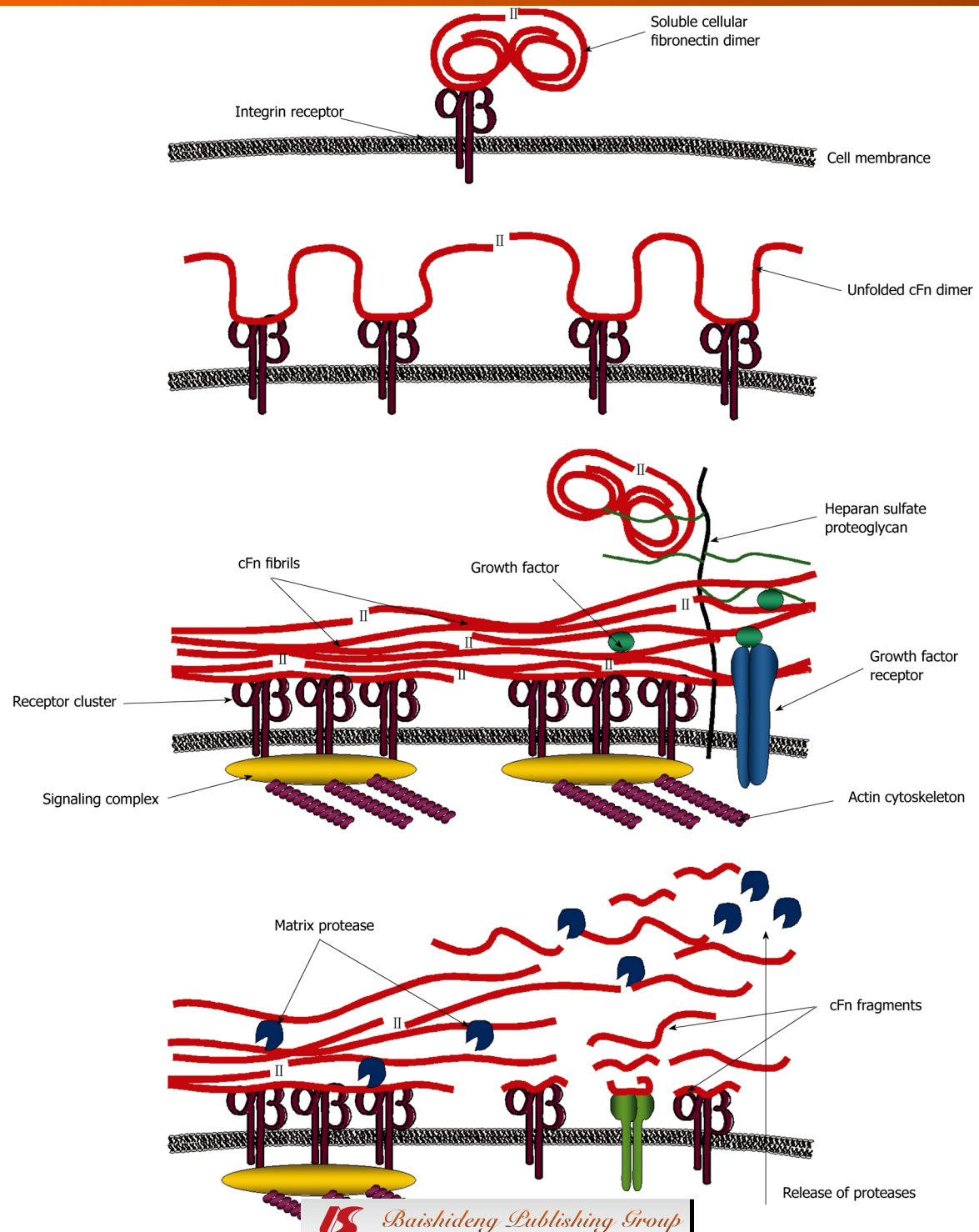

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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 20 May 28, 2011

EDITORIAL

2455 An annual topic highlight: Alcohol and liver, 2011
Osna NA

TOPIC HIGHLIGHT

2456 Epigenetic regulation in alcoholic liver disease
Mandrekar P

2465 Histone modifications and alcohol-induced liver disease: Are altered nutrients the missing link?
Moghe A, Joshi-Barve S, Ghare S, Gobejishvili L, Kirpich I, McClain CJ, Barve S

2473 Targeting collagen expression in alcoholic liver disease
Thompson KJ, McKillop IH, Schrum LW

2482 Fibronectin: Functional character and role in alcoholic liver disease
Aziz-Seible RS, Casey CA

2500 Mechanisms of alcohol-mediated hepatotoxicity in human-immunodeficiency-virus-infected patients
Szabo G, Zakhari S

2507 Involvement of autophagy in alcoholic liver injury and hepatitis C pathogenesis
Osna NA, Thomes PG, Donohue TM Jr

2515 Animal models for studying hepatitis C and alcohol effects on liver
Mercer DF

2520 Role of lipid rafts in liver health and disease
Dolganiuc A

2536 microRNAs: Fad or future of liver disease
Lakner AM, Bonkovsky HL, Schrum LW

2543 Hepatic stellate cells and innate immunity in alcoholic liver disease
Suh YG, Jeong WI

2552 Role of MGST1 in reactive intermediate-induced injury
Schaffert CS

2558 Proteasome inhibitor treatment in alcoholic liver disease
Bardag-Gorce F

ORIGINAL ARTICLE

2563 siRNA targeting Livin decreases tumor in a xenograft model for colon cancer
Oh BY, Lee RA, Kim KH

BRIEF ARTICLE

2572 Differential protein expression during colonic adaptation in ultra-short bowel rats
Jiang HP, Chen T, Yan GR, Chen D

CASE REPORT

2580 Non-cirrhotic portal hypertension with large regenerative nodules: A diagnostic challenge
Vespasiani Gentilucci U, Gallo P, Perrone G, Del Vescovo R, Galati G, Spataro S, Mazzarelli C, Pellicelli A, Afeltra A, Picardi A

World Journal of Gastroenterology
Volume 17 Number 20 May 28, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Aziz-Seible RS, Casey CA. Fibronectin: Functional character and role in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2482-2499 http://www.wjgnet.com/1007-9327/full/v17/i20/2482.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
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LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
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SPONSOR
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Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
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PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

PUBLICATION DATE
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ISSN 2219-2840 (online)

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doi:10.3748/wjg.v17.i20.2455

World J Gastroenterol 2011 May 28; 17(20): 2455
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

An annual topic highlight: Alcohol and liver, 2011

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Received: January 29, 2011 Revised: March 1, 2011
Accepted: March 8, 2011
Published online: May 28, 2011

Abstract

An annual topic highlight: Alcohol and Liver, 2011, covers the important and new aspects of pathogenesis of alcoholic liver diseases (ALD). It includes broad topics ranging from the exacerbation of ALD by infectious (viral) agents (hepatitis C virus and human immunodeficiency virus) to the influence of alcohol on liver fibrogenesis, lipid rafts, autophagy and other aspects. This issue is recommended for both basic scientists and clinicians who are involved in alcoholic liver research.

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Key words: Liver; Alcohol; Autophagy; Fibrogenesis; Immune cells; Lipid rafts; Mouse models; Hepatitis C; Human immunodeficiency virus

Osna NA. An annual topic highlight: Alcohol and liver, 2011. *World J Gastroenterol* 2011; 17(20): 2455 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2455.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2455

This annual topic highlight is the fourth issue of reviews devoted to alcoholic liver diseases (ALD) pathogenesis and treatment. It is one of the most respectful annual series of reviews in alcohol research, and now it has an established audience as well as the contributing authors, who are the leading specialists in alcohol studies, including experts from National Institute on Alcohol Abuse and Alcoholism. Here, we cover new aspects of alcohol research that were not addressed in our previous issues. Because alcohol exposure induces epigenetic changes in liver cells, two articles of this Topic Highlight[1,2] focused on the epigenetic regulation in ALD. Another two articles[3,4] addressed the mechanisms of pro-fibrotic changes in ALD. Several articles[5-7] summarized the available animal models for studying HCV infection based on "second-hit" effects of infections in ALD progression. In addition, other important aspects of ALD pathobiology, such as the role of lipid rafts, microRNAs, MSGT1, hepatic stellate cells and innate immunity and the proteasome inhibitor for ALD treatment are included in this issue[8-12].

This Topic Highlight provides an overview of the most modern literature/approaches in the alcohol research and is strongly recommended for gastroenterologists, hepatologists and scientists who work in this field.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2456

World J Gastroenterol 2011 May 28; 17(20): 2456-2464
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Epigenetic regulation in alcoholic liver disease

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Author contributions: Mandrekar P solely contributed to this paper.
Supported by PHS Grant # AA017545 (to Mandrekar P) and AA017986 (to Mandrekar P) from the National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health
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Received: January 20, 2011 Revised: March 2, 2011
Accepted: March 9, 2011
Published online: May 28, 2011

Abstract

Alcoholic liver disease (ALD) is characterized by steatosis or fat deposition in the liver and inflammation, which leads to cirrhosis and hepatocellular carcinoma. Induction of target genes without involving changes in DNA sequence seems to contribute greatly to liver injury. Chromatin modifications including alterations in histones and DNA, as well as post-transcriptional changes collectively referred to as epigenetic effects are altered by alcohol. Recent studies have pointed to a significant role for epigenetic mechanisms at the nucleosomal level influencing gene expression and disease outcome in ALD. Specifically, epigenetic alterations by alcohol include histone modifications such as changes in acetylation and phosphorylation, hypomethylation of DNA, and alterations in miRNAs. These modifications can be induced by alcohol-induced oxidative stress that results in altered recruitment of transcriptional machinery and abnormal gene expression. Delineating these mechanisms in initiation and progression of ALD is becoming a major area of interest. This review summarizes key epigenetic mechanisms that are dysregulated by alcohol in the liver. Alterations by alcohol in histone and DNA modifications, enzymes related to histone acetylation such as histone acetyltransferases, histone deacetylases and sirtuins, and methylation enzymes such as DNA methyltransferases are discussed. Chromatin modifications and miRNA alterations that result in immune cell dysfunction contributing to inflammatory cytokine production in ALD is reviewed. Finally, the role of alcohol-mediated oxidative stress in epigenetic regulation in ALD is described. A better understanding of these mechanisms is crucial for designing novel epigenetic based therapies to ameliorate ALD.

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Key words: Alcohol; Epigenetics; Histones; Acetylation; DNA methylation; miRNA; Genes

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Mandrekar P. Epigenetic regulation in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2456-2464 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2456.htm
DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2456

INTRODUCTION

Chronic alcohol consumption has a significant impact on human health and is identified as a major risk factor for development of liver disease. Alcoholic liver disease (ALD) is one of the leading causes of liver disease mortality worldwide. Alcohol-related liver injury includes a spectrum of pathological conditions in the liver including steatosis, steatohepatitis, cirrhosis and hepatocellular carcinoma. Of the molecular mechanisms studied, epigenetic mechanisms altered by alcohol appear to play a significant role in development and progression of disease. These mechanisms have been identified in parenchymal and non-parenchymal cells in the liver and contribute to induction of fatty liver, inflammation, as well as hepatocyte

apoptosis and necrosis. In the past decade, a number of studies have reported epigenetic alterations by alcohol in the liver including histone modification, DNA and histone methylation derived from the methyl group donating system, S-adenosyl methionine (SAMe), miRNA as post-transcriptional modifiers, and chromatin remodeling enzymes responsible for epigenetic regulation such as histone acetyltransferases, histone deacetylases and DNA methyltransferases[1,2]. Defining an epigenetic imprint in alcohol-induced liver injury will provide new insights into pathophysiological mechanisms and open avenues for potential novel epigenetics-based therapeutics.

The goal of this concise article is to review alcohol-mediated epigenetic alterations, in parenchymal and non-parenchymal cells of the liver. Specifically, alcohol-mediated alterations in epigenetic enzymes, miRNAs as epigenetic modifiers, chromatin modifications affecting immune cell function in alcoholic liver, and the role of oxidative stress in chromatin remodeling that regulates alcohol-mediated gene expression in the liver are described.

THE EPIGENETIC CODE

The classical view defines epigenetics as heritable changes that affect gene expression without altering the DNA sequence. Epigenetic regulation of gene expression is facilitated through different mechanisms such as DNA methylation, histone modifications, and RNA-associated silencing by small non-coding RNAs. All these mechanisms are crucial for normal development, differentiation and tissue-specific gene expression. These three systems interact and stabilize one another and can initiate and sustain epigenetic silencing, thus determining heritable changes in gene expression. Alterations in one or more of these systems leads to inappropriate target gene expression or silencing that results in epigenetic regulation of human diseases such as cancer, autoimmune diseases, and age-related as well as neurological disorders[3,4]. Epigenetic abnormalities are diverse, tissue-specific and can occur due to various environmental factors such as toxins and drugs, including alcohol.

Epigenetic regulation of gene expression primarily works through modifying the secondary or tertiary structures of DNA (chromatin), which makes it more or less accessible to transcription. Chromatin is made up of repetitive structural units called nucleosomes. Nucleosomes are comprised of a histone octamer and the DNA that wraps around it. Histones are globular basic proteins that are subject to various covalent modifications that occur primarily on the N-terminal tail[5]. The histone octamer contains two molecules of each of the histones H2A, H2B, H3 and H4, around which the DNA wraps. Histone H1 the "linker histone" along with "linker DNA" physically connects the adjacent nucleosome core particles. Covalent histone modifications appear to act sequentially or in combination to form a recognizable code that is identified by specific proteins to regulate distinct downstream events such as transcriptional activation or repression[5]. Histones are subject to various post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitinylation and sumoylation, all having an impact on gene transcription[5]. Histone acetylation is a transcription-activating modification that is achieved by addition of acetyl groups to lysine residues by enzymes called histone acetyltransferases (HATs). The major sites of acetylation in histone H3 are Lys4, Lys9, Lys14 and Lys28. Acetyl groups are removed by histone deacetylases (HDACs) and this is generally associated with loss of gene expression or silencing[6]. Mammalian HDACs have been classified into four classes. Class I HDACs (1, 2, 3 and 8) are found predominantly in the nucleus, whereas Class II HDACs (4 5, 6, 7, 9 and 10) shuttle between the nucleus and cytoplasm. Class I and Class Ⅱ HDACs have tissue-specific expression profiles. HDACs 1, 2 and 3 are expressed in various immune cells[7]. Class Ⅲ HDACs (SIRT1-SIRT7) form a distinct class of NAD-dependent enzymes, can be inhibited by nicotinamide, and are important in DNA repair and anti-apoptotic functions[8]. HDAC 11 possesses properties of Class I and Ⅱ HDACs and is classified as Class Ⅳ. Table 1 illustrates the histone modifications and enzymes linked to the changes.

Table 1 Histone modifications, enzymes and genes

Modifications	Enzymes	Target gene transcription
Acetylation		
H3	GNAT	Activating
H4	MYST	Activating
H3 and H4	CBP/p300	Activating
Deacetylation		
H3 and H4	HDACs 1-11	Silencing
Methylation		
H3K4	Set1	Activating
H3K36	Set2	Activating
H3K79	DoT1L	Activating
H3K27	EZH2	Silencing
H3K9	SUV39H1	Silencing
H4K20	SUV4-20H1	Silencing
Phosphorylation		
H3S10	RSK2	Silencing
Methylated CpG	DNMT1, DNMT3a-3b	Silencing

Histone methylation is catalyzed by histone methyltransferases at lysine and arginine residues on histone H3 and H4 and can be mono-, di- and tri-methylated. The major sites on histone H3 are Lys4, Lys9, Lys27, Lys36 and Lys79, whereas histone H4 is methylated on Lys20[5]. The methyl group donor is S-adenosyl methionine. The effects of alcohol exposure on SAMe-mediated epigenetic changes have been under investigation[9]. Arginine methylation is transcription-activating, and lysine methylation can cause either transcriptional activation or repression, depending on the lysine residue methylated. For instance, H3K9 can be acetylated as well as methylated and have opposite transcriptional consequences; H3AcK9 causes transcriptional activation whereas H3K9me (mono-, di- and tri-) leads to transcriptional repression. Thus, a

balance in H3K9 acetylation and methylation may be important in determining chromatin architecture and gene silencing or activation[5].

Histone phosphorylation is a transcription-activating modification achieved by kinases that catalyze the transfer of a phosphate group from ATP or GTP to the serine or threonine residue of histone H3. Besides phosphorylation, histone H1, H2A, H2B and H3 can be ubiquitinated at lysine residues that activate transcription. Sumoylation on the other hand occurs on lysine residues and is a transcriptionally repressive modification.

DNA methylation involves transfer of a methyl group to cytosine bases at the C5 position of CG dinucleotides, referred to as CpG dinucleotides, and may occur in clusters, known as CpG islands. By definition, CpG islands are genomic regions that are at least 200 base pairs long, with ⩾ 50% GC content and ⩾ 60% expected CpG ratio[10]. The methyl donor is SAMe and the enzyme involved is DNA methyltransferase (DNMT). Two groups of mammalian DNMTs, one that *de novo* methylates DNA, and the other that maintains the methylation status, are classified as four different types: DNMT 1, 2, 3A and 3B. Although DNMT 3A and 3B are *de novo* methylation enzymes[11], DNMT 1 maintains methylation status, whereas the function of DNMT 2 is not yet clear and it has weak methyltransferase activity. DNA methylation leads to transcriptional silencing due to chromatin condensation, increased recruitment of methylated CpG binding transcriptional repressor, and inhibition of DNA binding of transcriptional activators[11]. The unmethylated CpG islands are associated with transcriptionally active promoters, and how CpG islands remain unmethylated is still unclear[12].

Non-coding RNA (ncRNA) is another mechanism of epigenetic regulation and is driven by long or small ncRNAs. Long ncRNAs such as *Air*[13], *Kcnq1ot1*[14] and *H19*[15] exert their epigenetic effect by genomic imprinting, which involves DNA methylation. On the other hand, small ncRNAs such as miRNA affect translational repression of mRNA, mRNA degradation, DNA methylation, and chromatin modification[16]. miRNAs are short [~22 nucleotides(nt)] ncRNAs that regulate gene expression by binding to their cognate binding sites at the 3'-end of the target mRNA, and inhibiting their translation. miRNAs are transcribed from miRNA genes mostly by RNA polymerase Ⅱ into long primary miRNA (pri-miRNA) transcripts that often contain thousands of nucleotides and form hairpins (stem loops). The pri-miRNA is processed in the nucleus by Drosha-DGCR8 complex to produce 70-80-nt precursor miRNA (pre-miRNA)[16]. Pre-miRNAs are transported from the nucleus to the cytoplasm by exportin-5 and Ran-GTP complex, where they are further processed into ~22-nt long miRNA/miRNA duplex by Dicer, a RNAse type Ⅲ enzyme. One of the miRNA strands bind to the cognate binding site on the target mRNA with a ~2-nt mismatch, and it is binding of the RNA silencing complex at the 3' UTR of the target mRNA that represses its translation and results in gene silencing[16].

The crosstalk between various epigenetic mechanisms described above can determine downstream chromatin remodeling and gene expression. Mechanisms such as histone acetylation and methylation, DNA methylation and ncRNA-mediated modifications, are acquired throughout life, and persist, and influence the ability to deal with environmental factors such as nutritional factors, toxicants and lifestyle-related factors including tobacco smoke, alcohol, chemical carcinogens, infectious agents and UV radiation, thus influencing the clinical outcomes of health and disease[17].

EPIGENETIC DYSREGULATION IN ALD

The emerging role of epigenetic regulation of gene expression by alcohol and its effect on organ injury comes from studies in the past decade[1,2]. Here, we review mechanisms related to histone modifications and DNA methylation induced by alcohol exposure in liver cells, which contribute to ALD.

Histone modifications: acetylation, phosphorylation and methylation

Multiple lines of evidence from *in vitro* and *in vivo* studies have established that alcohol induces epigenetic modifications in various organs including the gastrointestinal system, brain and liver[2]. In the liver, alcohol alters histone acetylation, methylation as well as phosphorylation. Selective acetylation of histone H3 at lys 9 (H3AcK9) has been observed in primary rat hepatocytes exposed to alcohol *in vitro*[18]. On the other hand, other lysine residues H3 lys14, lys18 and lys23 were not acetylated. In the liver, H3 acetylation was modulated by alcohol *via* increased HAT activity and HDAC inhibition[19].

The status of histone acetylation depends on the activity of HAT and HDAC[20]. In some instances, the balance of HAT/HDAC ratio determines the acetylation of histone residues and influences gene expression[21]. Alcohol exposure appears to alter HAT and HDAC activity in hepatocytes[19]. *In vitro* alcohol exposure of hepatic cells impairs HDAC6 function, which directly affects microtubule dynamics[22]. The mRNA expression of class Ⅲ HDAC, sirtuin 1 (SIRT1) is reduced in alcohol-exposed hepatocytes[23]. Furthermore, an essential role for SIRT1 in mediating effects of alcohol on SREBP-1 and hepatic lipid metabolism in alcoholic fatty liver has been reported[24]. These results indicate that SIRT1 can be developed as a therapeutic target in ALD. Overall, alcohol seems to influence HATs and HDACs in hepatocytes. Studies are needed to determine the precise role of these altered enzyme activities in the context of ALD *in vivo*.

Similar to acetylation, phosphorylation of histones is crucial to chromatin modifications, and activates gene transcription downstream of cell signaling events[25]. Acute alcohol exposure modulates H3 phosphorylation at serine 10 and serine 28 in rat hepatocytes, and this is dependent on p38 mitogen-activated protein kinase activity but not extracellular signal-regulated kinase and C-Jun N-terminal

kinase[26]. Recent studies also have indicated that *in vivo* acute alcohol exposure induces H3 serine-10 and serine-28 phosphorylation which was transiently increased at 1 h, but decreased at 4 h after alcohol administration[27]. On the other hand, persistent H3K9 acetylation in the liver was observed at 4 h after acute alcohol exposure *in vivo*[27]. A relationship between acetylation and phosphorylation in context with gene activation has been examined[25]. For instance, cytokine-induced gene expression mediated by nuclear IKKα leading to nuclear factor (NF)-κB activation involves coupling of H3 phosphorylation at serine 10 and acetylation at lysine 14[28,29]. On the other hand, retinoic acid receptor-β and c-jun gene regulation is linked to phosphorylation of histone H3 and not acetylation, which indicates that these two epigenetic changes can occur independently[30-32]. Whether alcohol induces histone phosphorylation and acetylation synergistically or as independent pathways to regulate target gene expression in ALD remains to be investigated.

Specific methylated lysine residues on histones function as stable epigenetic marks that direct particular biological functions, ranging from transcriptional regulation to heterochromatin assembly[33]. Histone methyltransferases catalyze transfer of a methyl residue predominantly to H3 and H4 histones that impart biological functions such as transcription or epigenetic silencing[33]. Shukla *et al*[34] have shown differential methylation of H3 and H4 in rat hepatocytes exposed to alcohol *in vitro*. These studies have indicated that methylation at H3meLys9 is decreased and is associated with downregulation of genes such as Lsdh, whereas increased methylation of H3meLys4 is associated with upregulation of alcohol dehydrogenases (ADH1)[34]. Whether acute or chronic alcohol-mediated histone methylation serves as a stable genomic imprint that determines the transcriptional state of a gene contributing to disease is still unknown.

DNA methylation in ALD

Methylation of cytosine at C5 in the CpG dinucleotides silences transcription, whereas absence of methylation activates transcription. Although 80% of CpG dinucleotides are methylated, unmethylated CpG residues in promoter regions of constitutively active genes are referred to as CpG islands[35]. The predominant methyl donor, SAMe, which is important in DNA methylation, is depleted in alcoholic livers, which results in hyperhomocysteinemia that is commonly observed in patients with ALD[36]. Decreased SAMe in alcoholic livers also seems to affect DNA methylation. Rats fed an intragastric alcohol diet for 9 wk exhibited decreased methionine, SAMe, glutathione and loss of DNA methylation by 40%[37]. This DNA hypomethylation can lead to alteration in gene expression and chromatin structure, results in increased DNA damage and strand breaks[37,38], which predisposes cells to malignant degeneration[39]. An association between alcohol intake and hypomethylation of the O^6-methylguanine DNA methyltransferase gene has been noted in context with hepatocellular carcinoma[40]. Studies also have shown that alcohol-metabolizing enzyme, ADH1, is regulated by epigenetic mechanisms in hepatoma cells involving DNA hypomethylation[41]. In addition to direct effects of alcohol on DNA hypomethylation, direct effects of acetaldehyde on DNA methyltranferase[42] and methionine synthase[43,44] have been reported. Whether alcohol directly alters DNA methyltranferase activity and methionine synthase is yet unknown. Chronic alcohol consumption induces global DNA hypomethylation[45], whereas hypermethylation of DNA is observed in human peripheral blood cells after alcohol consumption in humans[46,47]. Alcohol-associated hypomethylation thus far is linked to decrease in SAMe, the methyl donor.

An interplay between histone acetylation and DNA methylation is involved in the process of gene transcription and/or silencing in diseased states[17]. For instance, hypermethylation of CpG islands in target gene promoters triggers deacetylation of local histones, whereas lower levels of histone acetylation seem to sensitize DNA to methylation. Although there is an intimate communication between these two epigenetic phenomena, it is still not clear whether there is any hierarchical order of these events. In chronic alcohol exposed livers, whether an interconnection exists between hyperacetylation of H3K9, loss of methylation of H3K9, and increased methylation of H3K4, along with global hypomethylation of DNA is unknown. Studies undertaken to identify the interplay of epigenetic events will provide new insights into mechanisms of ALD.

miRNAs AS EPIGENETIC MODULATORS IN ALD

The role of miRNAs as epigenetic modulators is apparent from its regulation of gene expression at the post-transcriptional level. miRNAs elicit degradation of target mRNA or hinder translational mechanisms such as initiation, elongation, degradation or segregation of mRNA into P bodies for translational inhibition[48-50]. The importance of miRNAs in liver diseases such as viral hepatitis, alcoholic fatty liver disease and non-alcoholic fatty liver disease (NAFLD), fibrosis, and hepatocellular carcinoma has recently been recognized[51]. Studies have shown that alcohol exposure regulates miRNAs that control post-transcriptional events and influence gene expression that are important in ALD[52]. miRNAs have been linked to lipid metabolism and inflammation in steatohepatitis[53-56]. Dolganiuc *et al*[52] have shown that alcohol feeding increases and decreases ~1% of known miRNA, whereas methionine/choline-deficient diet upregulates 3% and reduces 1% of known miRNAs. Common to ALD and NAFLD, miR-705 and miR-122 are increased with both diets. However, miR-182, miR-183 and miR-199a-3p are increased in NAFLD and decreased in ALD mouse models. The functional relevance of miRNAs in ALD and NAFLD remains to be determined. Using hepatocyte cultures, recent studies have shown that ~11 miRNAs are altered in hepatic lipid droplets[57]. MiR-181d is the most efficacious inhibitor and reduces lipid droplets by 60%, which confirms its role in cellular synthesis of triglycerides

and cholesterol esters[57]. More recent studies have shown that chronic alcohol induces miRNA-155 in liver macrophages[58]. Chronic alcohol exposure also alters miRNAs that affect intestinal permeability[59]. Alcohol intake results in induction of miR-212, which in turn downregulates tight junction protein zonula occludens 1[59]. miRNA-212 is also higher in colon biopsy samples of patients with ALD, which confirms the pathophysiological significance of miRNA-212 in altering intestinal permeability during ALD. The role of miRNAs as epigenetic modulators of gene expression and silencing is becoming evident in ALD. An improved understanding of miRNAs and subsequent post-transcriptional regulatory mechanisms will be of importance in advancing their application as diagnostic or prognostic markers, as well as therapeutic targets in ALD.

EPIGENETICS AND INFLAMMATION IN ALD

The first report on aberrations in the epigenetic code in an inflammatory disease condition such as rheumatoid arthritis was reported in 1990[60]. Anti-inflammatory effects of HDAC inhibitors used in a number of experimental models of inflammatory diseases have confirmed a role for epigenetics in immune function[61-63]. Epigenetic regulatory mechanisms are central to the immune response, allowing an appropriate gene expression pattern in immune cells[64,65]. Environmentally regulated or endogenously mediated epigenetic alterations contribute to environment-host interactions in various forms of inflammatory diseases[66].

Innate immune responses and macrophage function play a central role in ALD[67,68]. Epigenetic modifications that influence inflammatory responses have been reported in macrophages[69] during lipopolysaccharide (LPS) tolerance. This phenomenon is dependent on histone acetylation and H3K4 methylation, as well as chromatin remodeling enzymes such as SW1/SNF[70]. Acute alcohol exposure decreases LPS induced proinflammatory responses in human monocytes[71,72]. Whether acute alcohol mediates histone modifications and recruitment of nucleosome remodeling enzymes at the promoters of the cytokine genes is yet to be determined.

The transcription factor NF-κB is a master regulator of proinflammatory responses in macrophages and monocytes[73,74]. It is the organization of the chromatin structure that controls the kinetics of NF-κB recruitment to target gene promoters that represents a focal point in mediation of transcription cooperativity (Figure 1). Acute and chronic alcohol exposure modulates NF-κB DNA binding that regulates expression of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6[68,75]. A tailored immune response that is regulated by NF-κB *via* interaction with HATs and HDACs elicits epigenetic modifications[76]. For instance, HDAC-1 is known to form a complex with NF-κBp50 homodimers to repress gene transcription[77]. On the other hand, inflammatory stimuli such as LPS can induce phospho-

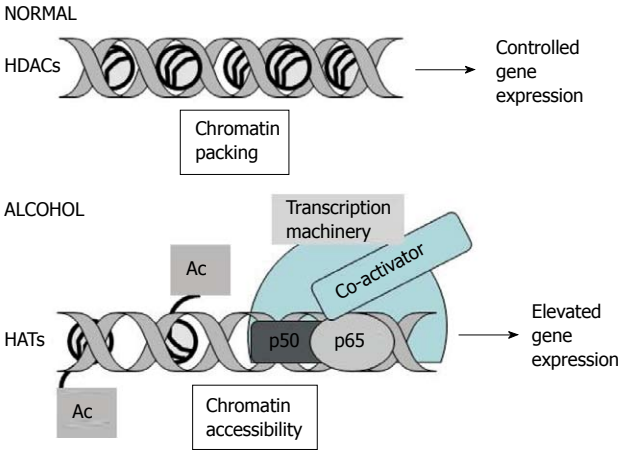

Figure 1 Epigenetic regulation of gene expression *via* histone modification. Under normal conditions, activation of histone deacetylases (HDACs) inhibits acetylation (Ac) of histones, which results in chromatin packaging, restriction of DNA accessibility for transcription, and controlled target gene expression. During alcohol exposure, histone modifications mediated by histone acetyltransferases (HATs) influence elevated transcription of target genes, due to chromatin accessibility, which leads to increased DNA occupancy of transcription factors such as nuclear factor-κB (p50/p65), co-activators and transcriptional complexes, and elevated target gene expression.

acetylation of histone H3 (K9/S10) at a subset of cytokine and chemokine gene promoters for increased NF-κB recruitment[29,78,79]. Reductions in H3 Lys9 methylation, along with increased H3/H4 acetylation are strongly correlated with RNA polymerase II recruitment, which results in transcription of NF-κB-inducible inflammatory genes[79]. Whether alcohol exposure affects epigenetic mechanisms to prolong or reduce DNA binding of NF-κB to the cytokine gene promoters in monocytes and macrophages is not yet known. Another environmental agent, cigarette smoke induces post-translational modification of phospho-deacetylases, HDAC2 and SIRT1, acetylation of p65-NF-κB and phosphoacetylation of histone H3 that leads to chromatin rearrangement and sustained proinflammatory gene transcription[80,81]. It is likely that chronic alcohol exposure modulates HDAC2 and SIRT1 in immune cells to regulate epigenetic events that promote prolonged proinflammatory responses in ALD.

In macrophages stimulated with viruses or bacteria, miRNA-155 is the only increased miRNA induced by both stimulants[82,83]. miRNA-155 regulates TNF-α production positively by enhancing its translation[84]. Bala *et al*[58] recently reported that chronic alcohol induces miRNA-155 in an NF-κB-dependent manner that increases mRNA stability and TNF-α expression in ALD. miR-146 is predominantly expressed in T regulatory and Th1 cells[85] and is upregulated in toll-like-receptor-stimulated macrophages in an NF-κB-dependent manner. miR-146 targets IRAK-1 and TRAF6 genes and is unaffected by chronic alcohol exposure in liver immune cells[58]. Overall, these findings suggest that miRNAs are capable of regulating alcohol-induced innate immune cell function and thus determining cellular memory *via* miRNA-mediated epigenetic modulation.

ALCOHOL, OXIDATIVE STRESS AND CHROMATIN REMODELING

Oxidative stress regulates chromatin remodeling by alteration of histone acetylation and deacetylation events *via* HAT/HDAC activity[86,87]. Acute and chronic alcohol exposure increases reactive oxygen species (ROS) production and lowers antioxidant levels that enhance oxidative stress in the liver[88]. Metabolism of alcohol through alcohol dehydrogenase and microsomal cytochrome P450 2E1 leads to enhanced production of ROS in the liver[88]. Acetylation of histone H3 by alcohol in rat hepatocytes is mediated by ROS[89]. Inhibition of NADPH-oxidase-mediated ROS results in decreased H3AcK9, whereas ROS inducers directly increase alcohol-induced acetylation of H3K9, along with induction of ADH1 mRNA expression[89]. A redox-sensitive class III HDAC molecule, SIRT1, is also decreased in alcohol-exposed rat hepatocytes and livers of alcohol-fed rats[24]. Whether alcohol-induced ROS[90] play an important role in modulation of SIRT1 to regulate steatosis remains to be determined. In addition to alcohol, acetaldehyde and acetate, which are products of alcohol metabolism, cause acetylation of H3K9. Pyrazole, an inhibitor of alcohol dehydrogenase and methyl cyanamide, an inhibitor of aldehyde dehydrogenase, both reduce H3K9 acetylation, which indicates that alcohol and its metabolites can trigger acetylation of histone residues. Similar to *in vitro* observations, *in vivo* acute alcohol exposure in rats also shows that H3K9 acetylation is significantly increased in the liver[91]. Studies have shown that H3K9 acetylation in hepatocytes due to alcohol exposure correlates with transcriptional increase in alcohol dehydrogenase (ADH1)[19]. It is likely that alcohol-induced acetylation is required for activation of alcohol-metabolizing enzymes, which induce oxidative stress. This, in turn, induces acetylation that creates an amplifying "autocrine loop" between alcohol metabolism and epigenetic events. Future studies to determine the precise role of alcohol-mediated oxidative stress in chromatin modifications in hepatocytes and liver macrophages will identify new pathophysiological mechanisms and epigenetic targets of gene expression in ALD.

Stress-induced heat shock transcription factor (HSF)1 plays an important role as a transcriptional repressor of proinflammatory cytokine genes[92,93]. Recent studies have suggested that HSF1 serves as a master regulator of global acetylation in normal cells, whereas, in stressed cells, HSF1 interacts with HDAC1 and HDAC2, which induce histone deacetylation and chromatin remodeling[94]. Studies from our laboratory have shown that alcohol exposure induces HSF1 DNA binding activity in monocytes and macrophages[95]. Whether alcohol-induced HSF1 induces chromatin reorganization that affects proinflammatory cytokine production is being investigated. Another key stress-induced molecule, heat shock protein (hsp)90, is characterized as an epigenetic "gatekeeper" that interfaces with the environment and may finally determine whether certain epigenetic markers succeed in downstream phenotypic expression[96-98]. Chronic alcohol exposure upregulates hsp90 expression in liver macrophages[95]. It is likely that hsp90 facilitates an alcohol-mediated "epigenetic code" in the liver. Studies to delineate epigenetic effects of alcohol-induced hsp90 on gene transcription in liver macrophages and hepatocytes are awaited.

Hydroxyl radicals generated by oxidative stress interfere with the ability of DNA to function as a substrate for DNMT, which results in global hypomethylation[99,100]. Recent studies have provided evidence for the role of endoplasmic reticulum stress pathways and epigenetic gene regulation[101]. It is likely that alcohol-mediated oxidative stress regulates epigenetic markers in ALD.

CONCLUSION

The interplay of epigenetic mechanisms and their influence on gene transcription in ALD is evolving. Epigenetic alterations associated with acute and chronic alcohol exposure of hepatocytes and immune cells in relation to ALD is discussed in this review. Studies thus far have shown that alcohol exposure, probably *via* oxidative stress, exhibits differential regulation of acetylation, phosphorylation and methylation of histones that regulate chromatin remodeling and gene expression. The effects of alcohol on DNA methylation in hepatocytes and miRNA regulation have been elucidated. An integrative approach of the various mechanisms that lead to genomic imprinting during alcohol exposure will identify novel pathways in the alcoholic liver and support epigenetic therapeutic interventions.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2465

World J Gastroenterol 2011 May 28; 17(20): 2465-2472
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Histone modifications and alcohol-induced liver disease: Are altered nutrients the missing link?

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Supported by The National Institute of Alcohol Abuse and Alcoholism grants AA014371 (to Joshi-Barve S), AA015970 (to McClain CJ), and Office of Dietary Supplements, NIH
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Received: January 17, 2011 Revised: February 12, 2011
Accepted: February 19, 2011
Published online: May 28, 2011

Abstract

Alcoholism is a major health problem in the United States and worldwide, and alcohol remains the single most significant cause of liver-related diseases and deaths. Alcohol is known to influence nutritional status at many levels including nutrient intake, absorption, utilization, and excretion, and can lead to many nutritional disturbances and deficiencies. Nutrients can dramatically affect gene expression and alcohol-induced nutrient imbalance may be a major contributor to pathogenic gene expression in alcohol-induced liver disease (ALD). There is growing interest regarding epigenetic changes, including histone modifications that regulate gene expression during disease pathogenesis. Notably, modifications of core histones in the nucleosome regulate chromatin structure and DNA methylation, and control gene transcription. This review highlights the role of nutrient disturbances brought about during alcohol metabolism and their impact on epigenetic histone modifications that may contribute to ALD. The review is focused on four critical metabolites, namely, acetate, S-adenosylmethionine, nicotinamide adenine dinucleotide and zinc that are particularly relevant to alcohol metabolism and ALD.

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Key words: Alcohol; Liver disease; Nutrients; Metabolism; Histone; Epigenetic modifications; S-adenosylmethionine; Acetate; Zinc; Nicotinamide adenine dinucleotide

Peer reviewer: Dr. Milan Jirsa, Laboratory of Experimental Medicine-building Z1, Institute for Clinical and Experimental Medicine, Videnska 1958/9, Praha 4, 14000, Czech Republic

Moghe A, Joshi-Barve S, Ghare S, Gobejishvili L, Kirpich I, McClain CJ, Barve S. Histone modifications and alcohol-induced liver disease: Are altered nutrients the missing link? *World J Gastroenterol* 2011; 17(20): 2465-2472 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2465.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2465

INTRODUCTION

Alcoholism is a growing health problem worldwide. In the United States, alcoholism is a major cause of liver-related disease and deaths. Recent statistics reveal 52% of US adults to be "current regular" drinkers (Summary Health Statistics for U.S. Adults: National Health Interview Survey, 2009) and the death rate for alcohol-

induced causes to be on the rise (National Vital Statistics Reports, May 2010). As a result, alcohol-induced liver disease (ALD) continues to be studied, with the objectives of elucidating the underlying mechanisms and discovering potential therapeutic targets.

ALD consists of the spectrum of pathological changes including fatty liver, alcoholic hepatitis and alcoholic cirrhosis. The clinical manifestations of these changes and the pathogenesis of this disease have been extensively studied and described. There is a growing body of evidence supporting the involvement of epigenetic mechanisms in response to environmental inputs in the development of human disease. Accordingly, in recent years, there has been increasing interest in understanding the role of epigenetic mechanisms in the initiation and/or progression of ALD.

Nutrient fluctuations can impact transcriptional activity and expression of selective genes by modulating epigenetic parameters including histone modifications, DNA methylation, and nucleosome positioning. In ALD, especially in chronic alcoholics, the combined effect of alcohol metabolism and compromised nutrition causes major nutrient disturbances that are likely to influence epigenetic mechanisms, gene expression and disease pathogenesis. Covalent modifications of the amino termini of the core histones in nucleosomes play a key role in regulating chromatin structure as well as DNA methylation status. This review interrelates alcohol-mediated nutrient disturbances and consequent histone modifications that may have a contributory role in ALD (Figure 1). Specifically, the review is focused on fluctuations in four critical metabolites, namely, acetate, S-adenosylmethionine (SAM, also known as SAMe or AdoMet), nicotinamide adenine dinucleotide (NAD^+) and zinc that are relevant to alcohol metabolism and ALD.

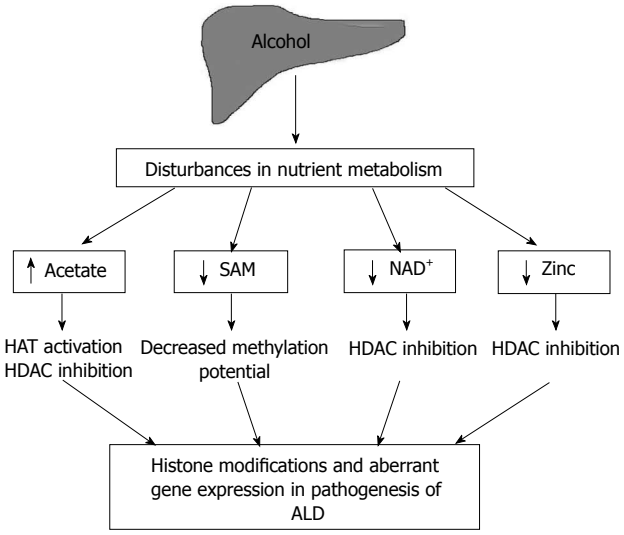

Figure 1 Nutrient disturbances, a potential link between alcohol metabolism and histone modifications in alcohol-induced liver disease. SAM: S-adenosylmethionine; NAD^+: Nicotinamide adenine dinucleotide; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; ALD: Alcohol-induced liver disease.

ALCOHOL AND ACETATE

The principal route of ethanol oxidation is through the enzyme liver alcohol dehydrogenase, which converts alcohol to aldehyde with the reduction of NAD^+ to NADH[1]. Acetaldehyde is then further oxidized by acetaldehyde dehydrogenase to acetate. The other major route of oxidation is through the microsomal ethanol oxidizing system (MEOS), in which the chief enzyme catalyzing alcohol oxidation is the cytochrome P450 mixed-function oxidase isoenzyme CYP2E1[1]. This route is engaged when alcohol is ingested in large quantities or in chronic alcoholics, who upregulate CYP2E1 expression. Thus, the end-product of both pathways of ethanol metabolism in the liver is free acetate[1]. This free acetate is then incorporated into acetyl-coenzyme A (acetyl-coA), by the catalytic action of the cytosolic and mitochondrial enzymes acetyl-coA synthetases[2]. Acetyl-coA is the substrate for histone acetylation, in addition to being utilized in the Krebs cycle, fatty acid synthesis and acetylation of other proteins[3].

Alcohol increases acetate production

Ethanol consumption has been shown to increase blood acetate levels significantly in several studies, dating back to the 1980s. Short-term ethanol administration in humans led to a sustained steady state concentration of 0.4 to 0.6 mmol acetate within 2 to 5 h following ingestion in a study consisting of healthy male and female volunteers[4]. Although this phenomenon was also seen in alcoholics, there were variations in the kinetics of acetate production, with chronic alcoholics eliminating alcohol faster and producing more acetate[5,6]. Acetate levels were significantly higher both in the hepatic vein (1.79 and 1.15 mmol) and peripherally (0.91 and 0.52 mmol) in alcoholics than in non-alcoholics respectively[6]. In another study employing an enzymic acetate detection method, acetate concentration in peripheral human blood increased to about 20 times the normal level with ethanol consumption, whereas neither fasting nor the intake of a fatty meal significantly influenced acetate concentration[7].

Acetate and histone modifications

Amongst the various modifications documented at the tails of histone proteins in humans, acetylation and methylation are the best characterized[8]. Generally, acetylation at the lysine residues of histones depicts a transcriptionally permissive state, allowing opening up of the chromatin structure and access to transcriptional machinery[8]. Hyperacetylation of histones has been observed after alcohol administration in both, cell culture and animal studies[9-13]. In a 2003 study by Park *et al*[12], ethanol increased acetylation at lysine 9 on histone 3 (H3K9Ac) in a dose- and time-dependent manner in isolated rat hepatocytes. There was a remarkable 8-fold increase in the amount of H3K9Ac at 24 h by 100 mmol ethanol without an increase in histone 3 protein expression[12]. Also, inhibition of the metabolism of ethanol to acetate largely abolished this effect, suggesting that production of the ethanol metabolite, acetate, was critical to the process of acetylation[12]. Similar experiments in rat hepatic stellate cells (HSCs) demonstrated an increase in H3K9Ac levels with

no changes at lysines 14 or 18[9]. An *in vivo* study of the effect of acute ethanol (binge drinking) in rats concluded that the ethanol-induced increase in H3K9Ac was largely restricted to the liver, lung and spleen, with the liver showing a maximal increase of ~6-fold in a 12 h period[10].

An increase in the acetylation of histones in response to ethanol may be brought about by an orchestration of events that (1) increase the substrate for the reaction, acetyl-coA, (2) and/or modulate the enzymes controlling histone acetylation (HATs, HDACs). A study by Shukla's group in 2005 demonstrated that ethanol increased histone 3 acetylation at lysine 9 by specifically modulating HAT(s) targeting lysine 9 in rat hepatocytes[13]. However, it was not determined whether ethanol induced this effect by increasing transcriptional expression of HAT(s) or by specifically augmenting their activity. Accordingly, H3K9Ac and HAT activity was also increased by acetate in these cells, again indicating it as the likely mediator of ethanol-induced histone acetylation[13]. Interestingly, signaling pathway analysis showed that mitogen-activated protein kinase kinase (MEK) and c-Jun N-terminal kinase (JNK) inhibitors reduced ethanol-induced acetylation without affecting ethanol-induced HAT activity. This suggests a role for MEK and JNK in histone 3 acetylation induced by ethanol; however, the mitogen-activated protein kinase (MAPK) cascades may influence histone 3 acetylation without involving HAT activity. In similar experiments, acetate-induced acetylation was not affected by MEK or JNK inhibitors further indicating that the MAPK pathway was not downstream of acetate in the process of acetylation. The precise role of MAPKs in ethanol-induced histone acetylation needs further investigation[13]. Another study in which rats were fed ethanol intragastrically demonstrated that levels of P300, a histone acetyltransferase, increased corresponding to the peaks in urinary alcohol levels, and this correlated with an increase in histone 3 acetylation at lysine 9[14].

A recent study by Day's group very elegantly demonstrated that the formation of acetate from alcohol is key to the process of alcohol-induced inflammatory gene expression by promoter histone acetylation in acute alcoholic hepatitis[15]. Treatment of Monomac6 cells (human macrophage cell line modeling Kupffer cell responses in ethanol) with ethanol increased global H3 and H4 acetylation and reduced HDAC activity significantly. Ethanol also induced the expression of acetyl-coA synthetases (ACSS1 and 2), the enzymes required for conversion of acetate to acetyl-coA, the substrate for acetylation reactions. Corresponding to this effect, increased acetylation was observed at the promoters of inflammatory cytokines IL-6 and tumor necrosis factor (TNF)-α, with an increase in their mRNA expression. Notably, when these experiments were performed using acetate, these effects could be reproduced. What underscores the critical role of acetate in these ethanol-induced effects is that inhibition of ethanol metabolism to acetate using 4-methylpyrazole (4-MP) completely abrogated the effects and histone acetylation remained at baseline[15]. This confirms that acetate is indeed, the mediator of alcohol-induced histone acetylation.

Although there are no published reports regarding the exact mechanisms by which acetate formed by ethanol metabolism may affect histone acetylation, some hypotheses have been suggested. Acetate may increase HAT activity simply by increasing substrate availability for the reaction. Since acetate is also the product of deacetylation reaction, free acetate may cause feedback inhibition of HDACs[15]. It should also be noted that most of the studies done with regard to ethanol-induced acetylation measure global acetylation and studies focusing on specific genes affected by alcohol metabolism are only beginning to be performed. One such gene examined in hepatocytes is ADH1 (Class 1 alcohol dehydrogenase)[13], while TNFα and IL-6 have been examined in Monomac6 cells[15] in response to alcohol. Also of interest are the findings that alcohol seems to have an effect on the acetylation at certain lysine residue, such as lysine 9 on H3 (H3K9), but not others (H3K14, H3K18)[9,12]. Further research is required to explore the significance and relevance of these findings.

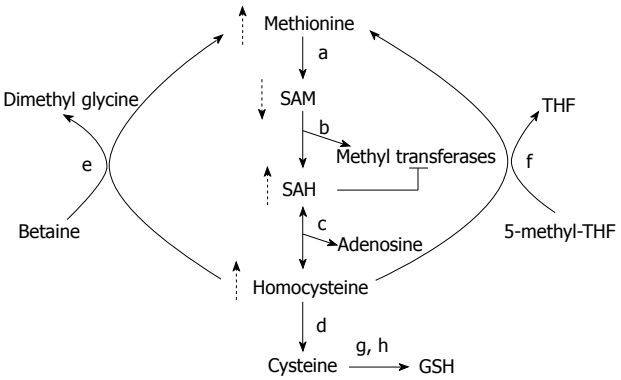

Figure 2 S-adenosylmethionine metabolism in alcohol-induced liver disease. Effects of alcohol are indicated by dotted arrows; a: Methionine adenosyltransferase; b: Enzymes involved in transmethylation reactions; c: S-adenosylhomocysteine (SAH) hydrolase; d: Cystathionine-β-synthase; e: Betaine homecysteine methyltransferase; f: Methionine synthase; g: glutamate-cysteine synthetase; h: Glutathione (GSH) synthetase. SAM: S-adenosylmethionine; THF: Tetrahydrofolate.

ALCOHOL AND SAM

SAM is the one of the most widely used cofactors in nature, probably second only to ATP[16]. The liver is the main source of SAM in humans, and is also largely the site where SAM is metabolized by methyltransferases to S-adenosylhomocysteine (SAH)[17,18] (Figure 2). SAH is a potent inhibitor of all methyltransferases, and needs to be promptly eliminated by the body by a reaction catalyzed by SAH hydrolase[19]. SAM is an essential molecule that is vital to numerous cellular processes and is the principal biological methyl donor required for methylation of histones; as also other proteins, DNA, RNA, biogenic amines and phospholipids. It gives away its high energy methyl group to methyltransferases in transmethylation reactions, and thus, plays a central role in the epigenetic regulation of genes that are controlled by histone or DNA methylation[19]. The ratio of SAM to SAH is a critical determinant of the efficiency of transmethylation reactions and hence this ratio is referred to as the cellular

methylation potential[20]. SAM dependent methyltransferases is a broad class of enzymes that contains over a hundred genes[21,22]. Besides, SAM also contributes to gene regulation by methylation of non-histone proteins such as tumor suppressor p53, transcriptional factor TAF10 and the receptor for angiogenic factor VEGF, VEGFR1[23].

Alcohol causes SAM deficiency

SAM deficiency in alcohol-induced liver disease was first described in the early 1980s[24]. Hepatic MAT activity was found to be subnormal in alcohol-dependent individuals, blocking the conversion of methionine to SAM and resulting in hypermethioninemia[25-27]. Alcohol-dependent individuals often display glutathione (GSH) deficiency because GSH synthesis requires SAM[27,28]. Hepatic SAM depletion in response to chronic alcohol consumption has been studied both in humans and in animal models. SAM deficiency has been associated with hepatitis in humans[29], and different stages of alcohol-induced liver injury in rats[30], baboons[31] and micropigs[32].

Ethanol may deplete hepatic SAM by more than one mechanism (Figure 2). Ethanol administration reduces hepatic MAT activity by the oxidation or nitrosylation of the cysteine residue at position 121 and this may be affected by the reactive oxygen and nitrogen species generated during ethanol metabolism[33,34]. MAT activity has also been shown to be reduced due to decreased gene expression of MAT1 (liver specific MAT) in alcoholic hepatitis patients[29] and ethanol-fed micropigs[35]. In another study in rats, chronic ethanol administration decreased SAM levels and glutathione concentration without affecting MAT and it was proposed that SAM consumption was increased to fuel glutathione synthesis[36]. In addition to increased consumption, SAM deficiency may occur because its synthesis may be inhibited by the unavailability of methionine, the endogenous precursor of SAM. Chronic ethanol administration can influence methionine synthesis by decreasing methionine synthase (MS) activity, and the hepatic levels of folate and betaine[35,37-39]. Thus, alcohol consumption can affect aspects of SAM production and metabolism at multiple levels to result in hepatic SAM deficiency in ALD.

SAM and histone modifications

Unlike histone acetylation, which is generally a transcriptionally permissive modification, histone methylation is known to exhibit differential effects that depend on the position of the particular residue that is modified. Methylation of the lysine at position 9 on H3 (H3K9) is a silencing event[40,41], and opposes the transcription-activating acetylation (H3K9Ac) of the same residue. On the other hand, methylation of lysine 4 on H3 (H3K4) activates transcription, and trimethylation at this residue (H3K4Me3) is strongly correlated with active transcription[42]. Also, in contrast to histone acetylation, histone methylation appears to be a more permanent mark and is relatively irreversible[43,44].

The effect of ethanol on histone and DNA methylation has been studied in cell culture and animal studies[9,10,45,46]. However, in comparison to histone acetylation, studies involving methylation changes with ethanol are only beginning to be documented. Pal-Bhadra *et al*[45] examined the effect of ethanol on H3 methylation in primary rat hepatocytes, and reported contrasting methylation patterns at H3K9 and H3K4 following ethanol treatment. H3K9 dimethylation was decreased whereas H3K4 dimethylation was increased. Further analysis showed that K9 methylation was associated with the promoters of ethanol-downregulated genes [L-serine dehydratase (Lsdh) and Cytochrome P450 2C11 (CYP2C11)] and K4 methylation with those of ethanol-upregulated genes [Alcohol dehydrogenase-1 (Adh-1) and Glutathione S-transferase Yc2 (GST-Yc2)] in these cells[45]. In earlier studies in rat liver and rat hepatic stellate cells, ethanol had been shown to increase acetylation at H3K9 with little, if any, change in methylation at the same residue[9,10]. In another recent study using a chronic rat model for ethanol (intragastric feeding model), a significant increase was noted in dimethyl histone 3 lysine 4 (H3K4Me2). Trimethylation of histone 3 lysine 27 (H3K27Me3), a transcriptionally silencing modification, also increased significantly after chronic ethanol feeding[46]. Similarly, the effect of SAM treatment on histone methylation[47,48] and gene expression[49] has been documented in some studies. When SAM was administered along with ethanol intragastrically to rats for a month (chronic model), SAM remarkably attenuated the ethanol-induced liver injury[48]. Histone 3 trimethylation at lysine 27 (H3K27Me3) was significantly increased with SAM treatment, irrespective of ethanol feeding. SAM also prevented most of the changes in gene expression caused by ethanol feeding. Since H3K27 trimethylation correlates with gene repression, it was postulated that SAM stabilized global gene expression and prevented the blood alcohol level (BAL) cycle through this epigenetic modification[48]. Other experiments in RAW and Kupffer cells show that SAM can inhibit the LPS-stimulated expression of pro-inflammatory genes such as TNF-α and i-NOS at the transcriptional level. In these studies, it was found that LPS increased the trimethylation of H3K4 at the promoters of these genes, and treatment with SAM reversed this effect[47]. Overall, research thus far indicates that SAM deficiency may be an important mediator of histone modifications in ethanol-induced liver disease.

ALCOHOL AND NAD$^+$

The ubiquitous biological molecule, NAD$^+$, was first discovered in 1906, and since then, has been widely studied for its ever-expanding repertoire of cellular functions. NAD$^+$ is best known for its role in oxidation-reduction reactions in cell metabolism[50]. NAD$^+$ is also the precursor of the important second messenger cyclic ADP-ribose[51], and more recently, has been found to be absolutely essential for the protein deacetylase activity of sirtuins[52,53]. Sirtuins or Sir2 proteins are a class of histone deacetylases and in addition to their role in gene transcription, are also involved in the regulation of ageing and metabolic processes[54]. Thus, from being a coenzyme in redox reactions, NAD$^+$ has come a long way, now being called

a critical metabolic regulator of transcription, longevity, calorie-restriction mediated life-span extension and several age-associated diseases, including diabetes, cancer and Alzheimer's disease[55].

Alcohol depletes NAD$^+$

Alcohol metabolism utilizes NAD$^+$ at the very initial steps of breakdown[1]. NAD$^+$ is used up both when alcohol dehydrogenase converts alcohol to acetaldehyde and when acetaldehyde dehydrogenase further converts it to acetate. In both these reactions, NAD$^+$ is reduced to NADH. Several acute effects of ethanol are caused by the reduction of the NAD$^+$/NADH ratio in the liver, as a consequence of ethanol metabolism. An increased NAD$^+$/NADH ratio in the liver disrupts fatty acid oxidation and can induce ketogenesis, lactic acidosis, hyperuricemia and hypoglycemia[56]. Even after conversion of ethanol to acetate, NAD$^+$ continues to be consumed. Acetate forms acetyl-coA, which reduces NAD$^+$ to NADH once it enters the Krebs cycle. Chronic ethanol consumption also recruits the CYP2E1 pathway for metabolism, which adds to the imbalance in the hepatic redox state by reducing NAD$^+$ and increasing hydroxyl radicals[57]. The oxidative stress and reactive oxygen species (ROS) caused by ethanol consumption are only aggravated by the poor nutritional status in chronic alcoholics, and play a major part in depleting NAD$^+$ and advancing liver injury[58].

NAD$^+$ and histone modifications

The NAD$^+$/NADH ratio in the liver likely plays a major role in the regulation of histone modifications and thus, gene transcription/silencing[59]. Histone deacetylases are categorized into three main classes, and class Ⅲ HDACs or sirtuins (SIRT), are activated only in the presence of NAD$^+$. In sirtuin-mediated deacetylase reactions, NAD$^+$ is hydrolyzed into nicotinamide and accepts the acetate moiety, O-acetyl-ribose. The nicotinamide formed is a potent inhibitor of sirtuin HDAC activity. Thus, the histone deacetylase activity of sirtuins is intricately controlled by NAD$^+$ metabolism, requiring NAD$^+$ for catalysis and being inhibited by nicotinamide. Ethanol, as discussed earlier, increases histone acetylation in the liver. The mechanisms underlying this effect of ethanol are not well characterized; however, there are reports of increased HAT activity[10] and/or decreased HDAC activity[11]. It may then be postulated that ethanol-induced inhibition of HDACs is due to the depletion of NAD$^+$ caused during its metabolism. Ethanol has already been shown to inhibit sirtuin expression and activity in other studies in the liver[60,61]. Chronic administration of ethanol in mice reduced hepatic SIRT1 protein levels and significantly inhibited its deacetylase activity in a recent study by You *et al*[61]. In an earlier report, ethanol was shown to increase SREBP-1c (Sterol regulatory binding protein-1c) lysine acetylation and transcriptional activity in rat H4IIEC3 cells, and this effect was at least in part, mediated by SIRT1 inhibition[62]. Results from another study reaffirm the link between ethanol consumption and SIRT1 reduction, and in this study ethanol reduced hepatic SIRT1 mRNA expression to half its baseline levels[60]. In addition, independent studies have shown that oxidative stress can inhibit HDACs[63-66].

In light of these findings, it can be hypothesized that the interplay between NAD$^+$ levels, reactive intermediates and oxidative stress will impact histone modifications and gene expression in ALD.

ALCOHOL AND ZINC

Zinc is an essential trace element and is vital in carbohydrate and protein metabolism, glucose control, wound healing, the immune system, digestion, fertility, and growth[67-69]. Zinc plays an important role in controlling gene expression, antioxidant defense and DNA repair. Abundant changes in gene expression in dietary zinc deficiency have been profiled in several tissues, including the liver[70,71], although the mechanisms have not been investigated. Zinc is an essential cofactor for over 300 enzymes; of particular relevance to this review are the zinc-requiring enzymes, namely (1) histone deacetylases (HDACs) that catalyze the removal of an acetyl group from lysines on histones protein tails, thereby increasing the accessibility of nucleosomal DNA and resulting in transcriptionally active chromatin; and (2) histone demethylases that demethylate Lys residues on histones and modulate gene expression.

Alcohol causes zinc deficiency

Zinc deficiency is often observed in chronic alcoholics and approximately 30%-50% of alcoholics are thought to have low zinc status[67,69], possibly due to decreases in intestinal absorption, increased urinary excretion and/or inadequate dietary zinc intake. Clinical studies have demonstrated that zinc concentrations in both serum and liver were significantly reduced in patients with alcoholic steatosis, hepatitis, and cirrhosis[72-75]. Indeed, zinc insufficiency is one of the most commonly observed nutritional manifestations of alcoholic liver disease[76]. Zinc depletion in the liver has also been documented in animal models of ethanol-induced liver injury[77-82]. Investigations of zinc metabolism in alcoholics has demonstrated that ethanol consumption leads to increased Zn excretion in urine[83] and decreased Zn absorption from intestine[84,85]; the latter is also seen in a chronic ethanol feeding animal model[86].

Zinc and histone modifications

While class Ⅲ HDACs require the cofactor NAD$^+$ for their deacetylase function, the class Ⅰ, Ⅱ and Ⅳ HDACs are structurally distinct and require zinc for their deacetylase function[87]. A suboptimal zinc concentration, as is observed in alcoholic individuals, is highly likely to significantly decrease the activity of HDACs, leading to altered gene expression. Indeed, the alcohol-induced decrease in HDAC activity observed by others[88] and us [unpublished results] may be attributed, at least in part, to lowered zinc levels. A direct effect of zinc on epigenetic histone changes was demonstrated in a recent study using chromatin immunoprecipitation assays, wherein zinc treatment rapidly decreased Lys4-trimethylated and Lys9-acetylated histone H3 in the metallothionein1 (MT1) promoter and

decreased total histone H3. Also, the micrococcal nuclease sensitivity of the MT1 promoter was also increased by zinc, suggesting that the chromatin structure in the MT1 promoter may be disrupted by zinc-induced nucleosome removal[89]. Another *in vitro* study showed that p21 transcription was downregulated by lowered zinc in HepG2 cells[90]. Zinc deficiency led to a reduction in acetylated histone-H4 on the p21 promoter resulting in reduced p21 promoter accessibility, which contributed to the decrease in p21 promoter activity and the downregulation of p21 mRNA and protein expression in zinc-depleted HepG-2cells. Specifically, the amounts of acetylated histone-4 associated with the proximal and distal p21 promoter regions were decreased in severe zinc-deficient (73% and 64%, respectively) and mild zinc deficient (82% and 77%, respectively) cells compared with zinc-normal (100% and 100%, respectively)[90].

A recently discovered histone lysine demethylase, LSD2, was shown to not only contain a CW-type zinc finger motif, but also to bind zinc with 3:1 molar ratio (zinc: protein), suggesting that zinc maybe important for its activity[91]. Also, a zinc finger motif located at the end of the conventionally defined JmjC domain of histone demethylases, such as jumonji-type JMJD2A, is thought to be essential for enzymatic function[92]. Changes in zinc status as seen with chronic alcohol consumption are expected to cause dramatic alterations in gene expression, leading to phenotypic changes. In addition to direct epigenetic effects, zinc deficiency is also known to reduce the utilization of methyl groups from SAM in rat liver, resulting in genomic DNA hypomethylation and histone hypomethylation[93,94]. This occurs due to the fact that the enzyme betaine-homocysteine S-methyltransferase (BMHT) is a zinc metalloenzyme[95]. Overall, we believe that alcohol consumption-induced zinc deficiency may greatly impact gene expression *via* direct and indirect epigenetic histone modifications and modulation of chromatin structure and gene expression.

In addition to the nutritional alterations mentioned in this review, alcohol is known to cause disturbances in other nutrients, which may also play a role in the epigenetic changes effected by alcohol. Alcohol has also been shown to influence other histone modifications, such as glycation[96] and phosphorylation[97]. The significance of these modifications in relation to gene expression is, however, not clear.

CONCLUSION

Alcohol metabolism is inextricably connected to the regulation of key nutrient metabolites in the liver. There is a growing body of literature suggesting a role for the complex interplay between alcohol-induced nutrient changes, histone modifications and gene expression. It is becoming widely accepted that specific aberrant patterns of histone modifications play a fundamental role in chromatin structure and function contributing to the development of disease processes. Epigenetic histone modifications provide a plausible link between alcohol-mediated nutrient alterations and pathogenic gene expression. However, in ALD, the precise contribution of histone modifications in the alteration of expression of specific genes remains largely unknown. Clearly more advances are needed and will be witnessed in this area that will enhance our knowledge about the epigenetic mechanisms underpinning ALD pathogenesis and lead to the development of relevant therapeutic strategies.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2473

World J Gastroenterol 2011 May 28; 17(20): 2473-2481
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Targeting collagen expression in alcoholic liver disease

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Received: March 22, 2011 Revised: April 17, 2011
Accepted: April 24, 2011
Published online: May 28, 2011

Abstract

Alcoholic liver disease (ALD) is a leading cause of liver disease and liver-related deaths globally, particularly in developed nations. Liver fibrosis is a consequence of ALD and other chronic liver insults, which can progress to cirrhosis and hepatocellular carcinoma if left untreated. Liver fibrosis is characterized by accumulation of excess extracellular matrix components, including type I collagen, which disrupts liver microcirculation and leads to injury. To date, there is no therapy for the treatment of liver fibrosis; thus treatments that either prevent the accumulation of type I collagen or hasten its degradation are desirable. The focus of this review is to examine the regulation of type I collagen in fibrogenic cells of the liver and to discuss current advances in therapeutics to eliminate excessive collagen deposition.

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Key words: Type I collagen; Fibrosis; Extracellular matrix; Hepatic stellate cell; Alcohol; Antioxidants; Endoplasmic reticulum chaperones; Matrix metalloproteinase; microRNA

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Thompson KJ, McKillop IH, Schrum LW. Targeting collagen expression in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2473-2481 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2473.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2473

INTRODUCTION

Liver fibrosis is an exacerbation of the generic wound-healing process of the liver and is defined by excess synthesis and deposition of extracellular matrix (ECM) components, of which type I collagen predominates[1]. Accumulation of ECM in the sub-endothelial space of Disse can disrupt liver microcirculation, leading to damage and death of parenchymal cells[2]. Liver fibrosis is a common sequela for a variety of insults, such as viral infection, industrial solvent exposure, autoimmunity, cholestasis, inborn errors of metabolism, and ethanol abuse.

In a setting of chronic fibrogenic stimulus, myofibroblast-like cells produce large quantities of ECM components. Extensive investigation has revealed several cell types that are potential myofibroblast precursors, including the hepatic stellate cell (HSC). HSCs are star-shaped, vitamin A-storing cells residing within the subendothelial space of Disse[3]. In fibrosis HSCs lose their vitamin A stores, transdifferentiate to a proliferative, myofibroblast-like cell, and produce or secrete excess ECM components[4]. Though considered the predominant source of ECM in hepatic fibrosis, other cell populations have been identified as sources of collagen and other ECM components. Portal fibroblasts and bone marrow-derived myofibroblast precursors have also been implicated as sources of ECM in fibrosis. Evidence also suggests that hepatocytes may undergo epithelial to mesenchymal transition to produce ECM, at least *in vitro*[5-7].

Globally, viral hepatitis is the leading risk factor for hepatic fibrosis; however, in highly developed nations chronic, high ethanol consumption is the principal risk factor for developing fibrosis. Nonalcoholic steatohepatitis (NASH) has also been identified as a growing cause of fibrosis[8]. Untreated, liver fibrosis is a major contributor of morbidity and mortality, as unresolved fibrosis may progress to cirrhosis and result in organ failure or progression to hepatocellular carcinoma (HCC). Despite increased understanding of fibrogenesis, there remains a dearth of effective anti-fibrotic treatments. This review will focus on type I collagen expression in fibrosis in alcoholic liver disease (ALD) and therapeutic strategies to limit or reverse its accumulation.

REGULATION OF TYPE I COLLAGEN

Excess ECM deposition in liver fibrosis can largely be attributed to members of three families of proteins - collagens, in particular types Ⅰ, Ⅲ and Ⅳ; proteoglycans, such as fibronectin, laminin, and hyaluronic acid; and glycoproteins, including heparin, chondroitin sulfates, and biglycan. Although multiple ECM components are dramatically upregulated in hepatic fibrosis, type Ⅰ collagen is the most abundant protein in the body and has been extensively characterized, making it an attractive target for the development of anti-fibrotic therapies.

Collagens are synthesized as a triple helix from three polypeptide α chains composed of continuous Glycine (Gly)-X-Y peptide repeats[9]. Glycine is essential in the first position as its side chain is the only one small enough to fit within the center of the coiled-coil triple helix[10]. Proline is frequently found in the X position and hydroxyproline in the Y position[11]. These amino acids limit rotation of the triple helical structure and their placement on the surface facilitates self-assembly and polymerization of collagen molecules through charge-charge and hydrophobic interactions[9].

In normal tissues, collagens are secreted into the ECM and help maintain the integrity of tissue by interacting with cell surfaces, with other ECM components, and with growth and differentiation factors[12]. Type Ⅰ collagen is an important component in the wound-healing process and is found in large quantities in scar tissue associated with a variety of pathological conditions[13,14]. In the liver, chronic damage stimulates activation of HSCs and other myofibroblast precursors, resulting in a phenotypic change towards excessive production and secretion of ECM products, particularly type Ⅰ collagen.

Transcriptional regulation of procollagens

Synthesis of type Ⅰ collagen is initiated by expression of the *col1a1* and *col1a2* genes, giving rise to α1(Ⅰ) and α2(Ⅰ) procollagen mRNAs, respectively. Levels of these gene products can be regulated at both the transcriptional and post-transcriptional level. Despite being located on different chromosomes, expression of these two genes are coordinately regulated in a tissue-specific manner giving rise to α1(Ⅰ) and α2(Ⅰ) procollagen mRNA products in a 2:1 ratio, respectively[15]. Numerous regulatory elements have been identified in the promoter and first intron of *col1a1* and *col1a2* that regulate expression of procollagen mRNA messages through interactions with transcription factors.

In ALD, ethanol consumption results in mediators that influence the expression of type Ⅰ collagen. Metabolism of ethanol by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) generates acetaldehyde and reactive oxygen species (ROS)[16]. Acetaldehyde treatment of HSCs increases binding of a kruppel-like transcription factor (KLF), basic transcription element binding protein (BTEB) to a region between -1484 and -1476 in the *col1a1* promoter in a *c-jun* N-terminal kinase (JNK)-dependent manner, enhancing α1(Ⅰ) procollagen mRNA levels[17,18]. Other KLFs, such as Sp1 and KLF6, have also been shown to upregulate transcription of procollagen mRNAs[19].

Transforming growth factor-β (TGF-β) has been described as the most potent fibrogenic cytokine for HSCs and is thus a common target for anti-fibrotic therapy[1]. TGF-β expression is upregulated in fibrosis and is secreted by HSCs and other cell types, such as Kupffer cells. Stimulation with TGF-β activates Smad signaling, which can upregulate procollagen expression *via* formation of a heterotrimeric complex of Smad2, 3, and 4, where Smad7 is inhibitory. TGF-β can also stimulate enhanced procollagen expression through the generation of intracellular H_2O_2 and subsequent activation of p38-mitogen activated protein kinase[1].

Nuclear factor κB (NFκB) is commonly associated with liver fibrosis, including ALD. Although NFκB is not required for the activation of HSCs, an increase in the p65/p50 heterodimer, with concomitant decrease in the NFκB inhibitory protein, IκBα, promotes survival of activated HSCs[20,21]. Overexpression of NFκB in activated HSCs, however, has been shown to inhibit α1(Ⅰ) and α2(Ⅰ) procollagen mRNA expression in culture-activated HSCs[22,23]. It is unclear, however, whether this observation occurs *in vivo*.

Post-transcriptional regulation of procollagen mRNAs

Upon activation of HSCs and other myofibroblast precursors, there is a > 50-fold increase in α1(Ⅰ) procollagen mRNA levels, with a concomitant increase in message half-life from 1.5 to 24 h[24]. Although fibrogenic stimuli, such as chronic ethanol consumption, increase the transcription rate of procollagen mRNAs, the major contributing factors associated with this increase are post-transcriptional. The α1(Ⅰ) procollagen mRNA possesses regulatory elements within both the 3' and 5' untranslated regions (UTRs), which influence the stability and translation of the message.

Heterogenous ribonucleoprotein particles (hnRNPs) are a family of RNA-binding proteins that have a variety of functions, including prevention of mRNA folding, transporting, association with the splicing apparatus, and mRNA stability. αCP is an hnRNP that has demonstrated

binding to the 3' UTR of several messages, including α-globin[25]. αCP binds to a C-rich segment of the 3' UTR of α1(I) procollagen mRNA located downstream of the stop codon, stabilizing the message and preventing degradation[24,26]. Though expressed in both quiescent and activated HSCs, αCP only has binding activity in activated HSCs. Furthermore, the cellular localization of αCP varies in HSCs, with αCP localized in the nucleus of quiescent HSCs and in both the nucleus and cytoplasm of activated HSCs, suggesting a yet to be identified post-translational event regulating the localization of αCP[27].

A well-conserved 5' stem-loop structure has been described in the message of collagen mRNAs, including α1(I) procollagen mRNA, comprising the translation initiation codon[28,29]. Mutation of this 5' stem-loop structure revealed improperly assembled procollagen I, as demonstrated by pepsin-sensitivity and diminished intermolecular disulfide bond formation[30]. Recent work has revealed La ribonucleoprotein domain family member 6 (LARP6) as a sequence-specific 5' stem-loop binding protein of α1(I) procollagen mRNAs[30]. Reporter experiments in the same study revealed that deletion of the 5' stem-loop or LARP6 resulted in diffuse accumulation of the reporter throughout the endoplasmic reticulum (ER), in contrast to the focal areas of translation associated with proper assembly of procollagens[31]. Further investigations by the same group demonstrated that LARP6 interacts with non-muscle myosins, and disruption of this interaction results in increased intracellular degradation of procollagen polypeptides and a preference towards α1(I) homotrimers[32]. Emergence of LARP6 as a collagen-specific regulator of translation may present a new therapeutic target for modulating excessive collagen synthesis in fibrogenic conditions like alcoholic fibrosis and cirrhosis.

Post-translational modifications of procollagen polypeptides

Most proteins begin folding at the N-terminus prior to completion of translation and translocation. For type I collagen, however, folding is initiated at the C-terminus following co-translational translocation into the ER[33]. Several proteins play an important role in facilitating the proper folding and trafficking of α chains into triple helix procollagen molecules. The 78-kDa glucose-regulated protein (Grp78) recognizes hydrophobic residues on polypeptide chains to help maintain solubility and may also bind the C-propeptide[34]. Protein disulfide isomerase (PDI) also plays a role in triple helix formation by catalyzing disulfide bonds between C-propeptide domains of the three α chains[35,36]. PDI also acts as a β-subunit for prolyl 4-hydroxylase (P4H) by keeping the catalytic α-subunits in a soluble state[37]. Further stabilization of the triple helix is accomplished through hydroxylation of select proline residues (typically in the Y position) by the P4H enzyme, which in turn facilitates hydrogen bonding and the formation of water bridges within and between collagen chains[38,39]. A 47-kDa heat shock protein (Hsp47) is a collagen-specific chaperone that also plays an important role in collagen trafficking[40]. Although the exact role of Hsp47 has not been clearly defined, studies utilizing Hsp47-/- mice showed they are embryonically lethal at day 11.5[41]. After procollagens traverse the Golgi apparatus and are secreted into the extracellular space, the C- and N-prodomains are cleaved by C- and N-peptidases, respectively[42]. This process decreases the concentration required for fibril formation and results in the self-assembly of collagens into fibrils[43].

THERAPEUTIC TARGETING OF TYPE I COLLAGEN

Removal (or suppression) of the underlying pathology is considered the most effective way to reverse liver fibrosis; however, removal of the causative agent is not always feasible. In ALD, patients often fail to comply with abstention programs and many patients do not respond well to casual treatments, or present with advanced fibrosis and/or cirrhosis. Thus, there is a need to identify and develop anti-fibrotic agents that can retard, or even reverse, liver fibrosis. To date there is no well-regarded or frequently used anti-fibrotic therapy in clinical practice.

Therapeutic strategies for established liver fibrosis can target type I collagen accumulation by employing one or more strategies: (1) decrease the secretion of type I collagen by disrupting either its transcription or assembly; or (2) stimulating fibrinolysis of type I collagen that has accumulated extracellularly. Therapies to reduce the pool of fibrogenic myofibroblasts are also a therapeutic strategy; however, these approaches are beyond the scope of the current review.

Antioxidants

Liver fibrosis caused by ALD has a well-established link with oxidative stress. Metabolism of ethanol by ADH and CYP2E1 generate ROS and acetaldehyde, leading to a variety of cellular defects including depletion of reduced glutathione (GSH), the main intracellular antioxidant, lipid peroxidation, acetaldehyde-protein adducts, and proteasome inhibition[44-46]. Oxidative stress from Kupffer cell activation or from damaged hepatocytes can promote HSC activation and procollagen mRNA expression. These findings led investigators to evaluate a variety of antioxidants as a way to limit production of type I collagen and other matrix components, with mixed results.

S-adenosyl-L-methionine (SAMe) is the principal biological methyl donor and is a precursor to GSH, thus it has received interest as a potential treatment for liver diseases. Liver fibrosis has been shown to be attenuated by SAMe administration in several animal models of fibrosis, including a rat model of ALD[47-49]. *In vitro* experiments reported that SAMe inhibits both basal and TGF-β-stimulated type I collagen expression in activated HSCs[50,51]. Additionally, studies by our group indicate that SAMe can enhance polyubiquitination of type I collagen, possibly suggesting a novel mechanism to prevent secretion of collagen (Thompson

et al 2011 DOI:10.1111/j.1478-3231.2011.02512.x). SAMe supplementation prevented oxidative stress and lipid peroxidation in ethanol- and ethanol plus LPS-fed animals as evidenced by normal GSH:oxidized glutathione (GSSG) ratio and diminished levels of 4-hydroxynonenal, respectively[47]. Despite attractive results *in vitro* and with animal models, SAMe has shown mixed results in modulating liver disease in human trials. A comprehensive review by Rambaldi *et al* revealed no clear benefit by SAMe in most trials analyzed; however, a well-designed study by Mato *et al* reported that SAMe administration could delay the need for hepatic transplantation in alcoholic cirrhotics[52,53]. However, the combination of SAMe with other antioxidants, such as dilinoleoylphosphatidylcholine (DLPC), has attenuated liver injury in a NASH model and *in vitro* studies reported decreased collagen and TIMP-1 expression in HSCs[54-56].

Turmeric has been used for centuries in Indian Ayurvedic medicine to treat a variety of ailments. Curcumin is a polyphenolic compound and the principal curcuminoid in turmeric. Curcumin in part owes its antioxidant properties to stimulation of nuclear factor erythroid-2-related factor 2 (nrf2), a transcription factor that binds several intracellular oxidant genes and enhances their transcription, including genes associated with production of glutathione[57,58]. In rodent models of cirrhosis, curcumin is reported to be protective; however, differences were noted depending on the model used. Curcumin can prevent thioacetamide (TAA)-induced cirrhosis, but no effect by curcumin was seen on established cirrhosis[59]. On the other hand, using bile duct-ligation (BDL) and carbon tetrachloride (CCl_4) models of established cirrhosis, curcumin improved liver histology and diminished collagen accumulation[59]. Studies utilizing curcumin on activated stellate cells revealed enhanced expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), disruption of TGF-β signaling, and diminution of collagen expression[60,61]. Additionally, curcumin has been demonstrated to improve alcohol-induced liver injury through prevention of oxidative stress and inflammation *via* downregulation of NF-κB[62-64].

Another antioxidant that has received attention as a potential therapy for hepatic fibrosis is resveratrol, a phytoalexin (a class of antibiotics produced in plants) naturally found in grapes and commercially in red wine. Resveratrol exhibits anti-inflammatory properties, anti-oxidant effects, and modulates metabolism of lipids[65]. In a CCl_4-mediated model of fibrosis, resveratrol prevented fibrosis with concomitant inhibition of NF-κB translocation and attenuation of TGF-β production[66]. Inhibition of NF-κB by resveratrol was also reported *in vitro* along with a decrease in pro-inflammatory cytokine production[67,68]. In a rat model of alcoholic liver injury, resveratrol blocked increased oxidative stress, as measured by malondialdehyde, through upregulation of superoxide dismutase, glutathione peroxidase, and catalase[69]. Resveratrol has also been shown to alleviate alcohol-induced fatty liver disease in mice through promotion of sirtuin 1 and AMP-activated kinase[70]. There is, however, a lack of human studies examining the efficacy of resveratrol as a treatment for liver disease.

Silibinin is an active flavinoligand derived from milk thistle, which has been used for several millennia as a treatment for a variety of liver disorders. Silibinin has demonstrated anti-proliferative, anti-fibrogenic, and anti-cancer properties with *in vivo* animal models and can inhibit TGF-β-induced collagen secretion in a human HSC cell line[71,72]. In addition to direct antioxidant properties, silibinin can inhibit CYP2E1 expression in the setting of chronic alcohol consumption, suggesting decreased ROS production in alcoholics[73]. Despite encouraging results in animal studies, enthusiasm for its use in humans to treat chronic liver disorders is limited by questionable success in clinical trials. A review of 13 randomized clinical trials revealed silibinin had no effect on mortality, liver histology, or liver-related complications, but there was a significant decline in liver-related mortality; however, others found no decrease in liver-related mortality in trials that were deemed to be of high quality[74].

MicroRNAs

MicroRNAs (miRNA) belong to a class of small non-coding RNAs involved with post-transcriptional regulation of gene expression, termed RNA interference (RNAi). These sequences are typically 18-25 nucleotides and are generated by processing of full-length primary transcript miRNAs, termed pri-miRNA, through enzymatic cleavage by RNase Ⅲ Drosha, generating pre-miRNAs. Subsequent transport to the cytosol permits additional processing by dicer to produce double-stranded miRNAs. One strand is loaded into the silencing complex and translation is disrupted by imperfect binding of the miRNA and elements within the 3'-UTR of target transcripts[75].

miRNAs have demonstrated roles in most biological events, including proliferation, differentiation, cell-fate determination, apoptosis, and signal transduction. Disregulation of miRNAs has been implicated in a number of disease states, including cancer and fibrogenesis in a number of solid organs including liver[76]. Comparisons of miRNA expression between quiescent and activated HSCs revealed several miRNAs that may be involved in liver fibrosis and are thus attractive candidates for targeting. Expression of miR-27a and miR-27b were shown to increase during activation of rat HSCs. Inhibition of these miRNAs reverted activated HSCs back to a quiescent state that was associated with an increase in retinyl ester storage and decreased proliferation[77]. Studies by Guo *et al*[78] suggested that miR-15b and miR-16 reduce Bcl-2 and increase caspase signaling, promoting apoptosis of activated HSCs. Overexpression of miR-150 and miR-194 in human LX-2 cells (a human activated HSC line) resulted in decreased expression of α-SMA and type I collagen, possibly through inhibition of c-myb and rac 1[79].

MiRNAs that specifically target collagen production have also been identified in a variety of tissues, including liver. Accumulating evidence implicates the miR-29-

family in the regulation of type I collagen expression in several disease states[80,81]. Examination of miR-29-family members in two models of liver fibrosis revealed downregulation of miR-29a, b, and c with associated increases in type I collagen expression. Cell-specific expression from isolated primary liver cells revealed high expression of miR-29b in HSCs, which was lost upon culture-activation[82]. *In vitro* experiments aimed to determine a mechanism revealed that TGF-β treatment downregulated miR-29b expression with a concomitant increase in type I collagen expression. Another striking observation was that miR-29a serum levels are downregulated in human fibrotic patients compared to healthy patients; the degree of fibrosis and cirrhosis correlated with the extent of miR-29a suppression, suggesting that miR-29a may be a novel serum marker of liver fibrosis in humans[82].

Small interfering RNA therapy

Small interfering RNAs (siRNA), like miRNAs, are a class of double-stranded RNA molecules 20-25 nucleotides in length that participate in the RNAi pathway and have received considerable attention as a therapeutic strategy for a variety of conditions. Several barriers exist to effective siRNA therapies in hepatic fibrosis, including targeting delivery to the intended liver cell(s) to avoid systemic consequences and overcoming the physical barriers that occur in fibrosis that limit exchange, including the loss of endothelial cell fenestration and accumulation of ECM components in the space of Disse.

Efforts to target TGF-β, the most potent pro-fibrotic cytokine, has led to the development of siRNAs that can inhibit TGF-β mRNA expression in a rat HSC cell line[83]. These siRNAs have been conjugated with galactosylated poly(ethylene glycol)(Gal-PEG) or mannose 6-phosphate poly(ethylene glycol)(M6P-PEG) and targeted to HCC (HepG2) and HSC (HSC-T6) cell lines, respectively[84]. M6P-PEG targets to HSCs *via* M6P/insulin-like growth factor-Ⅱ receptor-mediated endocytosis and Gal-PEG targets to hepatocytes *via* asialoglycoprotein receptor-mediated endocytosis[84]. Though specific targeting produced favorable results *in vitro* these strategies have yet to be validated *in vivo*.

A novel approach to deliver siRNAs against Hsp47 has recently been reported utilizing two models of liver fibrosis; CCl_4 and BDL, and a lethal model of dimethylnitrosamine (DMN)-induced cirrhosis. Investigators conjugated vitamin A to liposomes carrying siRNAs targeting gp46, a homolog of Hsp47, which rapidly resolved fibrosis and prolonged survival in DMN-induced cirrhosis[85]. Evaluation of radiolabeled vitamin A-coupled liposomes showed uptake predominantly in livers, demonstrating organ specificity. These data represent an exciting advance in siRNA-mediated treatment of fibrosis and demonstrate that targeting of collagen production, not just the underlying pathology, can be an effective anti-fibrotic strategy. However, further studies to assess the functional consequences of Hsp47 disruption need to be conducted, as the investigators revealed that Hsp47 repression stimulated collagenase activity[85].

Inhibitors of chaperone proteins

A novel approach to targeting type I collagen secretion is inhibiting the activity of one or more chaperone proteins associated with the numerous post-translational modifications procollagens undergo prior to secretion. As described in the previous section, one such approach utilized siRNA against Hsp47 to abolish fibrosis in two animal models[85]. However, systemic administration of inhibitors to collagen chaperones poses systemic risks, particularly in tissues with high normal expression of type I collagen, like skin and bone.

One such approach to prevent systemic consequences centered on the design of a pro-drug inhibitor of prolyl 4-hydroxylase, HOE 077, which would be converted to the active form by liver cytochrome P450 activity to pyridine-2,4-dicarboxylate (2,4-PDCA). Use of this drug attenuated liver fibrosis and collagen accumulation induced by CCl_4 administration[86]. However, *in vitro* mechanistic studies reported that HOE 077 prevented activation of HSCs as opposed to type I collagen production[87].

Hsp47 is an attractive target for the generation of small inhibitors, as type I collagen is the only reported target for this chaperone. Several inhibitors of Hsp47 have been reported; however, their efficacy in inhibiting collagen production by mediators of fibrosis, or in animal models, has yet to be demonstrated[88].

Matrix metalloproteinases

Regulation of the ECM is accomplished in part by a diverse family of calcium- and zinc-dependant endopeptidases called MMPs. There are 25 identified unique members of the MMPs, of which 24 are found in mammals, and they are capable of degrading a variety of matrix components. MMPs can be divided into five categories: interstitial collagenases, gelatinases, stromelysins, membrane-type collagenases, and a metalloelastase (MMP-12), although there is some overlap in function between these groups.

In liver, the main interstitial collagenases are MMP-1 in humans, and MMP-8 and MMP-13 in rodents. MMP-3 (stromelysin-1) is another interstitial collagenase expressed in liver; however, it exhibits weak proteolytic activity towards ECM components[89]. Liver fibrosis results from an imbalance between fibrinogenesis and fibrinolysis, with an increase in tissue inhibitors of metalloproteinases (TIMPs) primarily responsible for this imbalance. Four TIMPs have been identified (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), each consisting of 184-194 amino acids. TIMPs inhibit MMPs by directly binding to the catalytic domain of MMPs in a 1:1 stoichiometric ratio. TIMPs have been shown to inhibit the activity of each MMP, though with varying efficiency[90]. Besides the well-established direct role of TIMPs as inhibitors of MMPs, TIMP-1 is capable of indirect inhibition of HSC apoptosis[91]. Furthermore, reduction of TIMP-1 levels is associated with increased hepatocyte proliferation *via* degradation of fibrotic ECM to permit hepatocyte expansion and liberation of ECM-bound hepatocyte growth factor[92]. Thus, therapeutic strategies to improve fibrinolysis can focus either on in-

creasing the pool of active MMPs or reducing the expression of TIMPs.

Attempts to enhance the expression of fibrinolytic MMPs have been carried out in various animal models of fibrosis. Infection with an adenovirus carrying human MMP-1 gene attenuated fibrosis with concomitant decrease in α-SMA positive cells in a rat TAA model[93]. An adenoviral delivery strategy was also utilized to stimulate human MMP-8 expression to abrogate fibrosis in rats treated with CCl_4 or subjected to BDL[94].

A similar approach was taken to inhibit expression of TIMP-1 in HSCs, utilizing an siRNA against TIMP-1 packaged in an adeno-associated virus (AAV) vector. AAV vectors have the ability to infect dividing and non-dividing cells, to incorporate into the genome at a specific site (AAVS1) in human chromosome 19 for sustained expression, and are non-immunogenic[95]. TIMP-1 expression was suppressed for > 12 wk, suggesting AVV-delivered siRNA against TIMP-1 has the potential for long-term efficacy. The study also reported concomitant increases in MMP-13 expression, the rodent equivalent to MMP-1 in humans; however, the investigators did not assess MMP-13 activity by zymography and have not investigated the efficacy of this system *in vivo*[96].

MMPs and their inhibitors clearly play an important role in the development, progression, and resolution of hepatic fibrosis. However, the context of MMP and TIMP expression must be considered when developing therapeutic strategies to target their activity. In early stages of hepatic fibrosis, MMPs appear to play a deleterious role, whereas in the resolution of fibrosis, MMP activity is critical to reduce the scar and achieve restoration of the normal liver architecture. Additional work to evaluate their effectiveness in treating hepatic fibrosis should be conducted.

CONCLUSION

Liver fibrosis is a complex disease that represents a common pathology for a variety of liver insults, including ALD. Sustained fibrogenesis can be linked to an exacerbation of the wound-healing process and results in the accumulation of ECM products, which can impair oxygen and nutrient delivery, stimulate proliferation of fibrogenic cells and result in injury. To date, there are no established therapies for liver fibrosis outside removal of the causative agent. Type I collagen is the most abundant component of the extracellular scar in liver fibrosis and is an attractive target for anti-fibrotic therapies.

Therapies to limit pro-fibrotic mediators, such antioxidants to scavenge ROS, have produced promising results *in vitro* and with animal models of fibrosis; however, antioxidants have failed to consistently reduce fibrosis in human trials. This discrepancy is not understood, and therefore, attention should be made to developing new therapeutics. Regardless of the etiology, it is widely accepted that other factors (e.g. genetic and environmental) contribute to the development and progression of fibrosis, thus a therapeutic target directed towards the culprit of fibrosis (collagen) might be a more successful and comprehensive therapy. Attempts to reduce accumulated type I collagen through MMP-mediated fibrinolysis has generated attractive results in animal models; however, their efficacy has yet to be tested in human trials. Additionally, further work to refine targeting and delivery of MMP-based therapies needs to be performed, as non-specific delivery of MMPs could have unintended consequences in other collagen-rich tissues.

Recent work in targeted delivery of siRNAs against the collagen-specific chaperone Hsp47 represents exciting proof-of-concept therapy of tissue-directed suppression of type I collagen, potentially reducing deleterious effects of systemic type I collagen inhibition. Despite this promising finding, additional studies to determine the efficacy and safety of this approach in humans need to be conducted. Continued investigation into the molecular mechanisms of type I collagen production and secretion in fibrogenic mediators should be performed to produce new targets for anti-fibrotic therapy.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2482

World J Gastroenterol 2011 May 28; 17(20): 2482-2499
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Fibronectin: Functional character and role in alcoholic liver disease

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Author contributions: Aziz-Seible RS and Casey CA both contributed to the preparation of this manuscript.
Supported by The National Institute on Alcohol Abuse and Alcoholism and the US Department of Veterans Affairs
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Received: January 21,2011 Revised: April 7, 2011
Accepted: April 14, 2011
Published online: May 28, 2011

Abstract

Fibronectins are adhesive glycoproteins that can be found in tissue matrices and circulating in various fluids of the body. The variable composition of fibronectin molecules facilitates a diversity of interactions with cell surface receptors that suggest a role for these proteins beyond the structural considerations of the extracellular matrix. These interactions implicate fibronectin in the regulation of mechanisms that also determine cell behavior and activity. The two major forms, plasma fibronectin (pFn) and cellular fibronectin (cFn), exist as balanced amounts under normal physiological conditions. However, during injury and/or disease, tissue and circulating levels of cFn become disproportionately elevated. The accumulating cFn, in addition to being a consequence of prolonged tissue damage, may in fact stimulate cellular events that promote further damage. In this review, we summarize what is known regarding such interactions between fibronectin and cells that may influence the biological response to injury. We elaborate on the effects of cFn in the liver, specifically under a condition of chronic alcohol-induced injury. Studies have revealed that chronic alcohol consumption stimulates excess production of cFn by sinusoidal endothelial cells and hepatic stellate cells while impairing its clearance by other cell types resulting in the build up of this glycoprotein throughout the liver and its consequent increased availability to influence cellular activity that could promote the development of alcoholic liver disease. We describe recent findings by our laboratory that support a plausible role for cFn in the promotion of liver injury under a condition of chronic alcohol abuse and the implications of cFn stimulation on the pathogenesis of alcoholic liver disease. These findings suggest an effect of cFn in regulating cell behavior in the alcohol-injured liver that is worth further characterizing not only to gain a more comprehensive understanding of the role this reactive glycoprotein plays in the progression of injury but also for the insight further studies could provide towards the development of novel therapies for alcoholic liver disease.

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Key words: Fibronectin; Liver disease; Alcoholic liver disease; Endocytosis; Cellular fibronectin

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Aziz-Seible RS, Casey CA. Fibronectin: Functional character and role in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2482-2499 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2482.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2482

INTRODUCTION

Fibronectins are ubiquitous, multifunctional, high-molecular weight glycoproteins that have been implicated in a wide array of fundamental biological processes specific to their structure and distribution in the body. These proteins have been the subject of extensive study for over 60 years yet their physiological roles remain to be completely defined. Most reports emphasize their critical participation in biological phenomena involving the modulation of components in the extracellular environment. However, there is a growing body of evidence that reveals fibronectins may also be directly involved in regulating cellular behavior, particularly in injured tissue and under pathological circumstances.

HISTORY

Two classes of fibronectin exist *in vivo*, each discovered through widely different research initiatives. Plasma fibronectin (pFn), which is found primarily as a soluble dimer circulating in various body fluids, was first identified as "cold-insoluble globulin" during post World War Ⅱ studies on the fractionation of human blood plasma[1]. Twenty-five years later, the search for tumor markers led to the discovery of cellular fibronectin (cFn) described then as the "large external transformation sensitive (LETS) protein" or "galactoprotein", and later determined to also be the "surface fibroblast antigen"[2-4]. This fibronectin is found predominantly as an insoluble, multimeric, fibrillar constituent of extracellular matrices. Separate biochemical and cell biological analyses of these two glycoprotein types drew similar conclusions that eventually led to the convergence of such studies and the realization that these molecules are, in fact, related[5-7]. The common term of "fibronectin" was agreed upon to define these similar proteins, but it was only after detailed genetic and structural analyses could be made that this similarity was truly understood[8].

During a marked collaborative period, several other research groups became aware that their glycoproteins of interest, originating from sources other than plasma and fibroblasts, resembled fibronectin in character. It was revealed that these proteins were, in fact, variants of fibronectin, as it appeared they all derive from the same complex gene. This gene consists of more than 45 distinct coding (exons) and non-coding (introns) nucleotide sequences, which can be transcribed from a single promoter into alternatively spliced messenger RNAs that account for the multiple isoforms of fibronectin found in human tissue[9-11].

STRUCTURE

Generally, the functional protein is composed of two similar, but not always identical subunits of 220 to 250 kDa that are joined by disulphide bonds at the carboxyl-termini to create the characteristic fibronectin dimer. Greater than 90% of the structure of each fibronectin monomer is

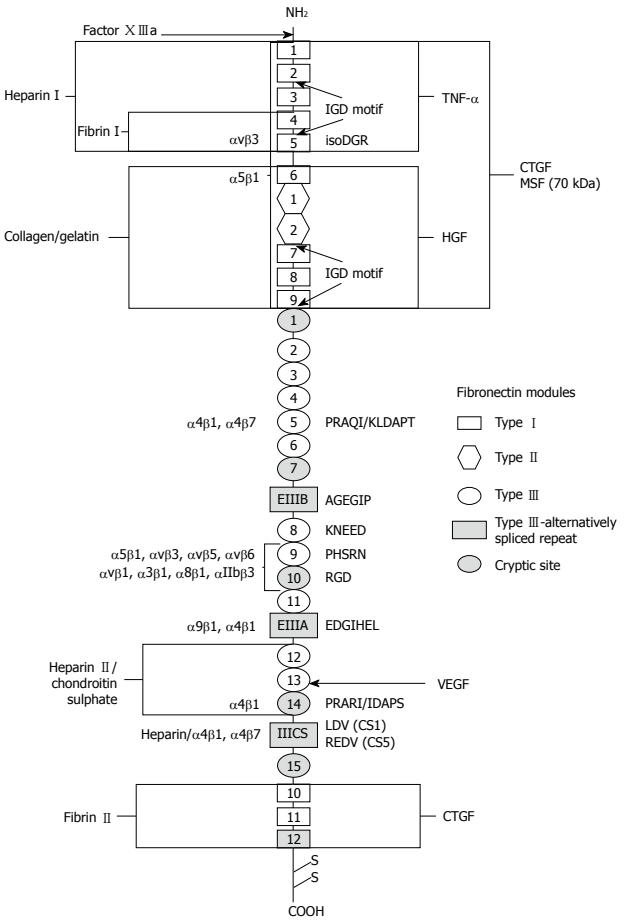

Figure 1 Domain structure and interaction sites of fibronectin. Fibronectin is a dimer comprised of two subunits which are covalently joined by two disulfide bridges near the COOH-terminus. Each subunit consists of three types of homologous structural domains called Ⅰ, Ⅱ, and Ⅲ. Recognition sequences, integrin binding sites, cryptic sites and interactive regions of the molecule are labeled.

defined by variable combinations of three different types of homologous repeating domains termed Types Ⅰ, Ⅱ and Ⅲ that are linked by short peptide segments (Figure 1)[8]. There are twelve Type Ⅰ (~40 amino acid residues) and two Type Ⅱ (~60 amino acid residues) homologous repeats in each fibronectin sequence that are individually folded to form sheets of β-strands stabilized by disulphide bonds. Type Ⅲ repeats (~90 amino acid residues) of which there are fifteen to seventeen per sequence flanked by Type Ⅰ and Type Ⅱ regions, are similarly organized into overlapping β-sheets. However, these modules lack disulphide bridges, thus have greater conformational latitude. Type Ⅰ and Type Ⅱ modules are each encoded by a single exon, while Type Ⅲ modules are coded for by 2 exons each with the exception of extra domains A and B (denoted EIIIA, EDA or EDI and EIIIB, EDB or EDII respectively) and the ninth Type Ⅲ domain (Ⅲ-9). Rather, these particular domains are each encoded by single exons of somewhat extended lengths[8-11].

Considerable molecular subunit diversity results from the complex splicing of the fibronectin primary transcript at three specific sites that code for the Type Ⅲ domains, EIIIA, EIIIB, and a region of the molecule towards the

carboxyl-terminus that links Type Ⅲ units, Ⅲ-14 and Ⅲ-15, referred to as the Type Ⅲ connecting segment (IIICS) or variable (V) domain[8]. Patterns of inclusion or exclusion of the sequences of these three alternatively spliced domains confer variability among species that express fibronectin[8, 11]. In fact, variations between the two biological forms of this glycoprotein are also largely attributed to differential splicing of pre-mRNA. Neither subunit of the pFn dimer contains the EIIIA and EIIIB sequences and only one of these subunits has a V-region. Alternatively, cFn contains variable proportions of all three domains[8,11]. Furthermore, the V-domain undergoes a more intricate tissue-specific splicing mechanism that results in its sequence being either entirely included or excluded, or only variable parts of it being present in the molecule. In humans, such splicing patterns generate five V-region variants. Altogether, these mechanisms can potentially produce greater than twenty human subtypes, which can be assembled to form a diverse array of fibronectin heterodimers. Such varied composition, particularly for cFn, is likely associated with a diverse array of functions.

Further structural complexity is established through post-translational modifications of the fibronectin molecule. Though both phosphorylation and sulfation have been observed, these modifications do not appear to account for significant differences among isoforms[12]. However, analyses have revealed considerable variation in the glycosylation profiles of fibronectin molecules derived from different sources[8,13]. All forms of fibronectin contain significant amounts of carbohydrate (5%-10%) that are predominantly in the form of biantennary asparagine-linked side chains that reside mostly among Type Ⅲ repeats. Some heterogeneity, with regard to the number and size of these carbohydrate side chains, is present among individual fibronectin polypeptide units. Studies have shown that domains with carbohydrate moieties are resistant to proteolysis and that glycosylation contributes to the conformational stability of the fibronectin molecule[14]. Interestingly, cFn variants have greater carbohydrate content than pFn which may serve to protect the function of cFn molecules in areas of active proteolysis and tissue remodeling where they are normally found. Additional evidence suggests that glycosylation may be involved in modulating the binding affinity of fibronectin to other matrix, as well as, cell-surface proteins[15]. Rotundo *et al*[16] revealed that the composition of the carbohydrate side chain determines whether fibronectin associates with a receptor on the surface of liver parenchymal cells that is involved in the clearance of endogenous glycoproteins from circulation. The presence of terminal galactose residues on the carbohydrate side chains of cFn make it a natural ligand for this receptor, termed the Ashwell or asialoglycoprotein receptor (ASGP-R), however, the fully sialylated chains of pFn are not recognized[16,17]. Approximately 80%-85% of the terminal carbohydrates of cFn are not capped by sialic acid[8].

Typically, the absence of sialic acid caps on its carbohydrate chains suggests that a protein is defective either as a result of normal catabolic mechanisms or pathogen induced sialidase activity[18]. These proteins may be harmful and induce a defensive response from the body if they are not rapidly removed. Perhaps cFn, as a naturally occurring desialylated glycoprotein, is intended to provoke a similar response from tissues that generate it in excess locally particularly during conditions of disease and disrepair.

FUNCTIONAL INTERACTIONS AND CHARACTER

The complex structure of fibronectins, their extensive presence in various tissues and fluids of the body, and their conserved expression across species, suggest that these molecules are important to fundamental biological processes. This is conclusively demonstrated by the lethality of *FN* gene inactivation during early murine embryonic development[19]. A greater understanding of this fundamental position can be obtained through a closer examination of its molecular architecture.

Fibronectin's functional properties are mapped by specific domains of modular repeats along the molecule itself[8,20,21]. Polypeptide regions linking these globular domains are particularly susceptible to proteolysis, thus are readily cleaved to form fibronectin fragments of defined structural and functional character. Analyses of these fragments have led to the identification of distinct interacting sites along the fibronectin molecule that provide some insight into the physiological role of this glycoprotein (Figure 1)[8,20,21].

The domain represented by the amino-terminal fragment of fibronectin is composed of type I homologous repeats that can bind to a variety of substrates including matrix heparin and cell-surface heparan sulfate proteoglycans, glycosphingolipids found in membranes of central nervous system tissues, as well as to bacteria[22]. Of particular relevance is this domain's strong affinity for fibrin, an insoluble plasma protein essential to blood clotting, to which fibronectin can be covalently stabilized *via* factor ⅩⅢa transglutaminase-catalyzed cross-linking[23]. This cross-linking mechanism can also facilitate other fibronectin interactions with asymmetric acetylcholinesterase and *Staphylococcus aureus*[24,25]. Thrombospondin, present in tissue matrices and implicated in platelet aggregation, also binds to fibronectin at its amino-terminal domain[26]. These interactions suggest the participation of fibronectin in such events as cell adhesion, blood clotting, as well as pathogen recognition and/or clearance.

Immediately adjacent to the amino-terminal domain is the highly glycosylated collagen/gelatin binding site of the fibronectin molecule[8,20,21]. Fibronectin has demonstrated variable affinity for the various types of collagen in their native forms, however, it also adheres quite effectively to the unfolded regions of the denatured collagen triple helix[27]. Under physiological conditions, it appears that Type I collagen, which is found at elevated levels in the matrices of injured tissue, is in an unfolded state thus could readily interact with fibronectin[28]. The same domain that adheres to collagen can also bind to the C1q component of the

complement system, facilitating fibronectin's involvement in the clearance of immune complexes and cellular debris during the body's defense response[29].

Situated at the carboxyl-terminal region of fibronectin is the molecule's major heparin binding domain[30]. It comprises Type Ⅲ repeats along with a variable segment that is determined by the tissue of origin. Nearest to the interchain disulphide bonds is a region consisting entirely of Type I modules that contains the molecule's second fibrin binding site. However, this domain does not exhibit the diverse interactions of its amino-terminal counterpart and plays a more minor role in fibronectin-fibrin binding[30].

Clearly, the structure-function relationships of the terminal regions of fibronectin are well defined and reflect similar substrate affinities and functional character. Conversely, the central, more variable region of the fibronectin molecule remains more obscure. This central region is made up entirely of Type Ⅲ homologous repeats that include the alternatively spliced EIIIA and EIIIB sequences positioned between repeats 11 and 12, and 7 and 8, respectively[8-10]. The heightened susceptibility of this section of the molecule to protease activity precludes it from the extensive fragmentation analysis that has been used to characterize the amino- and carboxyl-termini. Rather, the functional character of this large area of fibronectin must be determined through alternative analytical means. Accordingly, primary sequence data analysis has revealed the presence of a plausible DNA and heparin binding site adjacent to the collagen binding domain at the amino end of the central region; while the extra domains, EIIIA and EIIIB, have been implicated in a variety of roles based largely on *in vitro* analyses of their increased presence in fibronectin, particularly, under certain conditions of injury and disease[31-34].

Unlike the isoforms found in embryonic tissue, fibronectin molecules from healthy adult tissues include very low levels of EIIIA and EIIIB[35,36]. However, the variants with EIIIA and EIIIB will re-appear in abundance during such processes as wound repair and tissue regeneration[32,37]. Elevated levels of EIIIA and EIIIB fibronectin isoforms are also present under the pathological conditions of fibrosis and tumorigenesis[32,34,38]. Studies on malignant and benign remodeling activity in bone, as well as in human gingival tissue, show an increased presence of these extra domain containing forms of fibronectin[39,40]. In addition, these variants are also considered to be important mediators of the extensive interactions between participating cells and their environment during vascular morphogenesis[31]. Apparently, the EIIIA and EIIIB domains confer a role for fibronectin molecules containing them in processes that involve elaborate tissue modification and re-organization.

Interestingly, each fibronectin splice variant appears to be expressed in a tissue- and cell-specific manner, triggered by different stimuli at variable times. These distinctions have been demonstrated in studies on bone fracture repair that reveal a diffused expression pattern for EIIIA-containing fibronectin throughout the connective tissue that accumulates in the fracture gap during the granulation phase of healing, while the EIIIB-containing isoform remains localized in osteoblastic cells at the periphery of the newly differentiating tissue. Similarly, during the early stages of hepatic fibrosis, sinusoidal cells are the predominant source of EIIIA enriched fibronectin, which may be involved in the activation of hepatic stellate cells that subsequently produce the EIIIB inclusive fibronectin protein[32]. Additional evidence of such temporally and spatially distinct functions for EIIIA and EIIIB fibronectin splice variants can also be found in studies on chondrogenesis, renal fibrosis and various forms of lung cancer[41-43]. These observations suggest that the expression and function of fibronectin molecules with EIIIA and EIIIB segments may be regulated by specifically coordinated independent mechanisms that facilitate the transformative systems in various adult tissues.

More defined roles for the EIIIA and EIIIB domains have been identified in such events as matrix assembly, cell adhesion, migration and differentiation, as well as in cell cycle progression and mitogenic signal transduction, which are all relevant for tissue alteration, proliferation and development[42-45]. According to these *in vitro* studies both the EIIIA and the EIIIB domains appear to have equally essential, though different, roles in the aforementioned processes.

However, recent studies using genetically engineered mice seem to suggest a more critical function for EIIIA than EIIIB *in vivo*. Strains incapable of expressing EIIIA-containing fibronectin proteins have significantly shorter lifespans than their control counterparts[46]. Though these EIIIA knock-out mice exhibit wound healing defects, altered behavior and impaired motor coordination, they also develop fewer and smaller atherosclerotic lesions and appear to be protected from progressive fibrosis after bleomycin-induced lung tissue damage[34,46-48]. However, the *in vivo* function of the EIIIB domain remains obscure. No distinct phenotype has been observed in EIIIB knock-out mouse models aside from the impaired ability of extracted fibroblasts to form a significant pericellular matrix[44,49]. Nevertheless, this domain is highly conserved among vertebrates, thus it must have some biological importance. Perhaps the EIIIB domain plays a compensatory role in the absence of EIIIA during certain developmental processes, as mice devoid of EIIIA grow normally while the EIIIA and EIIIB double knock-out mice have lethal defects[19,46]. The EIIIB domain may have a significant function during the body's response to stress brought on by injury and disease, as these are the conditions under which the extra domain-inclusive fibronectin isoforms are upregulated.

Located in the tenth Type Ⅲ module (Ⅲ-10), on an exposed loop in the central region of the fibronectin molecule is a three-amino acid consensus sequence, Arg-Gly-Asp (RGD), that has been identified as the main site of cellular attachment to fibronectin[8,50]. Adjacent to this site, in the ninth Type Ⅲ module (Ⅲ-9) exists a Pro-His-Ser-Arg-Asn (PHSRN) sequence that acts synergistically to enhance the binding affinity of cells to the Ⅲ-10 RGD sequence[51]. These repeats, critical to cell-fibronectin con-

tact, lie in a region between the two alternatively spliced EIIIA and EIIIB domains. Several studies suggest that the inclusion or exclusion of these extra domains may affect the conformation of the fibronectin molecule in that region which, in turn, determines the interactions of the RGD and PHSRN sites with specific receptors on the surfaces of nearby cells[52,53]. These interactions can trigger a cascade of distinct intracellular signals that translate into a multitude of different responses.

The receptors largely responsible for mediating these fibronectin-induced effects belong to a family of heterodimeric transmembrane glycoprotein complexes known as integrins[54]. Each integrin is comprised of two non-covalently associated α- and β-subunits that link the fibronectin-rich extracellular matrix with the cytoskeleton of the cell[55]. Structural studies have revealed that residues along the fibronectin molecule, external to specific integrin binding sites, play a critical role in optimizing the specificity and stability of this receptor-ligand assemblage[56]. Moreover, binding affinity is determined not only by the external configuration of the fibronectin ligand, but also by internal mechanisms that modulate the condition of the receptors themselves. Certain intracellular events can affect the association of the integrin α and β cytoplasmic tails, thus also, the activation state and the affinity of the receptor for specific ligands[57]. As such, integrins can function both as sensors (inside-out signaling) determining the presence of fibronectin, then mediating cell attachment and matrix assembly; and upon ligation with fibronectin, they can function as effectors (outside-in signaling) promoting ligand-induced biochemical processes[58].

To date, a dozen members of the integrin family have been shown to interact with fibronectin[50,59]. Not surprisingly, the major cell-binding Ⅲ-10 RGD sequence on the fibronectin molecule is a key integrin-recognition motif and critical binding site for several of these cell surface receptors[8,50]. Of these integrin heterodimers, the prototype fibronectin receptor, α5β1, binds with greatest specificity. It is widely expressed, and likely serves as the major mediator of fibronectin-cell interactions in most tissues. Binding of α5β1 to the RGD motif is optimized through its association with the PHSRN synergy sequence on the adjacent Ⅲ-9 repeat[51]. This sequence is also recognized by the platelet integrin αⅡbβ3[60].

Other regions of fibronectin, besides repeats Ⅲ-9 and Ⅲ-10, have also been reported to interact with integrins. The α5β1 receptor exhibits a low affinity attachment to the N-terminal region of fibronectin, specifically to an Asn-Gly-Arg (NGR) sequence in the fifth Type I module (I-5) that has been converted through deamidation and isomerization to an isoAsp-Gly-Arg (isoDGR) sequence[61]. Though deamidated proteins typically undergo loss of function, this modification on the fibronectin molecule may bring about a gain of function. Studies have shown that the isoDGR sequence is also a high affinity binding site for the αvβ3 integrin, which is involved in regulating endothelial adhesion and blood vessel formation[61]. Protein deamidation is also linked to aggregate formation through a process resembling matrix assembly, thus the isoDGR sequence may be involved in fibronectin fibrillogenesis[62]. Tissue accumulation of proteins with atypical aspartyl residues is often observed during injury and disease[63,64]. Though unconfirmed, it seems likely that the prevalent form of fibronectin under such conditions would also contain a modified I-5 repeat.

Alternative splicing of the fibronectin primary transcript results in the production of structurally diverse molecules with very specific configurations and binding capabilities. The arrangement of alternatively spliced domains determines the level of exposure and accessibility of binding sites in involved regions of the protein[52,53,65]. However, the existence of internal integrin recognition sequences suggests that, in addition to structural considerations, these domains can also directly influence fibronectin's effect on cell behavior. The alternatively spliced Type Ⅲ connecting segment (IIICS) of the fibronectin carboxyl-terminal region contains two active binding sequences, Leu-Asp-Val (LDV, residues1-25) and Arg-Glu-Asp-Val (REDV, residues 90-109), that interact with the leukocyte integrin receptors, α4β1 and α4β7[66-68]. The sequence, Glu-Asp-Gly-Ile-His-Glu-Leu (EDGIHEL) in the EIIIA domain is recognized by integrins α9β1 and α4β1 during cell adhesion and wound healing[69]. Structural analyses have revealed the presence of a conserved Ala-Gly-Glu-Gly-Ile-Pro (AGEGIP) sequence on the EIIIB beta strand CC' loop (links beta strands C and C') that is part of an acidic groove created by the interface of the EIIIB domain with the adjacent eighth type Ⅲ module (Ⅲ-8)[52]. However, the specific binding partners for this site are yet to be identified. It is apparent that each variation in the splicing pattern of these alternate domains would produce distinct combinations of binding sites that would have a differential influence on cell behavior when engaged by the appropriate receptor.

Proteoglycan receptors, such as the integral membrane syndecan-1,-2 and -4, as well as, glycosyl-phosphatidylinositol (GPI)-anchored proteoglycans such as glypican-1, have been linked to the fibronectin mediated processes of cell adhesion, cytoskeletal organization and matrix assembly[70,71]. These proteins can link directly to fibronectin through their covalently attached flexible glycosaminoglycan (GAG) chains of heparan sulfate (HS) or chondroitin sulfate (CS). Although proteoglycans are capable of affecting cell behavior directly by independently engaging relevant intracellular pathways, most reports suggest that these receptors are more likely to be involved in complementary functions that support the activities of other fibronectin receptors, specifically integrins, that are considered to have a more significant role in certain cellular events[72,73]. The spatial arrangement of the respective binding sites for each receptor along the fibronectin molecule facilitates their cooperative regulation of the adhesive functions of the cell that influence movement and morphology, as well as, pericellular fibronectin fibril assembly[72-75]. Alternatively, the proximity of these binding sites could also allow for direct regulatory interactions between the different receptors themselves. Additional receptor collaboration may involve the strategic recruitment of distant fibronectin molecules, detected by the extended ectodo-

mains of the proteoglycan receptors, for closer positioning to the cell surface where more efficient integrin binding can occur. Cooperative mechanisms may also include the transduction of signals by activated proteoglycans to effect an appropriate distribution of integrins and influence their consequent function. A synergistic convergence of such signals could occur, which would reinforce a particular effect, as illustrated by the fibronectin-induced activation of both α5β1 and syndecan-4 to promote cell adhesion and matrix contraction in the unstable environment of a wound during tissue repair[73]. Evidently, the functional regulation of fibronectin-mediated cellular processes entails some degree of coordinated activity between participating proteoglycan and integrin receptors, however, the actual mechanisms behind these interactions remain obscure.

Considering the number of receptors from the integrin family itself, that are reported to interact with fibronectin and the diversity of such interactions, it is expected that cross-regulation between activated integrins must also occur to ensure appropriate receptor cooperation or antagonism. Examination of the complex sequence of events that lead to the directional migration of a cell along a fibronectin fibrillar matrix, has revealed a pattern of spatially and temporally-regulated binding and alternate signaling by the α5β1 and αvβ3 integrin receptors, both of which recognize the same fibronectin domain[76]. Though not considered to be a fibronectin-specific receptor under normal conditions due to its indiscriminate adhesion to a variety of ECM molecules, the neutrophil receptor, αMβ2, during the cellular response to inflammation, binds with greater than normal avidity to fibronectin to effectively hinder directed migration[77]. Coordinated communication between α5β1 that facilitates chemotaxis, and αMβ2 must take place to ensure the effective translocation of the neutrophil to the site of injury where its presence is secured by additional interactions between αMβ2 and the fibronectin-enriched matrix so that it may effect the appropriate defensive response. The adhesive properties of the cell are further enhanced by the collaborative signaling of α5β1 and α4β1, each of which binds to different sites along the fibronectin molecule and interacts differentially with the provisional matrix that forms with granulation tissue during wound healing and repair[73]. Clearly, effective regulation of these fibronectin-integrin mediated physiological processes involves some coordinated crosstalk between the respective signaling pathways.

Each event induced by a particular set or sequence of interactions with fibronectin is not only dependent upon cooperative receptor activity but also upon synchronous availability and accessibility of respective ligand binding sites. When fibronectin is first secreted into the interstitial space of tissues, it exists as a soluble dimer whose compact form, stabilized by intramolecular forces, conceals many of these ligand binding sites and regions of significant adhesive character[78]. Though this closed conformation under physiological conditions is programmed by fibronectin's inherent structure, environmental factors can effect changes to its shape that expose these otherwise embedded sites, to potential binding partners[79]. It is thought that upon ligation to integrins and other cell surface receptors, particularly α5β1, which recognizes the already accessible RGD loop, a cooperative unfolding and elongation of the respective arms of the dimer is initiated which then expose these sites to binding by key receptors involved in fibronectin fibrillogenesis[80]. Gradual extension of this originally tightly-folded molecule is largely attributed to cell-traction forces that are induced by cytoskeleton-dependent events and transmitted through adherent receptors[81,82]. The globular structure of individual Type Ⅲ modules is disrupted, revealing intradomain binding sites that readily interact with their counterparts on other dissociated fibronectin molecules to launch progressive self-association that can lead to fibrillar matrix assembly[83,84]. These uncharacterized hidden or cryptic sites, as they are termed, are only activated when exposed by conformational changes that counter the native configuration of fibronectin. This suggests that these sites are involved in functional interactions, other than self recognition, particular to modifications in the extracellular environment that challenge normal physiological conditions.

As previously mentioned, the native fibronectin molecule is vulnerable to proteolysis, particularly along the unfolded polypeptide links between the compact domains. Cell-derived tensile forces increase the incidence of unfolding, thus create more unprotected regions that can be acted upon by endogenous proteases. Fibronectin is a known substrate of numerous different proteases, particularly the aggrecanases such as ADAM (A Disintegrin And Metalloproteinase)-8 and ADAM-TS (with Thrombospondin Motifs)-4, as well as matrix metalloproteases (MMPs) such as the gelatinases, MMP-2 and -9, the metalloelastase, MMP-12 and the membrane-type (MT)1-MMP. Interestingly, studies reveal that fibronectin itself induces the release and activity of several of these proteases, likely as a homeostatic response during ECM maintenance, that is regulated in part by its association with integrins and membrane-anchored MMPs[85,86]. Ongoing studies have revealed the presence of numerous specific cleavage sites or neoepitopes along the molecule that suggests the proteolytic degradation of fibronectin is not an arbitrary process and may have some functional value[87]. Random mechanical fragmentation, however, does also occur, especially among the less resilient Type Ⅰ and Type Ⅱ domains that have more restricted conformations.

The fibronectin fragments that result from these proteolytic events would have distinct folding patterns from their intact forms on the native molecule, therefore variably exposed binding sites. These peptides are, consequently, able to interface with dissimilar binding partners than the intact molecule thus they may act quite differently. In fact, certain fibronectin fragments have been associated with bioactivities that are quite disparate from those of the parent molecule and likely serve a regulatory role[73]. Many studies report a similar competence between fragments and native fibronectin to modulate protease activity, while other reports reveal that such proteolytic potential exists in the fibronectin fragments themselves[88,89]. These fibro-

nectin-derived proteases or fibronectinases are capable of autodigestion but otherwise remain cryptic in nature, as no associated physiological role nor other mechanism has yet been identified. Other fragments can exhibit chemotactic activity, promote apoptosis, regulate anabolic and catabolic processes or induce the release of nitric oxide and cytokines[90-92]. Analyses of these fibronectin fragments and their specific functions can reveal distinct interactions that may provide further insight into the physiological role of the native protein itself. Studies of endogenous fragments that contain the EIIIA domain, for example, have revealed a unique interaction with Toll-like receptor (TLR)-4 that stimulates the release of proinflammatory cytokines[92]. These data suggest that fibronectin isoforms containing this alternatively-spliced domain may have a role in the physiological response to inflammation in injured tissue.

Fibronectin also adheres to a variety of different signaling molecules and regulates their distribution and access to other binding partners and cells[93,94]. Tumor necrosis factor (TNF)-α, upon release from activated cells at the site of inflammation, can complex with the amino terminal domains of fibronectin in the surrounding matrix[93]. This interaction confines this proinflammatory cytokine near to its source thus ensuring its availability to further stimulate cells in the region to release proteases as part of the defense response[95]. Fibronectin may also be involved in presenting a growth factor to its cognate receptor in a manner that will enhance a desired physiological effect. This is demonstrated when hepatocyte growth factor (HGF) or vascular endothelial growth factor (VEGF) attaches to fibronectin with a specific juxtapositioning that promotes the coordinated interaction and costimulation of the respective growth factor receptor and the fibronectin-binding α5β1 integrin, to amplify the proliferation and migration of endothelial cells[94]. Though fibronectin does not interact directly with transforming growth factor (TGF)-β, but with the latent TGF-β binding proteins (LTBPs) to which the TGF-β-confined small latent complex (SLC) is covalently bound, it is able to sequester TGF-β, and regulate its activation by proteases and matrix remodeling forces[96,97].

The detection of additional binding partners is ongoing, as is the recognition, with each associated function, that fibronectin is more than a mere component of biological scaffolds and conduits of cellular activity. It is a repository for both intrinsic ligands that can be proteolytically transformed into soluble signaling peptides, and extrinsic ligands that can be regulated through complex formation. Moreover, its pliable constitution hints at mechanotransducing capabilities. This glycoprotein has the potential to affect cell behavior in a myriad of different ways.

SIGNALING PROCESSES

The mechanisms by which fibronectin may affect signaling events, as indicated by the diversity in its interactions, are expected to be quite involved (Figure 2). The initiating influence on cell behavior may be conceived when the soluble fibronectin molecule is ligated by a cognate receptor, likely an integrin heterodimer, and activates the first recognition-dependent sequence of molecular signals within the cell. Cytoskeletal restructuring and the consequent stimulation of certain intracellular complexes promote cell contractility which causes conformational changes in the attached molecule[80]. Intramolecular dissociation ensues and the compact dimer unfolds to expose additional binding sites and recognition sequences, which upon engagement, can trigger simultaneous cascades of signals that further influence cell behavior. Fibronectin self-association between attached and extended molecules, modulated again by cellular events such as receptor clustering, takes place to form an adhesive template upon which additional fibronectin molecules can also unfold. The progressive layering and interweaving of these elongated and stretched out fibronectin fibrils eventually results in the formation of a connective web between neighboring cells. Other matricellular components, recognizable to fibronectin can also be incorporated into this structure. The diversity and distribution of these elements can affect the character and signaling propensity of this fibronectin matrix that may now behave as a cohesive unit or solid-phase ligand. Nevertheless, individual interactions can impose changes to matrix ligand architecture that may create a flow of signals through interconnected molecules. Mechanical stress brought about by such extracellular perturbations can also alter the connectivity between ligand and receptor, and change the composition of signals being relayed[98]. Mechanotransduction is further regulated by the composition and density of this matrix ligand. A dense and correspondingly rigid matrix creates more exogenous tension which can affect such cellular activity as spreading and directed motility[99]. Protease release is also up-regulated in response to matrix rigidity. The resulting cleavage of fibronectin fibrils produces fragments with signaling properties that differ from the intact molecule. New interactions are formed that stimulate a different array of signal transduction pathways to evoke a differential cellular response. Other molecules, whose association with fibronectin have been compromised by proteolytic remodeling, are now also accessible to responsive cells[100]. All of these mechanisms by which fibronectin may influence cell behavior comprise a dynamic system that has complex spatially and temporally regulated components. Signaling pathways, though individually engaged, are part of a collective communication network between fibronectin and the cell.

Most of these pathways are thought to be driven by integrin-mediated signaling processes. Focal adhesion, stress fiber formation, and cell translocation are determined by integrin recruitment of focal adhesion kinases (FAK) with subsequent activation of the phosphatidylinositol 3-kinase (PI3K) signal transduction pathways[101,102]. However, recent studies have also implicated other receptors and associated pathways in the regulation of these events. It has been shown that cell adhesion and migration, as well as cytoskeleton reorganization are also determined by syndecan-2 and -4 mediate mechanisms that involve protein kinase C

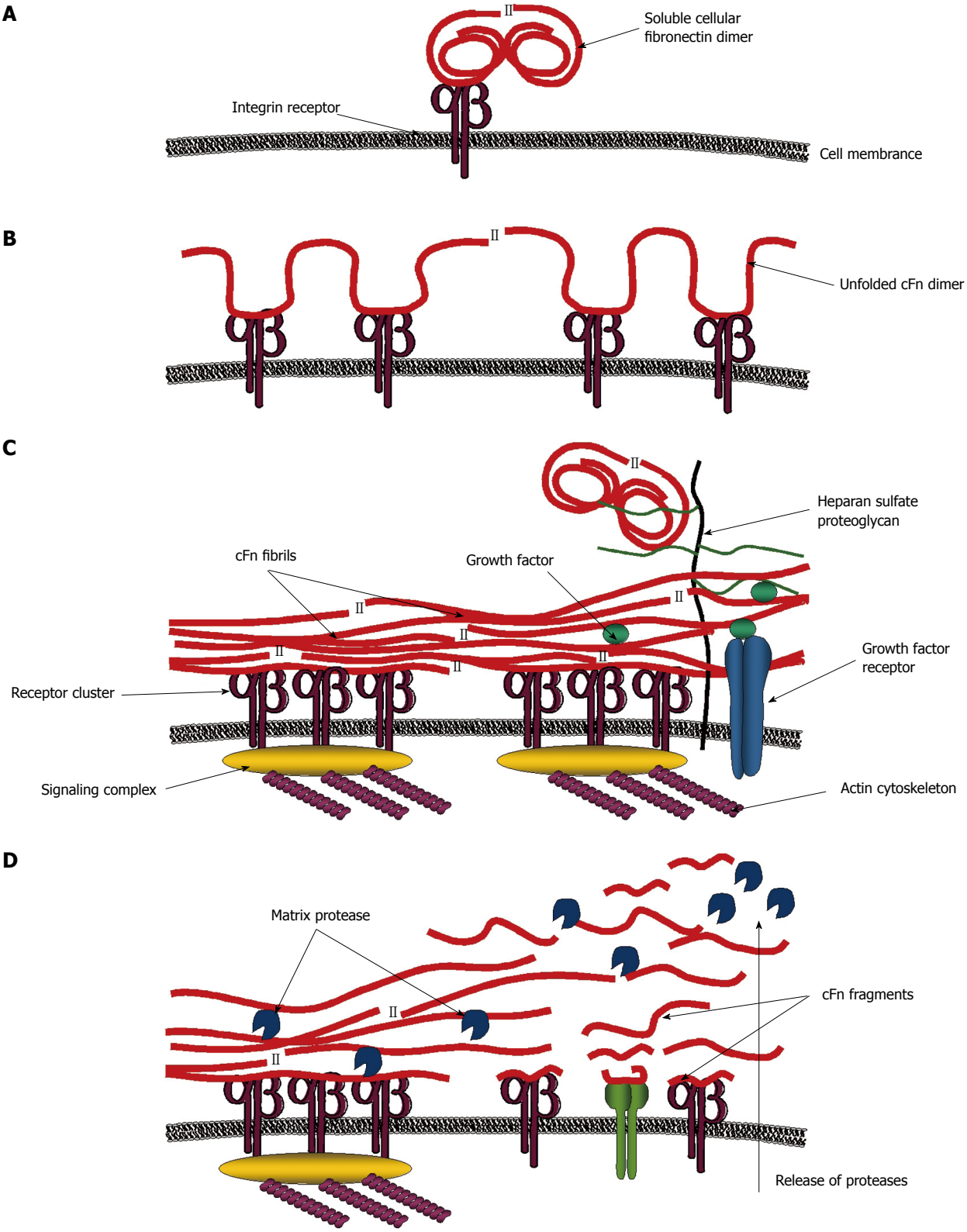

Figure 2 Model for signaling processes mediated by fibronectin. A: Initial signals are mediated when soluble compact cellular fibronectin (cFn) binds a cognate receptor; B: This causes cFn to unfold and interact with other receptors inducing further signals; C: Receptor clustering and the formation of signaling complexes lead to the reorganization of the actin cytoskeleton which creates tensile forces, conveyed through the integrin receptors to further stretch cFn into fibrillar form. Exposed cryptic sites interact with other cFn fibrils in matrix assembly. Access to growth factors and other molecules is regulated by cFn binding. Heparan sulfate proteoglycans also bind to cFn and recruit distant molecules closer to the cell surface; D: All of these interactions create a cascade of different signals, some of which promote matrix protease release. Resulting cFn fragments activate additional intracellular signaling pathways. Thus, cFn can regulate cell behavior *via* numerous different mechanisms.

(PKC)-dependent activation of the small GTPase molecules, Rac, Cdc42 and Rho[71,72,103]. Fibronectin-induced cell survival and proliferation are also regulated *via* the integrin-mediated FAK/PI3K pathway[104]. Other coordinat-

ed signals originating from a different set of fibronectin receptors may exist but have yet to be identified. Studies suggest that cytokine release and protease production that result from nuclear factor (NF)-κB activation or mitogen-activated protein kinases (MAPKs) signaling may also be mediated by toll-like receptors (TLRs) and MT-MMPs in addition to integrins, however, this needs to be further clarified[92,105]. Continued examination of the numerous interactions between fibronectin and cells, may reveal the identity of additional pathways and signaling mechanisms which would further our understanding of this multifaceted protein in its regulation of various cellular processes.

Clearly, the involvement of multiple ligand-receptor systems in fibronectin signaling requires intricate regulation and the appropriate integration of respective pathways to ensure optimal cellular activity. This elaborate network calls attention to the important role this glycoprotein plays in numerous biological processes. Disruption of these coordinated events could certainly have severe and deleterious consequences.

ROLE IN DISEASE

The aforementioned interactions and associated functions can generally be ascribed to both classes of fibronectin except where the alternatively spliced EIIIA and EIIIB domains are involved, as only the cellular forms of fibronectin contain these extra structures. Both pFn and cFn are secreted by cells as soluble globular proteins. Hepatocytes are the primary source of pFn, which is readily secreted into the bloodstream for distribution throughout the body. Cellular fibronectin, however, is produced locally in tissues, predominantly by resident fibroblasts and endothelial cells, to be deposited in the pericellular matrix. Nevertheless, it can also be taken up into the circulation. Conventionally, cFn is thought to be the main form of fibronectin found in the extracellular matrix of tissues, however, recent studies have determined that an almost equivalent fraction of matrix fibronectin is plasma-derived[106]. It appears that under normal physiological conditions, there is a balance of both types of fibronectin in intact tissues. It is not surprising then, to discover that unusually elevated levels of cFn are indicative of some underlying disturbance in the tissue of origin, which could very well have some pathological consequence.

Cellular fibronectin plays a critical role in tissue-specific morphogenesis and cellular differentiation during embryonic development[19]. These events recur in adult tissues during conditions that require regeneration or repair. Therefore, the accumulation of cFn at sites of injury and tissue perturbation where morphogenetic processes are again active is normal. Studies show that cFn regulates cell migration in damaged tissue, where it also stimulates fibroblast transitioning to its activated phenotype[73,107]. Other reports highlight the chemotactic activity of cFn and its regulation of growth factors during active wound repair[108]. Particularly convincing are data from knockout animal studies that reveal defective wound healing in cFn-deficient mice[46]. Clearly, this isoform is essential to the processes of tissue repair. Therefore, conditions involving any form of tissue damage would be marked by an increase in cFn production and release, particularly by cells near the site of injury. Under normal regulation, these events would culminate in the restoration of tissue function and integrity and a reduction in cFn to physiological levels.

However, under certain circumstances cFn levels remain elevated. Fibronectin-mediated cellular activity persists and may even be amplified. Otherwise uninvolved or down-regulated signaling mechanisms, eventually, become activated. Accordingly, cellular behavior adjusts and the maintenance of normal physiological processes changes to the promotion of pathological ones.

A prominent feature of many disorders associated with elevated levels of cFn is the persistent production and deposition of extracellular matrix proteins in affected tissue. This build-up of scar tissue may have started innocently, as a regulated wound healing response to chronic injury. However, it eventually becomes a fibroproliferative process that progressively destroys tissue integrity. Such fibrotic damage has been observed in many organ systems, particularly hepatic, pulmonary and renal systems[109-111]. Though it has been suggested that fibrosis may be reversible, most conditions do not improve but gradually progress to organ failure.

The pathological implications of cFn accumulation are considerably complex and widespread, reflecting the multifunctional capacity of this protein to influence cell behavior. The effects of cFn manifest in a tissue specific manner that may be exacerbated by other underlying factors unique to each disorder. For example, the cFn-induced fibrotic response may be complicated by factors involving the source of chronic injury. These parallel yet interdependent effects need to be recognized in order to achieve a deeper understanding of the molecular basis for these conditions and the specific role that cFn may play in their progression.

ROLE IN LIVER DISEASE

In the normal liver, the most abundant matrix protein is plasma fibronectin. This is not surprising, considering it is originally synthesized by hepatocytes. It can be detected in the subendothelial space of Disse where it comprises a major part of the low-density matrix that connects hepatocytes with the endothelial cells that line the sinusoids[112]. Cellular fibronectin, however, is present at very low levels throughout the liver. It is localized primarily in the pericellular matrix that surrounds the cells but also exists as bundles connected to the microvilli of hepatocytes in the space of Disse[113].

Naturally, most studies on fibronectin that reference the liver focus on the plasma isoform and of these, only a small percentage deal with disease. Most of those reports deal with the effects of hepatic insufficiency on pFn production and physiological function. Very few address a potential role for fibronectin itself in the incidence of liver damage that creates conditions of insufficiency. Such liver damage may result from a range of potential pathogenic

mechanisms accounted for by autoimmune diseases (autoimmune hepatitis), genetic disorders (Alagille syndrome, alpha-1 antitrypsin deficiency, hemachromatosis, Wilson's disease), viral infection (hepatitis A, hepatitis B, hepatitis C), disorders of uncertain etiology (cancer, primary sclerosing cholangitis, non-alcoholic fatty liver disease) and those attributed to systemic disease (Reye's syndrome, Budd-Chiari syndrome) and toxic insult (alcoholic liver disease).

Many of these conditions have acute and chronic presentation. Under conditions of acute liver damage, wound healing would not be a prolonged process. It would entail a quick remodeling of the ECM to create a cFn-rich provisional matrix, which would be involved in modulating repair activity to restore liver integrity. The onset of this response would be marked by a sudden surge in fibronectin production, which would just as suddenly diminish once the repair is complete. However, under conditions of chronic liver damage, wound healing would no longer be a finite process. The initial surge in cFn production may likely persist.

Accordingly, studies concerning such conditions of sustained and chronic liver damage report a considerable increase in patient blood plasma levels of cFn[114]. Immunohistochemical and RT-PCR analyses also reveal elevated amounts of cFn and its mRNA in the tissue of diseased livers[115,116]. These findings confirm that cFn production does persist as a likely response to mechanisms perpetuating liver injury under such conditions of chronic disease. The only role cFn has been considered to have in the course of these events is as an indicator of the onset of progressive damage. As such, it has potential utility as a biomarker for chronic liver disease.

Currently, a liver biopsy is the only means by which clinicians can accurately determine the extent of liver damage in patients suffering from chronic disease. However, it is an invasive, painful and inherently risky procedure that does not always provide information that would lead to significant alterations in treatment, especially if severe liver damage was already a concern[117]. Therefore, the development of additional biomarker tests that could reduce the prevalence of unnecessary biopsies is of great interest. Much consideration has been given towards developing a means to incorporate cFn as a marker in this system. However, elevated levels of cFn are not specific to hepatic injury, as similar concentrations have also been detected in the plasma of patients with no known hepatic pathologies, but who suffer from some other tissue-related chronic disease[114]. This lack of specificity diminishes the utility of cFn as a diagnostic indicator of liver disease. Nevertheless, increasing cFn levels remain a reliable indicator of sustained tissue damage. Perhaps tests for cFn could be incorporated in a panel that includes tests for the more specific markers of hepatic damage, alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (GGT), thus providing a means to determine whether a detected increase in cFn levels is, in fact, related to liver disease[118]. A ratio of ALT or GGT levels to cFn could also be instructive.

These efforts to determine a clinical application for cFn have led to further investigations of the pathophysiological events in chronic liver disease that result in its up-regulated levels. It was previously believed that such up-regulation was a response to injury and had no relevance to the progression of disease itself. However, recent studies suggest that cFn may participate in, and may even promote, the progression of injury that marks these chronic conditions.

Chronic injury of the liver may initially manifest as altered lipid metabolism that leads to the accumulation of fat deposits in hepatic cells. There is no evidence to date to suggest any cFn involvement in this process. However, as the injury persists, an inflammatory response is induced that could involve cFn-regulated wound healing activity. Studies that currently report a regulatory role for cFn during inflammation in the liver address acute rather than chronic injury conditions. Although studies from other systems suggest that cFn does affect the behavior of immune and inflammatory cells, thus may also be a potent mediator of the inflammatory response to chronic injury, further investigation is still required to confirm such a role for cFn in chronic liver disease[119,120].

In response to injury, hepatic sinusoidal endothelial cells (SECs) become activated and increase their production of cFn[32]. It has been suggested that this event occurs in the very early stages of damage, and could, in fact, be among the initial reactions to the detection of harmful stimuli. For example, the hepatitis B virus x antigen (HbxAg) has been shown to activate fibronectin gene expression in liver cells *via* an NF-κB-dependent mechanism[121]. The direct detection of HbxAg by SECs could, therefore, be an initiating event in the progression of hepatitis B-induced liver injury. The resulting upsurge in cFn production dramatically increases the total concentration of cFn in the liver to a level that is several-fold above normal[32].

Of particular interest, Jarnagin *et al*[32] showed that greater than 80% of the cFn produced by SECs during injury, 12-24 h after stimulation, contain the alternatively spliced EIIIA domain. As mentioned earlier in this text, the fibronectin EIIIIA domain has been implicated in cell adhesion and pro-inflammatory cytokine production through its interaction with cell surface receptors, α9β1 and α4β1 integrins, and TLR-4[69,92]. Moreover, studies have also shown that cFn activates α5β1 signaling *via* the RGD motif in its major cell binding domain to upregulate the production of MMPs. These MMPs are involved in ECM remodeling events that can also produce cFn fragments, which can further stimulate cell behavior. Thus, it would not be too bold to suggest that cFn may be involved in regulating many of the cellular responses to injury in the liver.

In fact, activation of hepatic stellate cells (HSCs) during injury is mediated by cFn[32]. Studies reveal that HSCs will transition to their myofibroblastic phenotype *via* a TGF-β1 regulated mechanism induced by EIIIA cFn[122]. Further studies show that TGF-β1 up-regulates the expression of the fibronectin receptor, α5β1 in HSCs, making them more responsive to cFn thus reinforcing its effect[123].

Once activated, HSCs are involved in mediating most

of the ECM remodeling activity that leads to fibrotic damage in the liver. These cells are also the major source of matrix protein constituents in connective scar tissue that manifests during disease progression. HSCs also produce cFn in continually increasing amounts, but of a different composition than the cFn secreted by SECs. The cFn secreted by HSCs is also predominantly of the EIIIIA variety, constituting 42% of the total, 7 d post-activation[32]. However, there is also a significant increase in the relative amount of cFn produced that contains the EIIIB domain (9% of the total after 7 d). The physiological significance of this increase in EIIIB variant levels and the regulatory relevance of timing its production during the advanced stages of chronic hepatic injury, are yet to be determined.

Each form of chronic liver disease may present with variable distinction between the stages of injury; however, should the incidence of damage continue along this general trajectory, fibrotic scarring will totally compromise organ function. Without a transplant, death is certain. It has become apparent that cFn has a functional role in this progression of liver injury, thus may warrant greater attention for its pathogenic nature than for any biomarker potential.

ALCOHOLIC LIVER DISEASE

As the body's major detoxifying organ, the liver is the primary site of alcohol metabolism, and is particularly susceptible to the detrimental effects of alcohol abuse. Though the association between liver injury and the excessive consumption of alcohol was established over 200 years ago, we are still unable to fully understand how such damage occurs, nor have we developed any thoroughly effective strategies to counter the progression of alcoholic liver disease (ALD).

ALD initially manifests as fatty liver (steatosis), a reversible condition characterized by increased fat deposition in the liver cells, which leads to hepatomegaly (enlarged liver) and can progress to alcoholic hepatitis, a more serious condition marked by inflammatory changes. Persistent damage prompts the development of scar tissue (fibrosis), which will eventually replace the functional tissue of the liver resulting in alcoholic cirrhosis, hepatic failure and death.

Evidence suggests that alcohol itself and its metabolites are direct hepatoxins that stimulate changes in the cells of the liver which result in a cascade of responses culminating in tissue damage[124-129]. The toxicity of alcohol is linked to its oxidation which is catalyzed mainly by the multi-variant cytosolic enzyme, alcohol dehydrogenase (ADH), to produce acetaldehyde, which is further processed in mitochondria to form acetate, most of which escapes to the blood[126,130]. Acetaldehyde binds reactive amino acid residues in proteins to form acetaldehyde-protein adducts which can impair secretion and enzymatic activity[128]. As the level of alcohol increases with ongoing consumption, microsomal enzymes, predominantly cytochrome p450 isozymes, as well as peroxisomal catalase (minor pathway), become involved in metabolizing alcohol with the additional creation of reactive oxygen species (ROS) and hydroxyl radicals that provoke lipid peroxidation events and the release of further harmful metabolites[131,132].

The conversion of alcohol involves the reduction of a co-enzyme intermediate, nicotinamide adenine dinucleotide (NAD^+) to generate NADH which increases the $NADH/NAD^+$ ratio and redox state within cells. A highly reduced intracellular environment sustained by persistent metabolism of alcohol will greatly impair the cell's ability to function normally. Under these conditions, hepatic cells are rendered more vulnerable to damage from the reactive metabolites of alcohol whose concentrations are correspondingly increased[124,127-129].

Typically, these cells would respond to the increasing levels of such harmful byproducts by releasing factors that stimulate mechanisms of tissue defense and repair. However, such mechanisms are impaired in liver tissue that has been subject to prolonged insult by alcohol. Rather than countering the progression of injury, the cellular response reinforces it.

For example, SECs respond to increasing levels of harmful adduct modified proteins formed during alcohol metabolism by up-regulating their output of the EIIIA variant of cFn that is involved in tissue repair[132]. Under a condition of chronic alcohol metabolism, this process will persist, resulting in an elevation in the levels of cFn in the liver. Restoration of homeostatic levels of this glycoprotein will usually occur towards the end of a wound healing response to injury. In the liver, this turnover of cFn is mediated, in part, by the hepatocyte specific ASGP-R[17]. However, studies have shown that the cellular processes of this receptor, particularly those that involve protein trafficking, are particularly susceptible to the effects of alcohol[133-135]. Several alcohol-induced alterations in ASGP-R activity have been identified that contribute to impaired receptor-mediated uptake of its ligands[136,137], which could include cFn. This coupling of persistent production with ineffectual clearance would lead to a build-up of cFn in the alcohol-injured liver. This has been demonstrated in studies using a rat model of alcohol consumption. Significantly elevated levels of cFn were detected in the livers of animals subject to prolonged alcohol administration which correlated with the inability of the hepatocytes from these animals to adequately internalize and degrade cFn[138,139]. Though these observations have not yet been corroborated in human liver tissue, a blood plasma study found elevated levels of cFn in patients suffering from alcoholic cirrhosis which suggests that the hepatic levels of cFn were also high[114].

The functional character of cFn suggests that its accumulation would exacerbate the deleterious effects of sustained alcohol abuse on the liver. A clearer understanding of how this reactive glycoprotein influences the cellular events that promote injury may reveal new targets for the development of effective treatments for ALD.

To this purpose our lab employed a rat model that is

extensively used in alcohol research. Male Wistar rats were pair-fed a nutritionally adequate Lieber-DeCarli liquid diet that contained 6.4% alcohol by volume as 36% of total calories or an isocaloric control diet. Animals maintained on this diet exhibit morning (i.e. 9 am CST) blood alcohol levels of 100 to 150 mg/dL (21.7 to 32.6 mmol/L)[140]. These concentrations correspond to levels found among chronic drinkers in the human population. After twelve weeks of feeding, 60% more cFn was detected in livers of the alcohol-fed animals than from pair-fed controls. Furthermore, we found that the hepatocytes from these alcohol-fed animals exhibited a diminished capacity to degrade cFn that correlates with its accumulation. As the purpose of this study was to ascertain whether cFn contributes to the development of advanced liver injury, animals were fed for a shorter duration of 4-6 wk, sufficient for the development of the early stages of alcoholic liver injury but not prolonged enough for substantial cFn accumulation to have already taken place[138,139]. Moreover, the Lieber-DeCarli rodent model rarely ever sustains injury beyond fatty liver, thus it is an appropriate system to investigate whether cFn could provoke further inflammation and/or a fibrotic response when added exogenously to cultured cells isolated from the livers of alcohol-fed animals.

The pro-fibrogenic propensity of cFn has largely been attributed to its observed effects on HSC activation and proliferation[32]. Moreover, studies have shown that fibronectin fibrils have a particular affinity for collagen Type I molecules that are synthesized by activated HSCs and are major constituents of the connective tissue that forms during fibrosis. Formation of the matrix during the fibrotic response to injury requires a stable ECM layer of cFn[141]. These reports imply that the build-up of cFn in the liver of chronic consumers of alcohol would be sufficient to initiate fibrotic damage.

However, cFn has also been implicated in the recruitment and activation of other cell types besides HSCs during the wound healing response. These cells may be involved in the pro-inflammatory activity that precedes HSC activation and may even prime the conditions in the liver for an HSC response. Kupffer cells (KCs), as the resident macrophages of the liver, are the primary mediators of such inflammatory activity in response to alcohol-induced injury that occurs during the early onset of damage. Anchored at strategic intervals throughout the hepatic sinusoid, these macrophages sentinel portal flow entering the liver lobule for incongruous and harmful substances. Accordingly, they can detect early changes in the hepatic environment arising from alcohol-induced injury such as the increasing levels of cFn. In healthy tissue, Kupffer cells orchestrate defensive and reparative processes through their phagocytic activity and production of soluble signaling molecules. However, under a condition of chronic alcohol administration, excess cFn provokes a response in Kupffer cells that actually promotes rather than protects against further tissue damage[142].

Kupffer cells are the major source of TNF-α and IL-6 during the liver's homeostatic response to tissue damage. Elevated levels of these pro-inflammatory cytokines are characteristically detected in the serum of patients with alcohol-induced liver injury. These cytokines stimulate autocrine and paracrine effects that result in the activation of other liver cell types during the injury process[143]. For example, both TNF-α and IL-6 are involved in the transition of HSCs to their myofibroblast-like phenotype. These cells in turn, accelerate the production of ECM proteins that heralds the fibrogenic response to injury[143].

Regions of the liver that first respond to the toxic effects of alcohol contain both cFn-enriched matrices and elevated numbers of KCs[144]. The behavior of KCs in these regions is likely influenced by the high concentration of cFn present. In fact, studies from our lab have shown that cFn has a profound effect on the KC secretion of TNF-α and IL-6, and therefore may be involved in promoting the KC-mediated activation of HSCs[142].

ECM remodeling poses a homeostatic challenge to cells that prompts the production of agents that can restore and maintain normal tissue architecture. MMPs and their inhibitors (TIMPs) are key agents that regulate this process. An imbalance in the relation of MMPs to TIMPs can lead to profound changes in the composition of the ECM such as is found in various pathological conditions including alcoholic fibrosis[113,145]. We believe that increasing levels of cFn, itself a constituent of the ECM, can prompt events leading to such imbalance in susceptible tissue.

Though HSCs are the most prolific source of factors that regulate the deposition of matrix components in the liver, during the early stages of fibrotic injury and prior to HSC activation KCs assume this role. Our studies have revealed that in response to increasing levels of cFn, cultured KCs from both control and alcohol-fed animals secrete significantly higher amounts of MMP-2 protein than their untreated counterparts. This increase in protease levels may be a regulatory response to excess cFn, a known substrate of MMP-2. However, we also found that the cells from alcohol-fed animals released significantly more of the associated inhibitor, TIMP-2, than matched control and untreated cells. Correspondingly, though the total MMP-2 secreted by the KCs from alcohol-fed animals was elevated, most of the enzyme detected was still in the less operational precursor form (pro-MMP-2)[142]. These results suggest that a higher degree of MMP-2 inhibition exists under a condition of alcohol administration. The consequent reduced degradative capacity of this protease could, in turn, contribute to the eventual build-up of ECM proteins characteristic of fibrotic injury.

These findings also imply that chronic alcohol consumption alters the homeostatic response of KCs to the build-up of proteins in the ECM. This may be another regulatory mechanism compromised by excessive alcohol metabolism that may contribute to the accumulation of cFn in the liver. The inhibition of matrix proteases is reinforced with each increase in cFn, creating a cycle that may later also facilitate the deposition of other matrix proteins involved in the fibrogenic process. Collectively, these studies suggest a role for cFn in Kupffer cell activation that

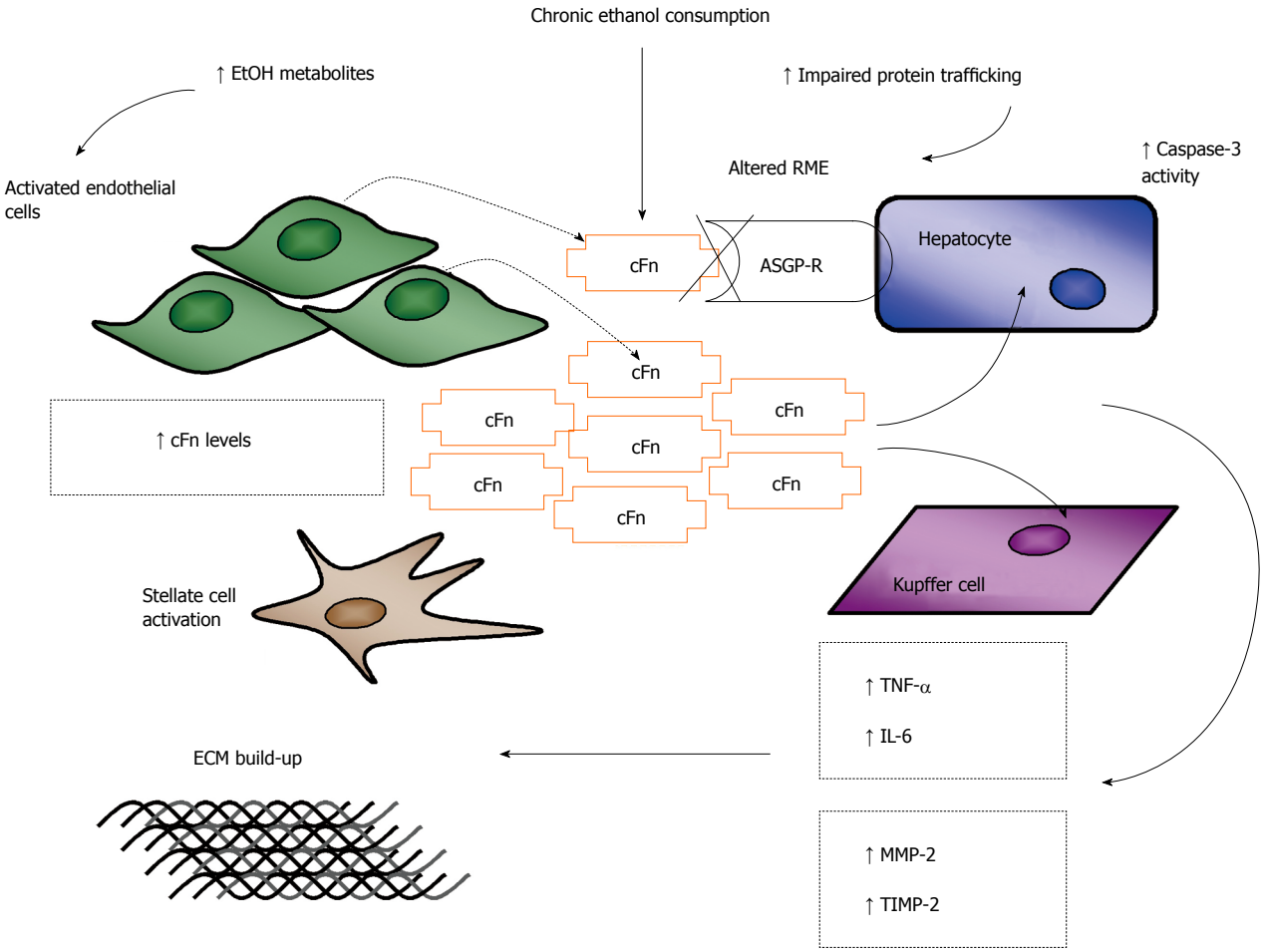

Figure 3 Schematic representation of the proposed model of ethanol-induced liver injury linking altered asialoglycoprotein receptor clearance of cellular fibronectin with hepatocyte and kupffer cell activation by the accumulating protein. The alcohol induced up regulation of cellular fibronectin (cFn) production by sinusoidal endothelial cells (SECs) and its impaired clearance by the hepatocyte-specific asialoglycoprotein receptor (ASGP-R) leads to the accumulation of cFn in the liver. Hepatocytes (HCs) and kupffer cells (KCs) are stimulated by cFn to produce the pro-inflammatory/pro-fibrogenic cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6, which further activate hepatic stellate cell (HSCs) stimulating their transformation to the pro-fibrogenic phenotype. HCs and KCs are also stimulated to produce the matrix degrading enzyme, matrix metalloproteinase (MMP)-2 and its corresponding inhibitor, tissue inhibitor of metalloproteinase (TIMP)-2. Greater levels of TIMP-2 are secreted resulting in the inhibition of MMP-2 activity and subsequent build-up of the extracellular matrix (ECM), characteristic of the early onset of fibrotic liver damage. RME: Receptor mediated endocytosis.

contributes to the progression of alcohol-induced liver injury that may lead to fibrogenesis (Figure 3).

Hepatocytes are the chief functioning cells of the liver and key targets for mediators of injury. Accordingly, studies on the effects of various hepatoxins focus on disruptions to hepatocytic processes, such as alcohol-induced impairments to ASGP-RME as previously determined by our lab. As an extension of this work, we examined whether the consequent accumulated cFn would itself also be toxic to hepatocytes[138]. We found that elevated concentrations of cFn induced a significant increase in caspase-3 activity, a marker of apoptosis (programmed cell-death), in hepatocytes from alcohol-fed animals after a 20-h incubation.

It was also observed, in these same cells, a corresponding increase in the secretion of TNF-α and IL-6. This treatment with elevated, pathology-associated, concentrations of soluble cFn also stimulated cultured hepatocytes from both control and ethanol-fed animals to secrete significantly higher amounts of MMP-2. As previously mentioned, the activity of MMPs is dependent upon their balanced relationship with corresponding TIMPs. We also found that in the presence of elevated levels of cFn, cultured HCs from ethanol-fed animals secreted more TIMP-2 protein than their control and untreated counterparts. These cells, much like the KCs from ethanol fed animals, also release TIMP-2 in excess of MMP-2. Again, this disparity in the relative levels of these proteins would lead to an inhibition of MMP-2 activity which contributes to a reduction in matrix protein degradation and the subsequent build-up of the ECM.

The response by the hepatocytes to treatment with high concentrations of cFn was, however, not particularly robust relative to KCs, suggesting that the secretion of these factors may have a more localized purpose. For example, TNF-α, in particular, has been implicated in both the inflammatory and apoptotic responses of cells[146,147]. It is thus plausible that the observed increase in both the release of IL-6, and in the activity of caspase-3, may be attributed

to autocrine TNF-α signaling after cFn induction. However, as demonstrated by other ECM molecules, cFn may also influence cell death directly[148,149]. Moreover, MMP-2 and TIMP-2 produced by these hepatocytes in response to cFn treatment may be involved in the immediate degradation of the surrounding matrix, producing reactive fragments of cFn which would have only a localized effect on hepatocyte behavior[150]. It is plausible that the hepatocyte response to excess cFn affects hepatocytes alone and is not to be included as part of the collective signaling pool. Thus these findings also suggest that hepatocytes may be more involved in reinforcing their own demise during a condition of alcohol-induced injury than previously assumed (Figure 3). The specific mechanisms underlying these observed responses will, however, require further examination.

This seemingly unconventional response observed in hepatocytes could be explained by recent reports that suggest that these cells possess an inherent plasticity that makes them more susceptible to changes in the tissue microenvironment that could compromise pure epithelial character[151,152]. This plasticity is essential to the regenerative capacity of the liver that allows for recovery from sustained damage, which is an inevitable consequence of its function as a detoxifying organ. However, this poses a unique challenge for the researcher studying a specific aspect of hepatocyte behavior, as current isolation procedures and culture techniques can also alter the character of these cells. These findings should be further explored using new techniques involving 3D cultures and liver slices that are more representative of *in vivo* conditions[153,154].

CONCLUSION

Under normal conditions, cFn is an ostensibly innocuous and minor component of the ECM that is produced locally by tissues where it concentrates in the pericellular matrix that surrounds cells. It debuts as a critical factor for embryonic development, after which its levels greatly diminish, and are only re-established in adult tissue during events that involve regeneration or repair. However, its elevated presence has also been associated with various chronic disorders that are characterized by extensive tissue damage. These conditions create doubts as to whether cFn is truly a mediator of healing processes or an instigator of disrepair.

In healthy tissue, where physiological processes are appropriately regulated, cFn production is stimulated in response to signs of injury. Its upregulation provokes extreme activity that is essential for the repair of damaged tissue. Once tissue integrity has been restored cFn production is reduced to homeostatic levels and normal cFn turnover is restored. However, during a condition of relentless attack by agents that compromise tissue function, the regulatory mechanisms that restrict cFn activity become impaired. A condition of unbridled 'wound healing' develops that actually causes more tissue damage rather than repair.

In the liver, chronic metabolism of alcohol leads to the build-up of harmful byproducts which cause the increased production of cFn by hepatic SECs, as well as its impaired clearance by the hepatocyte specific ASGP-R. Although the physiological relevance of the resulting accumulation of cFn in the liver parenchyma remains debatable, evidence suggests cFn is not a static component of the hepatic scaffold, but a dynamic mediator of cellular events that may promote the progression of liver damage associated with chronic alcohol abuse. This reactive glycoprotein stimulates specific cells in the liver to release pro-inflammatory and pro-fibrogenic factors which create further tissue damage and disrepair that lead to alcoholic fibrogenesis (Figure 3).

Despite the medical community's initiatives to educate and intervene, alcohol consumption rates world-wide continue to rise and with it the development of alcoholic liver disease with often fatal outcome. Currently, there are no effective measures to counter this epidemic. Therefore, the effects and underlying mechanisms of cFn-induced cell behavior in the alcohol-injured liver are worth further characterizing, not only to gain a more comprehensive understanding of the role this glycoprotein plays in the progression of alcohol-induced liver injury but also for any insight such investigation could provide towards the development of desperately needed novel therapies for alcoholic liver disease.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2500

World J Gastroenterol 2011 May 28; 17(20): 2500-2506
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Mechanisms of alcohol-mediated hepatotoxicity in human-immunodeficiency-virus-infected patients

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Received: January 21,2011 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: May 28, 2011

Abstract

Clinical observations have demonstrated that excessive chronic alcohol use negatively affects human immunodeficiency virus (HIV) infection and contributes to the liver manifestations of the disease, even in HIV mono-infection. HIV/hepatitis C virus (HCV) co-infection is associated with increased progression of HVC liver disease compared to HCV infection alone, and both of these are negatively affected by alcohol use. Recent data suggest that alcohol use and HIV infection have common targets that contribute to progression of liver disease. Both HIV infection and chronic alcohol use are associated with increased gut permeability and elevated plasma levels of lipopolysaccharide; a central activator of inflammatory responses. Both alcoholic liver disease and HIV infection result in non-specific activation of innate immunity, proinflammatory cytokine cascade upregulation, as well as impaired antigen presenting cell and dendritic cell functions. Finally, alcohol, HIV and antiretroviral therapy affect hepatocyte functions, which contributes to liver damage. The common targets of alcohol and HIV infection in liver disease are discussed in this mini-review.

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Key words: Hepatitis C virus; Hepatitis B virus; Liver; Intestine; Inflammation

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Szabo G, Zakhari S. Mechanisms of alcohol-mediated hepatotoxicity in human-immunodeficiency-virus-infected patients. *World J Gastroenterol* 2011; 17(20): 2500-2506 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2500.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2500

INTRODUCTION

The life expectancy of patients infected with human immunodeficiency virus (HIV) has been prolonged, therefore, liver diseases have assumed far greater importance as a cause of morbidity and mortality in these patients. Antiretroviral therapy (ART) used by patients who have HIV infection is often hepatotoxic. In addition, patients who have HIV often are co-infected with hepatotropic viruses such as hepatitis B and C viruses; factors that are damaging to the liver. Furthermore, chronic alcohol consumption causes injury to the liver and may result in more rapid progression to cirrhosis, end-stage liver disease, and hepatocellular carcinoma in co-infected patients. This review addresses the interactions between these factors that result in furthering liver damage.

HIV, VIRAL HEPATITIS AND ALCOHOL USE - EPIDEMIOLOGY AND NATURAL HISTORY OF LIVER DISEASE

The natural history of HIV infection has drastically changed since the discovery of its clinical manifestation, AIDS in advanced disease[1-5]. HIV affects about 40 million people worldwide, with most of the infections in developing countries. As a blood-borne pathogen, HIV infection is transmitted *via* blood, sexual contact, and maternal-newborn transfer. HIV primarily infects immune cells and it replicates in T lymphocytes and monocytes[4-7]. HIV mono-infection results in minimal hepatitis, however, co-infection with hepatitis virus results in increased liver damage. Hepatitis B virus (HBV) infection affects 370 million people and it is estimated that an additional 170 million suffer from hepatitis C virus (HCV) infection[8-13]. Both HBV and HCV can lead to chronic hepatitis and as a result, progressive liver inflammation and fibrosis lead to cirrhosis and liver failure. An additional insult to the liver is excessive alcohol consumption that is an ongoing social and medical problem in people with and without HIV infection.

Several studies have found significant alcohol problems in HIV-infected individuals. In a primary care setting, physicians have reported that alcohol consumption is a common habit in patients initiating care for HIV disease[14-16]. Heavy drinking among people with HIV infection under medical care has been found to be almost double that of the general population[17]. It has also been shown that heavy alcohol consumption has a negative impact on CD4 cell count in HIV-infected persons who are not receiving ART[18].

The interaction of HIV and HCV infection has received increasing attention in recent years. It has been well documented that liver disease progression is accelerated in HIV/HCV co-infected patients compared with HIV or even HCV mono-infection[11,13,19]. Furthermore, chronic heavy alcohol consumption acts as an additional insult in all of these forms of chronic hepatitis. With the new era of highly active antiretroviral therapy (HAART) that provides sufficient control of HIV replication, it has become evident that, in HIV/HCV co-infected patients, progression of HCV infection causes more lethality than HIV infection itself[11,13,19].

Excessive alcohol consumption is associated with fatty liver, and if persistent, it can lead to alcoholic steatohepatitis, liver fibrosis and cirrhosis[20]. Clinical studies have demonstrated that excessive alcohol consumption in individuals with comorbid conditions such as chronic HCV or HBV infection accelerates liver damage and progression to liver cirrhosis. In a study of a large cohort of HIV-positive and negative US veterans, investigators have found a trend toward increased liver injury manifested as inflammation and fibrosis, in patients with hazardous or binge drinking[21]. The same study has shown that alcohol abuse and dependence significantly increase the risk of advanced fibrosis and cirrhosis in HIV-mono-infected patients, as well as in those with HIV/HCV co-infection[21]. A cross-sectional study has investigated the impact of alcohol on liver fibrosis using aspartate aminotransferase (AST)-to-platelet ratio index (APRI) as a measure of liver function, and has found significant liver disease with an APRI > 1.5. This study also has found that hazardous alcohol drinking is an independent, modifiable risk factor for fibrosis[21], and the same investigators have concluded that the problem of alcohol abuse is not adequately addressed by health care providers in patients with HIV positivity[21].

In HIV-infected patients, 17% of death was related to end-stage liver disease; of those, 75% of patients had HIV/HCV co-infection and 48% heavy alcohol use history[22]. Alcohol use in HIV-infected patients was also shown to be associated with decreased adherence to ART, and not surprisingly, reduced HIV suppression[23].

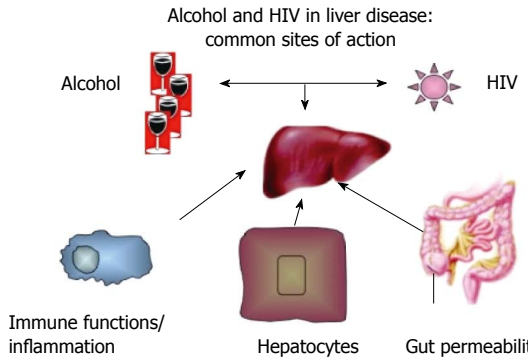

Figure 1 Alcohol and human immunodeficiency virus in liver disease. There are multiple common sites of action of alcohol and human immunodeficiency virus (HIV) infection that contribute to the development of liver disease. These include modification of immune functions and inflammatory responses, functions of hepatocytes and intestinal permeability.

ALCOHOL AND HIV: COMMON TARGETS IN LIVER DISEASE

Although the pathological mechanisms of alcoholic liver disease are relatively well explored[24-26], the mechanisms by which HIV infection affects the liver remain somewhat elusive. Based on current knowledge, several elements in the pathological mechanisms of alcohol-induced liver disease can by exacerbated by HIV infection, which results in the potentiation of the individual negative effects. These major categories of mutual actions and modulating effects of both alcohol and HIV infection are on gut permeability and gut-liver homeostasis, immune functions and inflammation, and alterations in hepatocyte functions and survival (Figure 1).

Gut permeability and the role of lipopolysaccharide in HIV infection and alcoholic liver disease

The important role of gut-derived lipopolysaccharide (LPS) has been demonstrated in alcoholic liver disease in humans, as well as in animal models of chronic alcohol administration[27,28]. Gut-derived LPS has been shown to have a causative role in alcoholic liver injury as demonstrated by experiments in which gut sterilization with antibiotics prevented alcohol-induced liver damage[29]. This

was associated with increased intestinal permeability in rats[30]. Alcohol feeding in rats and mice increases serum endotoxin levels after 1-2 wk, and these increases remain after continued alcohol administration[31]. Studies in mice as well as *in vitro* have demonstrated that alcohol damages the intestinal epithelial barrier by downregulating tight junction proteins[32-34]. The importance of the gut-liver axis has been implicated in alcoholic liver diseases and in other etiologies of liver injury[27,28].

The novel finding, that HIV infection is associated with increased plasma endotoxin levels was first reported by Douek's group in 2006[35]. Plasma LPS was significantly increased in patients with chronic HIV infection, as well as in patients with AIDS compared to uninfected controls or to those with acute infection[35]. LPS in the serum is bound to lipoprotein binding protein (LBP) and soluble CD14 (sCD14) that modulate the biological availability of LPS. The same authors have found increased plasma sCD14 and LBP levels even in acute HIV infection, as well as in those with progressive HIV infection[35]. Subsequent studies have evaluated the effect of ART on plasma LPS levels and have found that patients with ART have a moderate decrease in plasma LPS levels[36]. This is also associated with an improvement in plasma tumor necrosis factor (TNF)α levels, which suggests attenuation of inflammatory cell activation[36-38]. The source of the increased plasma LPS in HIV infection is presumably the gut. It has been speculated that HIV infection in the intestinal epithelial and immune environment permits increased translocation of gut microbiota and their components such as LPS[39].

Increased serum LPS levels have been noted in patients with chronic HCV infection, even in the absence of advanced liver disease[40]. Furthermore, in patients with HCV and HIV co-infection, serum LPS levels are higher compared to those in patients with HIV infection alone[41].

The biological significance of the elevated serum or plasma LPS in HIV infection is yet to be fully understood. It is generally accepted that chronic LPS exposure of immune and inflammatory cells results in a non-specific inflammation that may "reset" thresholds for specific immune responses[42]. It is tempting to speculate that constant and repeated exposure of innate immune cells, particularly tissue macrophages (Kupffer cells) in the liver from the gut-derived LPS in the portal blood, could result in loss of LPS tolerance, which predisposes to increased inflammatory responses[43]. We have found loss of Toll-like receptor (TLR) tolerance in circulating monocytes of HCV-infected patients, and this is associated with increased production of proinflammatory signals including TNFα[40].

In alcoholic liver disease, there is a large body of evidence in support of increased sensitivity of Kupffer cells to LPS and proinflammatory cytokine activation[44,45] (Figure 2). Early studies have demonstrated that elimination of Kupffer cells with gadolinium chloride in rats ameliorates alcohol-induced liver damage and that LPS/CD14 receptor signaling is involved[44]. Recently, we have shown that deficiency in TLR4, the receptor for LPS, or deficiency in the TLR4 downstream signaling by interferon regulatory factor 3, are crucial for development of alcohol-induced liver damage[31,46]. The combined effect of alcohol and HIV infection on LPS signaling in the liver awaits further investigation.

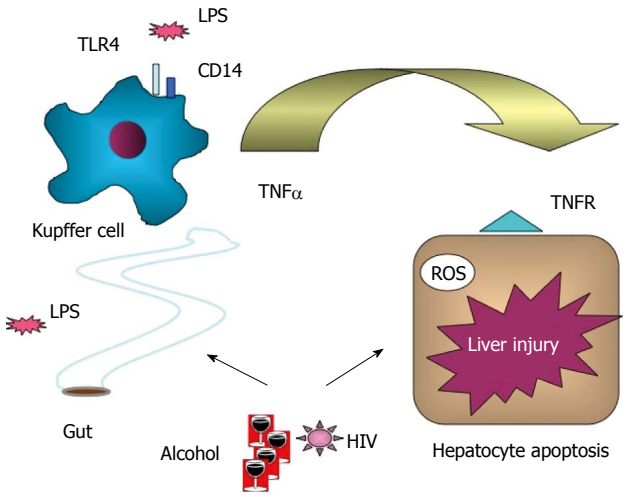

Figure 2 Alcohol-induced hepatocyte damage is amplified by human immunodeficiency virus infection. The current model of alcohol-induced liver disease includes increased gut-derived lipopolysaccharide (LPS) entry into the liver where Kupffer cells are activated *via* LPS/Toll-like receptor (TLR) 4 and produce tumor necrosis factor (TNF)α. Human immunodeficiency virus (HIV) infection also increases gut-derived LPS levels in the circulating blood. Increased production of TNFα will act on alcohol-exposed hepatocytes to induce apoptosis. ROS: Reactive oxygen species.

Inflammation and immune functions

Activation of the inflammatory cascade and increased proinflammatory cytokine production is a hallmark of both alcoholic steatohepatitis and chronic HIV infection[26,45]. The immunological consequences of such proinflammatory cascade activation are not fully understood. The clinical observations of increased susceptibility to infections in alcoholic steatohepatitis and chronic HIV infection demonstrate that, although non-specific proinflammatory activation is present, pathogen-specific innate immune responses are defective. Furthermore, antigen-specific immune responses as well as antigen presenting function of monocytes and dendritic cells are severely dampened by alcohol exposure, as well as in chronic HIV infection[47-49]. Our previous studies have demonstrated that acute alcohol consumption in humans inhibits the antigen presenting function in human monocyte-derived dendritic cells and monocytes *via* mechanisms that involve decreased interleukin (IL)-12 and co-stimulatory molecule expression and/or impaired dendritic cell differentiation/maturation[48-50]. The effects of prolonged alcohol also inhibit myeloid dendritic cell functions in humans as well as mouse models[51].

Defects in myeloid and plasmacytoid dendritic cell function have been extensively studied in HIV infection. It has been shown that HIV infection results in impaired differentiation and maturation of monocyte-derived dendritic cells in humans and that these dendritic cells have impaired antigen presentation capacity. Chronic HCV infection is also associated with decreased antigen presenting function of dendritic cells and HIV and HCV infection affect im-

Table 1 Common mechanisms of liver/hepatocyte damage

Effect	HIV	HCV	Alcohol	HAART
Increase ROS	√	√	√	
Increase proinflammatory cytokines	√	√	√	
Oxidative stress	√	√	√	√
Lipid peroxidation	√	√	√	
Mitochondrial damage	√	√	√	√
Steatosis	√	√	√	√
Glutathione depletion	√		√	√
Proteasome dysfunction			√	√

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; HAART: Highly active antiretroviral therapy; ROS: Reactive oxygen species.

mune responses[52,53]. Although the combined effects of alcohol exposure and HIV infection are yet to be evaluated on dendritic cell functions, we have previously demonstrated that alcohol treatment has an additive inhibitory effect on the already dampened accessory cell function in dendritic cells of HCV-infected patients[54]. This is associated with reduced expression of the T cell co-stimulatory molecules, CD80 and CD86, low IL-12 and increased IL-10 production[54]. The role of dendritic cells in HIV/HCV co-infection in relation to the liver disease needs further exploration. However, the importance of dendritic cells in liver tolerance and inflammation is emerging[42,43].

Hepatocyte function and cell death

There are multiple mechanisms by which alcohol, HIV infection and even ART could directly affect hepatocytes. As summarized in Table 1, reactive oxygen radical production, mitochondrial damage and steatosis can all be induced independently by alcohol, HIV or HCV infections alone or in combination with HAART[55]. However, these factors together will most likely cause combined damage in exposed hepatocytes. Some of the mechanisms involved include depletion of the intracellular glutathione stores by chronic alcohol and HIV infection that predisposes to increased reactive oxygen species (ROS) production and increased susceptibility to hepatocyte death[55]. In addition, alcohol, HCV, and HAART cause mitochondrial damage, fatty liver, and increased levels of proinflammatory cytokines, particularly, TNFα, which results in hepatocyte death. This mechanism has been investigated in alcoholic liver damage as well as in HIV infection, and HIV/HCV co-infection[55].

Although the introduction of HAART for HIV infection has resulted in a significant decrease in the mortality rate of AIDS, a sizable proportion of patients receiving HAART develop liver toxicity. For example, patients treated with the antiretroviral nucleoside reverse-transcriptase inhibitors (NRTIs) zidovudine, zalcitabine, didanosine and stavudine have been reported to develop hepatic steatosis[56,57]. Heavy alcohol consumption by HIV patients on HAART results in liver decompensation in about 2% of cases[58], and increases the risk of liver injury 5.8 times in 10% of patients receiving NRTI or non-NRTI regimens[55]. The enhanced liver toxicity due to alcohol, HCV, and HAART could result from shared mechanisms such as fat accumulation and mitochondrial damage.

Fat accumulation and insulin resistance

Alcohol and HCV-induced fatty liver is well documented. Several mechanisms have been reported for alcohol-induced fatty liver including increased hepatic uptake of fatty acids and *de novo* lipogenesis, impaired peroxisome proliferator-activated receptor α signaling, reduced mitochondrial fatty acid oxidation and reduced secretion of triglycerides[59-61]. These effects could be attributed to reduced adiponectin secretion by the adipose tissue and elevated expression of TNFα[62-64].

Some AIDS patients develop HAART-associated lipodystrophy (LD). A study by Sutinen *et al*[65] has shown that, in comparison to HIV-negative subjects, HAART+LD patients show: (1) higher liver fat content (regardless of alcohol consumption); (2) for a given amount of liver fat, serum-free insulin concentration correlated with liver fat, which suggests that fat accumulation in the liver is crucial for development of insulin resistance; and (3) significantly lower leptin concentrations than the other two groups. Others have also demonstrated low plasma leptin concentrations in patients with HAART-associated LD[66]. Hypoleptinemia and subsequent insulin resistance could favor fatty liver accumulation in patients suffering from NRTI-induced lipoatrophy[67-69].

Although Sutinen and co-workers did not observe lactic acidosis, others have reported lactic acidosis and hepatic steatosis in some patients using HAART, which was attributed to mitochondrial toxicity induced by nucleoside analogs[70,71]. In addition, co-infection with HCV increases the risk for severe liver damage during HAART[72], and end-stage liver disease is the primary cause of death in HIV/HCV co-infected patients under HAART[73,74]. Seth[75] has reported that, in HIV co-infected patients, the HCV load is higher by an average of 0.5-1.0 log than the mono-infected patients due to immune depression. Studies on liver fibrosis show conflicting results. Although some studies have found that early HAART in HIV/HCV co-infected patients may slow liver fibrosis progression[76], others have found that HAART regimens including nevirapine accelerate liver fibrosis progression in HIV/HCV co-infected patients[77].

Mitochondrial damage

Alcohol and HCV can induce mitochondrial DNA (mtDNA) damage through the production of ROS and/or reactive metabolites. It has been reported that prolonged administration of antiretroviral NRTIs increases the risk of mitochondrial damage in the liver[78]. NRTIs can cause the accumulation of the oxidized base 8-hydroxydeoxyguanosine (8-OH-dG) in liver mtDNA[79,80]. In addition, NRTIs can cause mtDNA point mutations in some patients, which may result from interaction with mtDNA replication (*via* misreading of 8-OH-dG by DNA polymerase c) and/or impairment of polymerase c repair capacity[81]. It has been reported that NRTIs inhibit mtDNA replication by

undergoing phosphorylation as the cognate endogenous nucleosides, which are subsequently incorporated within the mitochondrial genome by DNA polymerase c[81].

CONCLUSION

Although increasing evidence suggests enhanced combined effects of alcohol and HIV infection on the liver with and without viral hepatitis, a number of questions remain unanswered. What are the interactions between alcohol use, HIV, HCV and HBV infections? Is the crosstalk between organs, such as the homeostasis between the liver and gut, affected by combined insults of alcohol, HIV and viral hepatitis? What is the effect of the cumulative effects of alcohol and HIV infection on cross-regulation between various cell types in the liver? How can new therapeutic targets be developed in the light of knowledge about the interactive effects of HIV and alcohol on the liver? All of these questions are pertinent to the status and defects observed in immune functions, gut permeability, Kupffer cell activation, hepatocyte function and liver fibrosis/stellate cell function in patients with HIV infection and excessive alcohol use and/or viral hepatitis.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2507

World J Gastroenterol 2011 May 28; 17(20): 2507-2514
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Involvement of autophagy in alcoholic liver injury and hepatitis C pathogenesis

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Author contributions: All authors contributed equally to this manuscript; Osna NA and Donohue TM Jr reviewed the current literature; Thomes PG provided much of the preliminary data on alcohol and autophagy mentioned in the text.

Supported by NIAAA, R21AA017232 and Dean's Reviewed Research Grant of the University of Nebraska Medical Center

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Received: January 7, 2011 Revised: March 23, 2011
Accepted: March 30, 2011
Published online: May 28, 2011

Abstract

This review describes the principal pathways of macroautophagy (i.e. autophagy), microautophagy and chaperone-mediated autophagy as they are currently known to occur in mammalian cells. Because of its crucial role as an accessory digestive organ, the liver has a particularly robust autophagic activity that is sensitive to changes in plasma and dietary components. Ethanol consumption causes major changes in hepatic protein and lipid metabolism and both are regulated by autophagy, which is significantly affected by hepatic ethanol metabolism. Ethanol exposure enhances autophagosome formation in liver cells, but suppresses lysosome function. Excessive ethanol consumption synergizes with hepatitis C virus (HCV) to exacerbate liver injury, as alcohol-consuming HCV patients frequently have a longer course of infection and more severe manifestations of chronic hepatitis than abstinent HCV patients. Alcohol-elicited exacerbation of HCV infection pathogenesis is related to modulation by ethanol metabolism of HCV replication. Additionally, as part of this mechanism, autophagic proteins have been shown to regulate viral (HCV) replication and their intracellular accumulation. Because ethanol induces autophagosome expression, enhanced levels of autophagic proteins may enhance HCV infectivity in liver cells of alcoholics and heavy drinkers.

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Key words: Autophagy; Iysosome; Autophagosome; Hepatitis C virus; Hepatitis C virus replication cycle; Ethanol

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Osna NA, Thomes PG, Donohue TM Jr. Involvement of autophagy in alcoholic liver injury and hepatitis C pathogenesis. *World J Gastroenterol* 2011; 17(20): 2507-2514 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2507.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2507

INTRODUCTION

Alcohol is known to exacerbate the pathogenesis of hepatitis C virus (HCV) infection and makes chronic hepatitis C patients less sensitive to antiviral treatment. Recent investigations indicate that autophagy is involved in the regulation of HCV replication and infectivity. Here, we provide basic information about the role of autophagy in liver and how ethanol modifies the autophagic response in liver cells. In addition, to underline a link between autophagy and viral replication, we review the data about HCV structure, replication cycle and autophagy in HCV-infected liver cells. Since alcohol consumption exacerbates HCV pathogenesis, we propose possible mechanisms that lead to liver failure in HCV-infected patients who drink

excessively, which may in part be regulated *via* autophagy-mediated accumulation of HCV in liver cells.

AUTOPHAGIC MACROMOLECULAR DIGESTION IN THE LIVER

Lysosomes and autophagy

In most tissues, the majority of intracellular proteins are degraded by the proteasome, an integral component of the ubiquitin-proteasome system (UPS)[1]. In tissues such as liver that respond quickly to the composition of the plasma, the lysosome has a large part in degrading extracellular proteins (e.g. obsolete plasma proteins) brought by endocytosis into the hepatocyte. Lysosomes also degrade the remaining fraction of intracellular proteins that are not proteasome substrates and break down complex lipids (e.g. triglycerides), complex carbohydrates (e.g. glycogen) and nucleic acids. All the latter hydrolytic steps are done by autophagy, which literally means "self eating"[2]. While autophagy always terminates by degrading macromolecular substrates in lysosomes, it can begin *via* one of three distinct cellular pathways. The most well-characterized process by which autophagy is defined is macroautophagy, the vacuolar sequestration by endoplasmic reticulum (ER) membranes of bulk portions of cytoplasm, forming a double membrane-enclosed body known as an autophagosome or autophagic vacuole (AV). The nascent AV, which contains both soluble constituents and particulate organelles, fuses with existing lysosomes to form a hydrolytic organelle called an autolysosome. Here, the sequestered substrates are hydrolyzed and the degradation products are released. Microautophagy, which is less well characterized than macroautophagy, is the uptake of smaller cytoplasmic particles by the lysosomal membrane followed by their degradation in the lysosome interior. Chaperone-mediated autophagy (CMA) is the uptake and degradation by lysosomes of single protein substrates that bear a KFERQ peptide motif. The latter is recognized by soluble chaperonins of which the most prominent is the heat shock constitutive protein 70 (HSC 70), which binds to and "directs" the protein substrate to lysosome-associated membrane proteins (LAMP). Most significant is LAMP-2A, which recognizes substrate chaperonin complexes and facilitates entry of the protein substrate into the lysosomal matrix for degradation. CMA was formerly considered a minor autophagic pathway, but recent findings indicate its heightened importance in liver cell maintenance and hepatic performance. CMA activity declines with age due to a gradual loss of LAMP-2A on the lysosome surface. Artificial restoration of this protein in aged LAMP-2A transgenic mice significantly improves liver function[3]. CMA also has a compensatory function when macroautophagy in liver cells declines after artificial knockdown of macroautophagy gene products[4].

Autophagy is important for liver cell survival, particularly in times of nutrient deprivation, when autophagy swiftly responds to fasting. The short half-life of autophagosomes (< 10 min)[5] indicates that the protein components required for their formation are readily available for recruitment. Like the proteasome, autophagy has a key housekeeping and backup function as it degrades misfolded, aggregated proteins that are resistant to proteasome-catalyzed degradation[6]. The importance of removing such aggregated proteins is clearly illustrated by the association of aggregate accumulation with disease pathogenesis. Patients with α-1-antitrypsin (A-1AT) deficiency are more susceptible to cirrhosis and liver cancer because of a propensity of the mutated, unsecreted A-1AT protein to aggregate within hepatocytes[7].

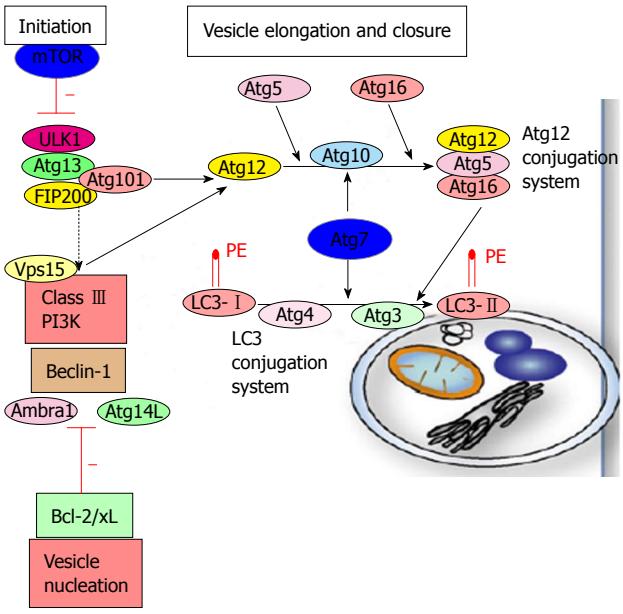

Figure 1 Molecular aspects of autophagosome formation. Reproduced with permission from[11].

Mechanism of autophagy

Identification of the major autophagy genes in yeast[7-10] designated "Atg" has contributed significantly to our understanding of how autophagy proceeds in mammals. In all eukaryotic cells, nutrient deprivation is an inducer of autophagy. The mechanism of autophagy is described below in three steps and depicted in Figure 1.

Inititation of autophagosome formation: Inititation of autophagosome formation is regulated by the class Ⅲ phosphoinositol-3-kinase (PI-3K) which triggers an upstream signaling cascade to inhibit the activity of the mammalian target of rapamycin (mTOR), a major anabolic kinase and a potent suppressor of autophagy. Inhibition of mTOR triggers the autophagic response by activating Atg proteins. In mammals, a complex is formed among the serine/threonine-protein kinase, ULK1 (a homolog of the yeast Atg1), the focal adhesion kinase family interacting protein 200, (FIP200), Atg17-like protein and Atg13, to begin the process of AV nucleation[11]. Autophagosome (autophagic vacuole) formation begins when Beclin-1 (Atg6) forms a complex with Atg14. This subsequently

brings about formation of a double membrane vesicle that is likely derived from the ER[12]. Coordinated complexes of Atg proteins catalyze vacuole (vesicle) elongation, notably the Atg12 ubiquitin-like conjugation system, consisting of Atg 5, 12 and 16, catalyze cleavage of Atg8, the microtubule light chain-3 (LC3I), and its conjugation to phosphatidylethanolamine (PE) to form the autophagic marker, LC3 Ⅱ. The latter subsequently localizes to the autophagosome membrane, while its unlipidated precursor, LC3I remains in the cytoplasm (Figure 1).

Docking and fusion of cargo-filled autophagosomes: Docking and fusion of cargo-filled autophagosomes with lysosomes follows autophagosome formation to form the hydrolytic autolysosome. Fusion is believed to be highly dependent upon the lysosomal membrane protein, LAMP-2.

Breakdown of the autolysosome contents occurs: The mechanism that triggers this hydrolysis is incompletely understood but it is likely that the collective action of lysosomal hydrolases brings about autolysosome dissolution.

Lysosomal proteolysis during ethanol consumption: Liver enlargement (hepatomegaly) is common in alcoholics and in alcohol-fed laboratory animals. The rise in hepatic protein and fat each accounts for half the net increase in liver mass[13]. The net protein gain very likely contributes to the more severe alcohol-induced liver pathologies, because some of the accumulated proteins are damaged by oxidants generated from ethanol metabolism, from mitochondrial leakage and from secondary reactions that enhance production of reactive species[14,15]. In early studies, we showed that ethanol-induced protein accumulation reflects slower degradation of long-lived proteins, which are generally degraded in lysosomes[16]. We later confirmed that ethanol feeding diminishes the proteolytic capacity of liver lysosomes[17]. This is due to a reduced capacity for their acidification[18] and lower contents of cathepsins B and L[19]. The latter deficiency results from disruption of cathepsin precursor trafficking to lysosomes[20], owing to declines in the ligand-binding activity, content, and synthesis rate of the mannose-6-phosphate receptor[21,22]. This protein recognizes and binds cathepsin precursors for placement into the lysosomal compartment[23].

Because lysosomes degrade the contents of autophagic vacuoles, we considered it likely that ethanol suppresses the initial stages of autophagy. Others demonstrated that livers of ethanol-fed rats exhibit volume densities of autophagosomes and autolysosomes substantially lower than controls[24]. Other lines of evidence support autophagic suppression, including ethanol-induced down-regulation of AMP kinase, a catabolic regulator[25] and ethanol-elicited disruption of cytoskeletal proteins[26] that are essential for delivery of AV cargo to lysosomes.

Ethanol effects on intracellular autophagosome content in vitro and ex vivo

Using the marker, LC3-Ⅱ we quantified autophagosomes in recombinant HepG2 (VL-17A) cells which metabolize ethanol *via* ADH and CYP2E1. Parental HepG2 cells express neither enzyme[27]. Contrary to our expectations, exposure of VL17A cells to 25 or 100 mmol/L ethanol for 12 to 72 h enhanced autophagosome content over that of untreated cells. AV elevation by ethanol was blocked by simultaneous exposure to 4-methyl-pyrazole (4MP), an inhibitor of ethanol oxidation. Furthermore, exposure to ethanol of non-metabolizing HepG2 cells showed no differences from untreated controls in autophagosome content, to suggest that the initiation of autophagy depends on the generation of ethanol metabolites[28]. These results are consistent with those reported recently[29]. Interestingly, they contrast with another report[30] demonstrating that in CYP2E1-expressing E-47 (HepG2 recombinant) cells, ethanol exposure enhances steatosis but only slightly increases autophagy by 50%. In non-metabolizing C34 cells (similar to HepG2 cells) ethanol exposure causes a four-fold rise in autophagy but only a slight elevation of steatosis. The apparent disparity may be explained by differences in the duration of ethanol exposure (12 to 72 h reported here, compared with 120 h in the Wu *et al*[30] study) and the high degree of cellular steatosis, which is reciprocally regulated with autophagy in liver cells[31].

We further tested the autophagic response in control and ethanol-exposed precision-cut rat liver slices (PCLS)[32]. LC3 Ⅱ levels in PCLS exposed to 50 mmol/L ethanol were increased two-fold over controls after 24 h of incubation. Simultaneous exposure to 4MP blocked this response[28].

***In vivo* studies:** After acute ethanol administration to mice, we observed a 35% enhancement in autophagosome content. This coincided with a decline in reduced glutathione (GSH) and a significant elevation in hepatic lipid peroxides, both indicators of oxidant stress. Chronic ethanol feeding to transgenic LC3-green fluorescent protein (GFP) mice caused a five-fold increase over controls of fluorescent puncta, indicating enhanced autophagosome formation. It is noteworthy that ethanol-fed mice in this study exhibited hepatomegaly and elevated liver protein over pair-fed controls[28] consistent with our previous findings in rats[16,33].

Summary

Our results are consistent with those recently published[29] but are inconsistent with our hypothesis that ethanol suppresses the autophagic pathway. An obvious paradox emerges from these recent findings in view of our previous work, showing slower protein degradation in livers of ethanol-fed animals because of a disruption of lysosome function and biogenesis[17-22]. An explanation for this disparity is that our recent investigations focused on autophagosome formation while our earlier studies emphasized lysosome function. We now postulate that ethanol metabolism enhances autophagosome formation while it disrupts the distal, degradative step of autolysosome formation. Previous work in alcohol/endotoxin-induced

pancreatitis, revealed that LAMP-2 protein is severely reduced in pancreata of ethanol-fed/endotoxin-treated rats. This alteration reduces autolysosome formation and subsequent degradation of autophagosome cargo[34]. We postulate that this also occurs in liver during ethanol metabolism. Finally, we do not know the exact mechanism by which ethanol metabolism induces autophagosome content but we surmise that acetaldehyde, the primary oxidation product of ethanol metabolism and/or other reactive species generated by reactions secondary to ethanol metabolism, is/are probably responsible for this activation. Further, chronic alcohol consumption causes oxidant stress, which enhances the unfolded protein response (UPR) in the ER[35]. Because AV membranes are derived from this organelle[12], ER stress could enhance the formation of autophagosomes during ethanol oxidation.

HCV, ETHANOL AND AUTOPHAGY

Ethanol-induced oxidative stress and HCV infection

HCV is a well-established second hit, which drives the progression of alcoholic liver disease. Worldwide, 170 million people (about 2.2% of the world's population) are infected with HCV, and in about 80% of cases, infection persists for many years. Chronic viral hepatitis provides the potential risk for cirrhotic liver disease and life-threatening complications of portal hypertension and hepatocellular carcinoma[36]. The ability of HCV proteins to induce oxidative stress *via* generation of reactive oxygen species (ROS) by mitochondrial electron transport complex I plays an important role in HCV infection pathogenesis. Therefore, ROS release results in decreased mitochondrial GSH and mitochondrial depolarization, which can be augmented by simultaneous ER oxidative stress[37-39]. Cytochrome P450 (including CYP2E1) is involved in evolution of hepatitis to HCV-associated hepatocarcinoma[40]. Furthermore, alcohol abuse associated with CYP2E1 activation strongly accelerates the progression of HCV infection by increasing fibrosis as well as the risk of death from cirrhosis in HCV patients. It is believed that immunosuppressive effects of alcohol impair viral clearance[41,42]. The main mechanisms of HCV infection development, including immune dysfunction, apoptosis, steatosis and hepatic iron overload, can be triggered by heavy alcohol consumption in HCV patients[43,44].

Hepatitis C virus and viral proteins

HCV, an enveloped virus of Flaviviridae family, has a positive-sense and single-stranded RNA. Its genome is 9.6 kb, encodes a polyprotein of 3010 amino acids and consists of a single open reading frame (ORF) flanked by untranslated regions[45]. The NH_2-terminal part of the polyprotein includes three structural proteins: core (capsid protein) and two envelope proteins, E1 and E2[46]. The polyprotein is processed by cellular and viral proteases into distinct structural and non-structural viral proteins. The COOH-terminal portion of the polyprotein consists of the non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) that form the replication complex for synthesis of viral RNA and regulate virion assembly[47-49]. The release of structural proteins from the nascent polyprotein is catalyzed by cellular proteases, while NS proteins are produced by viral proteases[50].

The HCV replication cycle

To initiate the viral life cycle, HCV binds to specific cell receptors on the hepatocyte surface and is internalized by clathrin-dependent endocytosis. Although the exact mechanism of HCV cell entry is unknown, it is believed to be a multi-step process involving at least the tetraspanin CD81[51], tight junction molecules, claudin 1 and occludin 1, as well as scavenger receptor SR-B1[52,53]. Since extracellular HCV also circulates bound to lipids and very-low-density lipoproteins (VLDLs)[54], the low density lipoprotein receptor may have a role in facilitating viral attachment. Initial capture of HCV particles by lipoprotein receptors is followed by interactions with SR-B1, CD81 and tight junction receptors leading to uptake and intracellular penetration of HCV *via* low-pH endosomes[55]. Most receptors for HCV entry are species specific, which prevents infection of mouse tissue with human virus, thereby limiting or preventing cross-species infections. After attachment to the receptors and penetration inside the cells, HCV viral and cellular membranes are fused at the acidic compartment and an HCV RNA is released into cytoplasm. Viral RNA replication requires the formation of a complex contacting NS proteins, replicating RNA and cellular membranes, and an enveloped HCV virion carrying a newly-synthesized viral genome is formed by budding into the ER lumen. After assembly of the viral particle on lipid droplets (LDs), HCV leaves the cells through a secretory (VLDL) pathway[56].

Association of HCV with lipid droplets

HCV core protein released from the polyprotein after host signal peptidase cleavage undergoes further maturation by proteolytic processing by signal peptidase[57,58]. *In vitro* expression of core protein is associated with ER membranes and the surface of LD[59]. The immature form of core protein cannot attach to LD. After maturation, core protein is directed from the ER to LD, and it attaches *via* a D2 domain, which includes a 55 amino acid protein containing two amphipathic α-helices (H Ⅰ and H Ⅱ) separated by a hydrophobic loop[60]. D2 is important for core protein stability and its subsequent associations with cell membranes. The neighboring core segment, D1, has important functions in RNA binding and protein-protein interactions[46]. LDs are directly involved in the production of infectious HCV particles[61]. They are surrounded by unique membranes called "membraneous web", allowing a high amount of plus- and minus-strand HCV RNA as well as NS proteins (NS5A) to attach to LD covered with HCV core protein. NS5A-core interactions require the serine phosphorylation toward the C-terminal end of NS5A and are essential for the association of replication complex to the periphery of LDs, which is a pre-requisite for infectious virion assembly[56]. Importantly, disruption of the core protein-LD association causes defects in HCV RNA and NS localization

and results in a loss of infectious viral particle assembly[61], demonstrating that core protein is responsible for recruitment of NS proteins to LD. Attachment of core protein to LDs leads their aggregation toward the periphery of the nucleus, suggesting that the core modifies the microtubule-dependent mobility of LDs; it also displaces adipocyte differentiation-related protein (ADRP), the major LD surface protein[62]. The modulation of the microtubule network by core protein is extremely important, as microtubule disruption reduces virus production. Thus, core protein changes the intracellular localization of LDs, securing the contacts between the sites of RNA synthesis and LDs. Due to core-NS5A protein-protein complex formation, these two proteins form a bridge between LDs and the sites of HCV RNA replication[46]. The targeting of the viral components to LDs establishes the link between assembly and release of HCV virions, VLDL production and apoprotein B lipidation[63].

HCV and autophagy

Some viruses, including HBV and HCV, subvert autophagy and use it to their own benefit. The link between autophagy and HCV replication is supported by several studies. Knockdown of autophagic proteins, beclin-1, Atg4B, Atg5 and Atg12 suppresses HCV replication at the onset of infection[64]. Specifically, Atg7 knockdown decreases the production of infectious viral particles without affecting HCV viral protein expression[65]. By inducing ER stress, HCV infection stimulates autophagosome formation regardless of HCV genotype[64,66]. Recently, we have shown that this autophagosome formation and specifically, LC3 lipidation can be further enhanced by exposure to ethanol of JFH1-infected ethanol-metabolizing hepatoma cells (unpublished observations). Nevertheless, a co-localization of LC3-Ⅱ protein in autophagosome and HCV proteins has not been found[65,67]. However, a recent study of Guevin *et al*[68] demonstrated a transient link between Atg5 and HCV NS5B (RNA-dependent RNA polymerase) protein at a very early stage of infection. This study was conducted in the frame of yeast two-hybrid analysis on the cells co-expressing Atg5 and NS5B. Interaction between these proteins is required for the onset of viral replication. Atg5 is indeed involved in other positive-strand RNA virus replications *via* formation of a double membrane vesicle[69]. This Atg5-NS5B interaction suggests that autophagy proteins play some additional, autophagy-unrelated role. Induction of autophagosome formation in HCV infection does not necessarily mean activation of autophagic protein degradation because HCV induces an incomplete autophagic response by impairing autolysosome function[66,70]. One of the possible reasons is either defective autophagosome-lysosome fusion or impaired postfusion proteolytic degradation of autophagosome contents induced by alkalization of lysosome due to HCV p7 protein[71]. The reduced capacity for lysosomal digestion may further prolong HCV survival.

HCV replication and ethanol

As revealed from numerous studies, exposure of liver cells to ethanol affects HCV replication. Zhang *et al*[72] demonstrated that ethanol produces a concentration-dependent increase in HCV replication and related that effect to the activation of NF-κB promoter. In addition, exposure of Huh7 cells and primary human hepatocytes to increasing concentrations of ethanol up-regulates intracellular and extracellular HCV RNA[73]. However, the role of ethanol metabolism in HCV replication is still controversial. Recent studies in full genomic and subgenomic HCV replicon cells have shown that alcohol increases HCV replication 4-fold in a CYP2E1-dependent manner and these effects are blocked by N-acetyl cysteine[74]. As demonstrated by Seronello *et al*[75], replication of both HCV genotypes 1b and 2a depends on lipid metabolism, is enhanced by acetaldehyde and requires elevated NADH/NAD+ ratio. On the other hand, extracellular and intracellular ROS suppress HCV replication. In addition, the effect of ethanol on HCV RNA replication in ethanol-metabolizing JFH1-infected Huh7.5 CYP2E1-expressing (RLW 2-9) cells depended on when ethanol was applied to the cells; application of ethanol before infection of cells with JFH1 enhanced HCV RNA replication, while exposure of RLW 2-9 cells to ethanol 3 d after infection suppressed HCV RNA levels[76].

The mechanisms by which ethanol interferes with or activates the HCV replication cycle are not clear. While the literature on viral entry develops rapidly, very little is known about how ethanol affects the expression of receptors for HCV entry. These effects may depend on the ability of cells to metabolize ethanol. Thus, by using ethanol non-metabolizing cells, it has been shown that the expression of CD81 is up-regulated on monocytes of HCV-infected alcohol-consuming patients[77], while ethanol exposure decreases claudin1 expression on alveolar epithelial cells[78]. Recently, we showed that ethanol exposure did not affect CD81, but enhanced claudin 1 expression on ethanol-metabolizing CYP2E1$^+$ HCV replicon and JFH1-infected infected hepatoma cells[76,79]. Other mechanisms explaining the enhancement of HCV replication/expression by ethanol exposure to hepatocytes, including the regulation of autophagy, are under investigation.

HCV, fatty liver and autophagy

Expression of PPARα, a nuclear receptor that modulates the expression of oxidative enzymes and fatty acid import into mitochondria, is impaired by HCV[80]. HCV core protein reduces expression of PPARα as well as its transcriptional activity. In addition, HCV core protein activates fatty acid synthesis by affecting SREBP1c. Sterol response element-binding proteins (SREBPs) are transcription factors that are bound to ER and regulate the activity of enzymes that support cholesterol and fatty acid synthesis. Genes for these proteins are transcriptionally induced by HCV[81]. The phosphorylation and activation of these proteins *via* the MAP kinase or PI3-K-Akt pathways leads to induction of fatty acid synthase. Activation of SREBP1 by HCV core protein requires the participation of PA28γ, a nuclear protea-

some activator[82]. Lipid droplets accumulate fat and are the place of HCV full particle assembly[83]. Since lipid droplet content is regulated in part by autophagy[84] and autophagic degradation of the substrates (including lipids) is compromised by HCV and ethanol, this defect in autophagy may further promote fat deposition in the liver of HCV alcohol-consuming patients.

CONCLUSION

Autophagy is mediated by autophagosomes that "capture" autophagic substrates (proteins and lipids) and deliver them to the lysosome for further degradation. Ethanol up-regulates the upstream part of autophagy (autophagosome formation), but it suppresses lysosomal function, thereby negatively affecting protein degradation. By creating ER stress, HCV also enhances autophagosome formation; however, some HCV proteins (p7) interfere with normal lysosome function. In addition, autophagic proteins are involved in regulation of HCV replication. Autophagosome content hepatoma cells can be further increased by exposure to ethanol. The effects of ethanol on HCV replication are redox-dependent (require elevated NADH/NAD+ ratio). Thus, HCV- and alcohol-modified autophagy apparently plays a role in enhanced viral replication, intracellular virus accumulation and steatosis progression in HCV-infected alcohol-consuming patients.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2515

World J Gastroenterol 2011 May 28; 17(20): 2515-2519
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Animal models for studying hepatitis C and alcohol effects on liver

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Received: January 11, 2011 Revised: March 8, 2011
Accepted: March 15, 2011
Published online: May 28, 2011

Abstract

Chronic consumption of ethanol has a dramatic effect on the clinical outcome of patients with hepatitis C virus (HCV) infection, but the mechanism linking these two pathologies is unknown. Presently, *in vitro* systems are limited in their ability to study the interaction between a productive wild-type HCV infection and chronic ethanol exposure. Mouse models are potentially very useful in dissecting elements of the HCV-ethanol relationship. Experiments in mice that transgenically express HCV proteins are outlined, as are experiments for the generation of mice with chimeric human livers. The latter models appear to have the most promise for accurately modeling the effects of chronic ethanol intake in HCV-infected human livers.

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Key words: Mouse models; Hepatitis C; Ethanol; Transgenic mice

Peer reviewers: Dr. Shivananda Nayak, PhD, Department of Preclinical Sciences, Biochemistry Unit, Faculty of Medical Sciences, The University of The West Indies, Building 36, EWMSC, Mount Hope, Trinidad and Tobago; Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Mercer DF. Animal models for studying hepatitis C and alcohol effects on liver. *World J Gastroenterol* 2011; 17(20): 2515-2519 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2515.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2515

INTRODUCTION

Chronic consumption of ethanol has a dramatic effect on the clinical outcome of patients with hepatitis C virus (HCV) infection. HCV-infected patients who also abuse alcohol have higher levels of HCV RNA[1], accelerated progression of fibrosis[1] and clinical disease[2,3], and an overall increased risk for development of hepatocellular carcinoma (HCC)[4,5]. Although both chronic alcohol use and HCV infection are individually injurious to the liver, when combined, their effects seem to be multiplied. Despite the well-known deleterious consequences of this combination, however, the mechanism that links these two pathologies remains obscure.

Complicating the study of HCV and ethanol is the fact that *in vitro* systems that support viral replication are based on the HCV strain JFH-1[6], a genotype 2a virus that caused an acute self-limited viral hepatitis in a young Japanese male[7]. Although unquestionably valuable in advancing the field of HCV biology, care must be taken in extrapolating results from this unique viral strain into wild-type strains in the community. Furthermore, the hepatoma-derived cell lines that are capable of supporting HCV replication, such as Huh-7, are inherently deficient in main ethanol-metabolizing enzymes such as CYP2E1 and alcohol dehydrogenase, although this has been overcome in part through the engineering of cell lines to metabolize ethanol and support HCV replication[8].

As pointed out by McCartney and Beard[9], progress

in this area has been significantly hampered by lack of a small animal model system. Species-restriction of HCV to humans and chimpanzees has impaired the ability to study HCV and ethanol use *in vivo*. Researchers have attempted to overcome this block through the development of transgenic strains of mice that express key portions of the HCV genome, and through the creation of mice with chimeric human livers. In this review, we explore the studies in transgenic systems, and examine humanized mouse models as potential platforms for HCV/ethanol studies.

TRANSGENIC MOUSE SYSTEMS

Transgenic mice that express portions of or the entire HCV genome have been created[10-12], and some strains have been used in experiments that have explored the relationship between ethanol administration and viral protein expression. In core-expressing mice exposed to 5% ethanol feeding for 3 wk, total reactive oxygen species were significantly elevated as compared to control animals[13]. In the absence of ethanol, these same mice showed activation of the mitogen-activated protein kinase pathway (which led to enhanced cellular proliferation signaling), which was significantly enhanced by the addition of 3 wk ethanol[14]. These murine experiments suggest that hepatocarcinogenesis mediated through the expression of HCV core protein is enhanced by medium-term administration of ethanol, and that this model system is appropriate for assessing the core protein/ethanol interaction.

In another series of experiments that have examined the HCV/ethanol relationship, core-expressing mice were subjected to chronic ethanol feeding (20% ethanol for 10 mo), and examined for effects on lipid oxidation and peroxidation, hepatic lipoprotein secretion or cytokine expression[15]. No interaction was seen between core expression and ethanol ingestion for lipid oxidation or secretion of lipoproteins, but an additive effect was seen on lipid peroxidation and a synergistic effect on expression of hepatic transforming growth factor-β and tumor necrosis factor-α. The latter effect mimics the accelerated fibrosis that is seen in HCV-infected patients who abuse alcohol, and supports the validity of a core-expressing transgenic mouse model for long-term experiments.

The interaction between NS5a expression and ethanol ingestion in carcinogenesis has been explored in an elegant study by Machida *et al*[16], who have used mice on a C57BL/6 background that transgenically expressed NS5a with either wild-type or knocked-out Toll-like receptor (TLR)4 expression. When fed ethanol by intragastric infusion for 4 wk, alcoholic steatohepatitis was significantly increased in NS5a-expressing mice compared with non-expressing controls; an effect that was largely ameliorated by TLR4 knockout. In a longer-term experiment, NS5a-expressing mice with or without TLR4 fed a control diet for 1 year showed no development of HCC, whereas, when they were fed a 3.5% ethanol Lieber–DeCarli diet for the same time, HCC developed in 23% of NS5a TLR4$^{+/+}$ mice but not in NS5a TLR4$^{-/-}$ mice. These findings suggest that NS5a expression and ethanol ingestion affect hepatic inflammation and carcinogenesis, which are mediated through the TLR4 pathway. In similar experiments using 12-mo ethanol feeding in NS5a-expressing mice (wild-type TLR4), upregulation of oncogenic pathways such as RNA pol Ⅲ dependent transcription and TBP and Brf1 expression were induced in animals that were chronically fed alcohol[17]. Taken together, these studies support a role for chronic ethanol use enhancing inflammation and carcinogenesis in livers that express NS5a.

Although they are seemingly valid and useful for studying the interaction between virally expressed proteins and ethanol ingestion, it is important to stress that transgenic models are not models of infection, and the expression of viral proteins is not under the same controls as would be seen in naturally infected hepatocytes. Although some investigators have demonstrated that expression of viral proteins is similar to that seen in human tissue sections[16], the expression is indiscriminate in all hepatocytes, which differs from the variable regions of replication seen in human liver. Additionally, the intracellular location of expression might differ from that in wild-type infections. Transgenic models are undoubtedly useful, but cannot yet evaluate the interaction of ethanol and HCV within the context of a full viral reproductive cycle.

CHIMERIC MOUSE MODELS

Given the species-restriction of HCV and the general inability to infect and maintain primary human hepatocytes in culture reliably, researchers have turned to alternate approaches to develop a model that is capable of supporting HCV infection *in vivo*. The establishment of murine models that support engraftment and expansion of non-transformed human hepatocytes within the liver has led to the term "chimeric mice", which here refers to mice with livers that are composed of substantial numbers of human hepatocytes. Two separate models, the Alb-uPA and the FAH-deficient mouse, appear to be capable of sustained support of human liver cells, and demonstrate many properties that make them useful for the study of HCV and ethanol.

Alb-uPA model

The first success in this area was the development of the SCID/Alb-uPA mouse[18]. The Alb-uPA transgene is a tandem array of murine urokinase genes under the control of the albumin promoter, which target overexpression of urokinase to the murine liver *in utero* and after birth[19,20]. Expression of the transgene causes a bleeding diathesis and hepatic toxicity, and produces a chronic stimulus for regeneration to which the mouse is incapable of responding. After spontaneous somatic deletion of portions of the transgene[20], cells are no longer restrained by expression of the transgene, and rapidly proliferate to fill the liver with non-transgenic cells, which reverses the liver and bleeding defects. By transplanting either mouse or rat hepatocytes into the portal venous system (*via* the

spleen), rapid expansion of the transplanted cells leads to similar reversal of the Alb-uPA phenotype[21,22].

Mice from the Alb-uPA strain have been crossed with an immunodeficient strain (c.b17-SCID-bg) and the transgene bred to homozygosity. These mice can then be transplanted intrasplenically with human hepatocytes, and have been shown by multiple groups to be capable of supporting high levels (up to 90%) of human chimerism within the liver[18,23,24]. In mice with sufficient human chimerism (typically > 20%), after inoculation with HCV, infections are established at levels identical to those seen in infected humans, and the infected state persists to beyond 16 wk after inoculation, often to the life of the infected animal[18]. The virus can be serially passaged between mice, which confirms that fully formed and infectious particles are produced, and the mice are capable of being infected with virus passaged through cell culture. Infections have been successfully established using viral genotypes 1a, 1b, 2a, 3a, 4a, and 6. The system has been confirmed by multiple groups to model accurately entry, replication, packaging and release of infectious particles[25], respond to human interferon $\alpha 2\beta$[26,27], putative antiviral agents[27,28], and blockade of infection by passive immunization[29].

Important in the study of ethanol-HCV interactions is the similarity between chimeric mouse and normal human liver metabolism. Similarities in the genomic response to HCV infection between human and chimeric mouse livers have been demonstrated by Walters *et al*[30], which suggests that not only does the system support the viral life cycle, but it also models the normal human response. In a series of experiments by a Japanese group, it has been shown that chimeric mouse livers express a wide variety of mRNA for drug-metabolizing enzymes and transporters[31], and that the levels of protein expression of these enzymes are very similar to those from source human liver tissue[32]. The expression of specific cytochrome P450 enzymes has also been studied, and shown to be appropriately expressed and induced (CYP3A4[33]), and inhibited (CYP2D6[34]). Although there have been no published studies on the metabolism of ethanol in chimeric livers, generalizing from other enzymes systems, it would appear likely to be very similar to that in humans.

FAH-deficient model

An alternate model of repopulation has been developed based on mice rendered deficient in the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (FAH)[35]. FAH mutant mice are protected by administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) in their water source, and develop liver disease when it is withdrawn, hence allowing for conditional expression of the phenotype. After withdrawal of NTBC, there is a stimulus for proliferation that can be exploited to expand a population of transplanted hepatocytes, similar to what happens in the Alb-uPA model. By crossing the FAH-deficient trait onto an immunodeficient background (Rag2/common γ-chain knockout), a strain of mice has been produced that is capable of supporting expansion of human hepatocyte grafts[36]. This model requires temporary introduction of the uPA gene *via* an adenoviral vector[37] to initiate engraftment. This model has the advantage of being useful for transplantation at any age (Alb-uPA mice are typically transplanted between days 7 and 28 of life), however, usable engraftment (serum human albumin level > 1 mg/mL) was achieved in only seven of 43 transplants (16%); somewhat lower than that seen in the Alb-uPA model. However, very high level engraftment has been achieved in some cases.

Chimeric FAH-deficient mice have been shown to express drug-metabolizing genes (CYP1A2, CYP3A4) at levels typical of adult human liver, and when hepatocytes have been isolated from chimeric livers and plated in temporary cultures, they have been found to be indistinguishable from primary human hepatocytes in standard drug metabolism assays[36]. In experiments by another group, these chimeric mice have been shown to be capable of supporting HCV infection, and of responding to standard antiviral therapies, including pegylated interferon (peg-IFN), peg-IFN plus ribavirin, and the cyclophilin inhibitor Debio 025[38]. Based on these experimental findings, it appears that chimeric FAH-deficient mice should also be appropriate for the modeling of HCV/ethanol interactions in the human liver.

Pilot studies of HCV and ethanol in Alb-uPA mice

We have conducted preliminary studies on the feasibility of using chimeric mice in the study of HCV/ethanol interactions, based on the Alb-uPA model. Potential concerns about tolerability of an ethanol regimen, as well as the ability to model the human response to ethanol exposure have been addressed by feeding a cohort of chimeric mice a diet including 20% ethanol in water for 5 wk. These mice tolerated the ethanol protocol with a slight decrease in weight and fluid consumption, as compared with mice on a control diet, and no evidence of increased mortality. At completion of ethanol feeding, liver samples from two of these mice were taken and analyzed by HPLC for glutathione (GSH) and SAM levels. These samples demonstrated a 40% decrease in SAM levels and an 83% decrease in GSH levels; both indicative of chronic ethanol toxicity on the livers. Studies of ethanol administration in HCV-infected mice are ongoing.

CONCLUSION

Based on the state of knowledge presently available, study of the complete interaction between ethanol and natural HCV infection will require the use of mouse models. Transgenic models have proven useful to study the relationship between ethanol exposure and viral protein expression, but have limitations in how accurately they can model HCV infections in humans. Studies based on chimeric mice appear to be the most promising, but have their own complexities which include technical challenges in establishing these models and the ability to extract the human response of a chimeric liver from within its murine

background. However, in well-engrafted mice, the overall response seems to mimic that of humans so closely that the murine background might not matter.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2520

World J Gastroenterol 2011 May 28; 17(20): 2520-2535
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Role of lipid rafts in liver health and disease

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Received: January 6, 2011 Revised: February 24, 2011
Accepted: March 3, 2011
Published online: May 28, 2011

Abstract

Liver diseases are an increasingly common cause of morbidity and mortality; new approaches for investigation of mechanisms of liver diseases and identification of therapeutic targets are emergent. Lipid rafts (LRs) are specialized domains of cellular membranes that are enriched in saturated lipids; they are small, mobile, and are key components of cellular architecture, protein partition to cellular membranes, and signaling events. LRs have been identified in the membranes of all liver cells, parenchymal and non-parenchymal; more importantly, LRs are active participants in multiple physiological and pathological conditions in individual types of liver cells. This article aims to review experimental-based evidence with regard to LRs in the liver, from the perspective of the liver as a whole organ composed of a multitude of cell types. We have gathered up-to-date information related to the role of LRs in individual types of liver cells, in liver health and diseases, and identified the possibilities of LR-dependent therapeutic targets in liver diseases.

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Key words: Hepatocytes; Stellate cells; Kupffer cells; Endothelial cells; Signaling; Therapeutic; Viral; Hepatitis C virus; Metabolism

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Dolganiuc A. Role of lipid rafts in liver health and disease. *World J Gastroenterol* 2011; 17(20): 2520-2535 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2520.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2520

INTRODUCTION

The liver is the largest parenchymal organ of the body and has a multitude of important and complex functions; among the most notable are the metabolism of fat, proteins, and carbohydrates, synthesis and secretion of bile, microelement recycling and detoxification of products resulting from body metabolism (bilirubin, ammonia, *etc.*) and from exogenous toxins (drugs, alcohol and environment)[1]. The liver is also an immune[2] and an endocrine[3,4] organ and functions as a blood capacitance reservoir[5,6]. To accomplish these many assignments, the liver accommodates a wide variety of cell types, including those that call liver "home", namely liver progenitor cells, hepatocytes, Kupffer cells (KCs), stellate cells (SCs) and the cellular components of vasculature, and those that use the liver as a temporary and/or terminal station, such as blood cells[1,7]. Liver diseases are the 10th leading cause of death and account for significant morbidity across the entire age and gender spectrum of the US population[8,9]. The mechanisms of liver diseases are extensively researched but not fully understood. More recently, research data have emerged proposing that some pathogenic mechanisms, such as inflammation, tissue death and regeneration, and organ remodeling, are shared across a large spectrum of liver diseases of distinct etiologies[10-14], with the suggestion that common denominators of cell structure or function may be involved. Here we have focused on a common denominator of the cellular structure, the lipid rafts (LRs), as players in liver physiology and pathology.

Cellular membranes separate each cell and define their entity. The main components of the cellular membrane are lipids and proteins[15]. Structurally, the membrane lipid molecules consist of a polar globular head and a straight non-polar hydrophobic backbone region. Each row of lipids aligns in a leaflet at the level of globular heads and hydrophobic areas of the leaflet attract similarly charged structures, such as the straight region of another lipid layer. Thus, the plasma membrane (PM) consists of two leaflets with the non-polar regions pointing inward and the polar heads pointing to the water rich-zones (intra- and extra-cellular spaces); architecturally this structure portrays a lipid bilayer[16] (Figure 1). Besides serving as warrant of cellular identity and integrity, the lipid bilayer assures that the membrane is flexible. At lower temperatures, the lipid bilayer forms a gel state and is tightly packed; as the temperature rises the bilayer favors tridimensional movement of the lipid molecules and the interior space between the two leaflets becomes more fluid. It is the gel/fluid exchange state that allows horizontal and vertical movement of lipids, and also of other components of the membrane[15-17]. Proteins are embedded into the lipid bilayer, either by spanning through, as transmembrane integral structures that could cross in a single- or multi-pass fashion, or as attached structures; the latter exist as peripheral proteins, attached to the membrane exclusively on either the extracellular or cytosolic face, or as glycosylphosphatidylinositol (GPI)-anchored proteins on the extracellular face of the membrane[18]. At the end of the 20th century, the LR hypothesis of cellular membrane function emerged: this proposed that certain regions of the lipid bilayer modify their chemical composition to become more rigid, thus allowing some membrane proteins to physically segregate depending on their interaction with the bilayer components and with other proteins[19,20]. This theory combined the biological knowledge about cellular activation and the biochemical knowledge about protein folding with biophysical approaches to the dynamic cellular membrane structure and attempted to answer the puzzling question about how proteins cluster and what contributes to signal transduction.

LRS: STRUCTURE, FUNCTIONS AND RESEARCH METHODOLOGY

LR structure and functions

LRs are areas of cellular membranes with a signature composition rich in sphingolipids and cholesterol phospholipids. Saturated fatty acids are preferentially enriched in the side chains of the membrane phospholipids, which allows closer packing and thus increased rigidity, more order, and less fluidity of the LRs compared to the surrounding membrane[15,19-21]. LRs act as a unique platform that aids co-localization of proteins involved in sensing (receptors), reacting (pores) and triggering/sustaining cell activation (intracellular signaling pathways) (Figure 1). Besides their structural signature, LRs exhibit two more unique characteristics: they are small, ranging from several to about 500 nm, and are dynamic, with a variable life span in the order of milliseconds (msec). It is not fully understood whether the LRs form and disintegrate constantly or maintain a longer lifespan when a cell is in its steady state, because a relatively constant number/composition of proteins can be identified in the LRs at this stage. However, once the cell is activated, the LRs undergo a radical change in composition by adding or eliminating certain proteins and thus accommodating the cellular needs for formation of signaling platform in a matter of msec. While they are well documented to be in the PM of the cells, it has been suggested that LRs can also reside in the intracellular structures, such as endoplasmic reticulum (ER), peroxisomes, mitochondria, and endosomes[22-26]; to date it is not clear whether such findings are truly due to LR presence in these intracellular compartments or if they are an artifact of LR isolation/investigation techniques. LRs are present throughout the entire evolutionary chain, from the viruses and bacteria[27] to mammals; the purpose of the LR in evolution is yet to be fully understood.

LR research methodology

Studying the LRs is challenging. With sizes ranging up to 500 nm in diameter, the LRs are beyond the resolution of optical microscopes. For this reason the overlay of one or more stains tagged to the proteins of interest revealing a significant co-localization with the coalesced LRs, labeled indirectly with a fluorescent choleratoxin B, is usually suggestive but never a definite indication of the LR localization of the studied proteins. In addition to being small, the rafts diffuse in msec across the cell membrane and thus require additional efforts for timely detection. The transmission electron microscope can analyze a 5-nanometer-thick cell membrane and captures short time frames; the more dynamic and short-lived rafts can only be observed with advanced imaging techniques such as atomic force microscopy (AFM)[28]. Fluorescence lifetime spectroscopy, including Förster resonance energy transfer (FRET), is becoming more accepted in the LR research field due to the fact that the capacity of energy transfer in FRET is about the size of the rafts and it occurs in the range of msec, thus covering both the size and the life span of the LR. Single point microscopic decay and fluorescence lifetime imaging microscopy are often used in combination with FRET for more comprehensive analysis of LR-related time-sensitive processes[29]. Flow cytometry analysis of LRs is emerging; however, it provides very limited resolution both for the size and for the timing of LR-localized events[30]. Fluorescence correlation and cross-correlation spectroscopy (FCS/FCCS) deliver information of fluorophore mobility and dynamics of lateral heterogeneity in the membrane and can be used in single or multiple colors[31]. Single particle tracking (SPT) can be employed to define the translational trajectory of very small particles, including quantum dots, to identify cluster size confinement based on intensity. The thinning out clusters while conserving stoichiometry of labeling (TOCCSL) technique is employed for

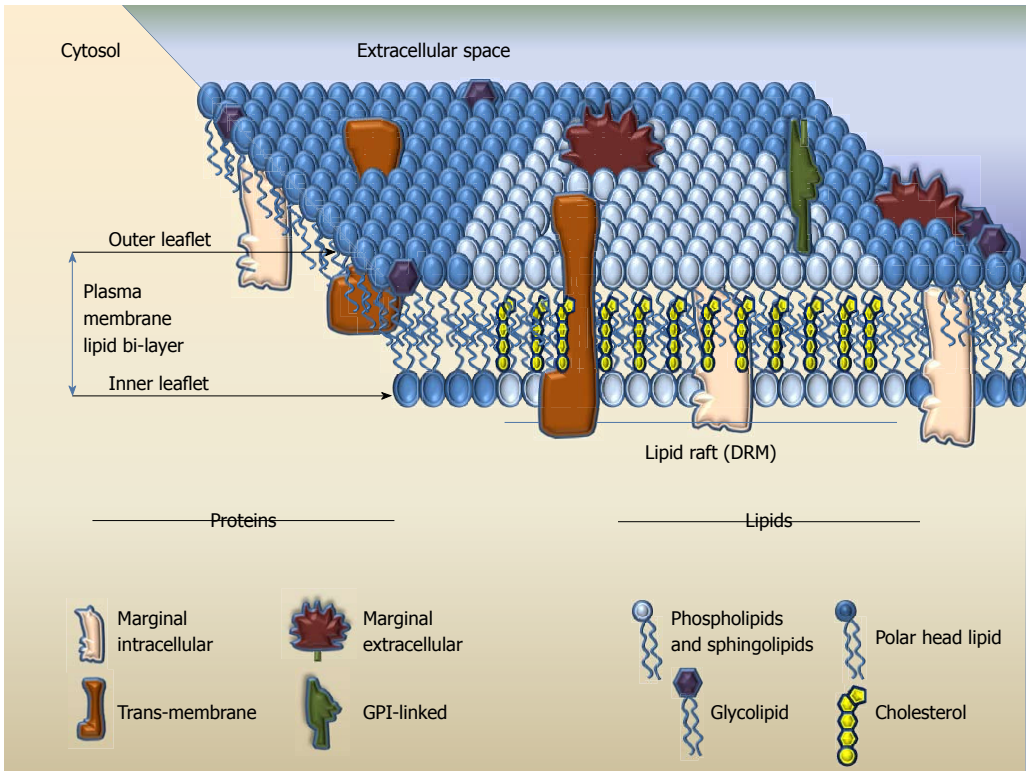

Figure 1 The theoretical model of lipid bilayer and lipid rafts in cellular membranes. GPI: Glycosylphosphatidylinositol; DRM: Detergent-resistant domain.

LR analysis because of its proficiency in discriminating between clusters and monomers[32]. The additional advantage of FRET, FCS/FCCS, SPT and TOCCSL is their high spatial/temporal resolution, and thus their ability to register rafts as they form and dissociate in time. Labeling of living cells with the fluorescent probe 6-acyl-2-dimethylaminonapthalene (Laurdan), which exhibits a 50-nm red shift as membranes undergo phase transition from gel to fluid due to altered water penetration into the lipid bilayer, followed by two-photon microscopy, is often used as another indirect method of LR visualization[33].

Translocation of proteins to or from the LRs is often analyzed by sucrose-density gradient fractionation of the membranes and subsequent Western blot analysis; LRs are defined by their insolubility in diverse nonionic detergents [thus named detergent-resistant domains (DRMs)] and by floating in the low-density sucrose fractions (interface between 5% and 30%) upon ultracentrifugation[34]. Diverse detergents can be employed, yielding diverse composition of DRMs[34-39]. Furthermore, some researchers construct step sucrose gradients and further sub-divide the DRMs into light and intermediately-light fractions, which may have distinct protein composition. It is still debatable as to how closely the DRMs represent the true identity of the LRs; however, this method yields consistent results and is the only one available that allows enrichment and analysis of large protein complexes at reasonable cost and using old-fashioned technology.

The functional assessment of the LRs *in vitro* is usually performed using reduction and enrichment approaches. Treatment with agents leading to cholesterol sequestration, including amphotericin, filipin or nystatin, inhibition of cholesterol synthesis using HMG-CoA reductase inhibitors, or cholesterol wash-out with mβCD, are among the well-accepted approaches to disrupt putative LRs; they are largely based on cholesterol manipulation[40,41]. Another method commonly utilized for LR destabilization is treatment with fumonisin B1, which removes sphingolipids[40]. The advantage of using mβCD over fumonisin B1 is based on the fact that the former acts rapidly, while the latter requires pretreatment of cells for about 72 h, which is longer than the time some polarized cells, including hepatocytes, can remain truly polarized in isolated cell culture[42]. Cholesterol replenishment and ceramide supplementation, which displaces cholesterol, are often employed to modulate the fluidity of the LRs and thus affect their function.

In vivo LR modulation has gained recent popularity based on findings that dietary lipids can modify lipid composition of cell membranes. In this context, multiple studies have attempted to establish how dietary factors or modulation of blood lipids, including cholesterol, affect LR-based signaling in various cell types[43,44]. Comprehensive experimental-based conclusions about the effects of dietary lipids in regulating LRs in non-liver systems and targeted evaluations of the liver in this regard are still awaited. Reliable methods of *in vivo* visualization of LRs also await development.

The significant difficulties in analysis of LRs in primary biological membranes have led to development of model systems. Diverse artificial membranes and models have been created over time, with different ranges of

spatial and temporal orders, diverse lipid and protein compositions, protein/lipid ratio or thickness of the lipid bilayer[45,46]. While helpful to elucidate the basics of the membrane function and structure, these models lack the combination of the proximity of the membrane with the cytoskeleton and regulatory/signaling proteins that are recruited in diverse ratios and in a time-dependent manner in natural biological membranes[45]; to date there is no comprehensive artificial model of LR-containing membranes.

COMPOSITION OF LIVER LRS

An extensive number of proteins have been identified as residing in LRs in the liver[36-39]. Depending on the method of raft isolation and protein analysis, it is currently estimated that at least 300 proteins reside in the LRs in the normal liver; these data include analysis of both human and rodent livers[36-39]. Bae *et al*[36] identified 196 proteins and pointed to a relatively large content of mitochondrial proteins in the rat liver membrane LRs. Zhang *et al*[38] reported 175 non-redundant gene products identified in mouse liver PM, isolated from mouse liver by floating in the sucrose density gradient upon ultracentrifugation, which largely resemble the DRM fraction enriched in LRs[23,34]. Zhang *et al*[38] also identified that about 50% of the LR-associated proteins were integral membrane proteins with one to seven transmembrane domains (TMDs), 40% represented enzymes, 12% were receptors and 9% were proteins with unknown function. He *et al*[39] identified 104 proteins in human liver membranes with about one third being of cytoskeletal affiliation, including proteins in fodrin-based meshworks, adhesion proteins involved in inter-cellular junctions, focal adhesions, desmosomes, hemidesmosomes and tight junctions, proteins that regulate F-actin dynamics, and motor proteins; most of these proteins usually affiliate with LRs in other cell types. Mazzone *et al*[37] pointed to the differential protein identity from the apical and basolateral LRs of the PM in normal rat hepatocytes. These data indicate a great diversity in the types of proteins affiliated with LRs in the liver (Figure 2) and indirectly suggest the importance of the LRs in the liver.

The analysis of the composition/function of LRs in the liver performed so far has involved whole liver and does not take into account that liver, as a whole, accommodates a wide variety of cell types. Nevertheless, fractioning of LRs from the whole liver has revealed novel information and thus spearheaded the interest in the field and targeted focused investigations towards specific liver cell populations.

LRS IN RESIDENT LIVER CELLS

LRs in hepatocytes

LRs aid polarized sorting and trafficking of apical proteins in both directed and trans-cytotic pathways in hepatocytes: Two structurally and functionally distinct PM

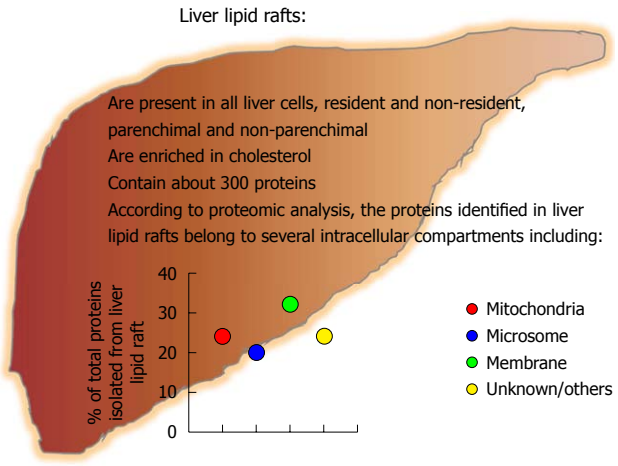

Figure 2 Estimation of the protein origins identified in the liver lipid rafts. Adapted, in part, from Bae *et al*[36], with permission.

domains, i.e. the apical and basolateral domains, determine the tri-dimensional architecture of the hepatocyte as true polarized cells; these domains are separated by the tight junctions. While the molecular signature of apical and basolateral domains is relatively well known, the maintenance of such identity requires sophisticated protein sorting and trafficking, the mechanisms of which are yet to be fully defined. Specific membrane-sorting signals have identified many involved proteins[47]. For example, GPI-membrane anchor and TMD target the proteins to the membrane[48]. Moreover, Tyr- and diLeu-based amino acid motifs, which are recognized by distinct molecular subunits of adaptor complexes, seem to direct the proteins to the basolateral membrane[49].

Two membrane-sorting pathways are known: direct and indirect. Unlike many polarized cells, hepatocytes employ both pathways to guide their proteins to the desired location. In "direct" pathway of membrane targeting, the newly synthesized proteins are primarily sorted in the trans-Golgi network (TGN) and then delivered directly to the membrane; this pathway serves both apical or basolateral membrane domains without advantaging either of them[50]. Membrane multispanning ATP-binding cassette (ABC) transporters, multidrug resistance protein 1 (MDR1), and P-glycoprotein, to name a few, employ the direct sorting mechanism in hepatocytes[51]. In "indirect" pathway, the new protein cargo travels from the TGN to the basolateral surface, from where it is endocytosed and subsequently transported to the opposite apical surface of the membrane, as reviewed by Nelson *et al*[52]. This laborious, energy-inefficient and very lengthy process is also called the trans-cytotic pathway and its purpose is not fully understood. The GPI-linked proteins and those with a single TMD preferentially employ the indirect pathway for apical trafficking[53].

Until recently, the factors that directed the proteins into direct *vs* indirect pathways of membrane-sorting were largely unknown. Nyasae *et al*[54] reported that cholesterol and glycosphingolipids are required for delivery from basolateral early endosomes to the subapical compartment.

Slimane *et al*[55] identified that the protein structure and its lipophilic characteristics sort the differential apical *vs* basolateral targeting of the proteins in a sphingolipid/cholesterol-enriched LRs-dependent manner. These authors also reported that the liver apical proteins that contain multiple membrane-spanning domains are selectively incorporated into lipid microdomains in the TGN and are then transported directly to the apical membrane[55]. However, direct apical sorting is not solely driven by incorporation into LRs *per se*[55]. Conversely, proteins that have only one TMD or tether to the membrane using their GPI anchor are also sorted to the LRs in TNG; they travel first to the basolateral membrane, where LR-based mechanisms are again key in their delivery to the subapical compartment and further exposure on apical membrane. Slimane *et al*[55] additionally suggested that lipid composition of the LRs is also critical for protein sorting. Thus LRs play a key role in both direct and indirect membrane targeting of the proteins and act as sorting platform for directing the proteins *via* one pathway or another.

LRs in hepatocyte polarity: Hepatocytes are classical polarized cells; the adequate function of the liver as an organ is ensured by the spatial setting and by maintenance of hepatocyte polarity. The complex polarity of the hepatocyte is characterized by the existence of several basolateral and apical poles per cell. The complex mechanisms of establishment and maintenance of hepatocyte polarity are not fully understood; however, a role for calcium waves has been proposed. In hepatocytes the Ca^{2+} waves are polarized, thus occurring in an apical-to-basal fashion[56]. The polarity of the Ca^{2+} signal is largely due to the increased density of inositol 1,4,5-trisphosphate receptor (InsP3R) in the pericanalicular region; redistribution of InsP3R from the apical to the basolateral region is associated with concomitant slowing of the onset and speed of Ca^{2+} waves in hepatocytes[57]. The pericanalicular accumulation of InsP3Rs in not exclusive to hepatocytes, as it is also observed in other types of polarized cells such as pancreatic acinar cells, cholangiocytes, salivary acinar and duct cells[58]. The pericanalicular area has been named the "trigger zone," because it defines the subcellular region that triggers the formation of Ca^{2+} waves[59,60]. The pericanalicular area is rich in LRs and it is also a preferred localization of InsP3Rs; most importantly, recruitment of InsP3Rs to this area is highly dependent upon the presence of intact LRs[57]. Similar to InsP3R redistribution, LR disruption by cholesterol depletion substantially slows Ca^{2+} waves in hepatocytes[57]. The expression of apical InsP3Rs is decreased or absent in cholangiocytes from patients with primary biliary cirrhosis, sclerosing cholangitis, common bile duct obstruction, and biliary atresia, suggesting that InsP3Rs deficit and/or misplacement may represent a final common pathway for the development of cholestatic disorders[61]. Given the key role of LRs in housing the InsP3Rs on the apical hepatocyte membrane, it is easy to envision a role for LRs and/or hepatocyte de-polarization in cholestatic disorders; this area remains open for investigation.

LRs as signaling platforms: The hepatocyte PM houses a wide variety of proteins that function as sensors, receptors, and/or transporters; these proteins allow the hepatocyte to react to a rapidly changing extracellular environment and accommodate the needs of homeostasis. Among the best researched hepatocyte receptors for which the intracellular signaling pathways are well defined and the ties to LRs are well established are hepatocyte growth factor (HGF) receptor (HGFR), epidermal growth factor (EGF) receptor (EGFR), angiotensin Ⅱ type 1 (AT_1R) and insulin receptor (IR) receptors.

The HGFR, also called c-Met, is activated by HGF and triggers hepatocyte proliferation, morphogenesis and survival. Defects of HGFR are a cause of hepatocellular carcinoma[62]. The HGFR is a heterodimer that exhibits tyrosine kinase activity and associates with a multiprotein complex to trigger downstream signal systems, including src, Grb2/SOS, PI3 kinase, Gab1 and focal adhesion kinase; the adequate function of these adaptors/signal transducers is largely dependent on LRs[63,64]. To date, it is not clear if the HGFR itself is localized in the LRs. However, HGF promotes HGFR association with CD44 and recruitment of this multi-protein receptor complex into caveolin-enriched LR microdomains[65]. The LR-dependent HGF/HGFR signal plays a role in several pathologic processes, including protection from lipopolysaccharide-induced vascular hyper-permeability[65]; its role in hepatocyte function remains to be clarified.

EGF binds to the EGFR and regulates hepatocyte growth both *in vivo* and in primary culture. EGFR signaling activates a group of signal transducers and activators of transcription, which increase the transcription of a characteristic set of early growth response genes thus leading to hepatic DNA replication[66]. In normal adult liver, EGFR expression is low, whereas EGF is virtually nondetectable. In contrast, in advanced cirrhosis, a continuous EGFR synthesis is accompanied by EGF expression that is localized to hepatocytes and proliferative bile epithelium[67,68]. EGF treatment is followed by rapid ubiquitination of the EGFR in hepatocytes[69] and plays a key role in trafficking of the EGFR between early and late endosomal compartments[70]. Ubiquitinated EGFR is internalized almost exclusively *via* a non-clathrin LR-dependent route[70]. In this context, EGFRs recruited to LRs in response to EGF derive almost exclusively from endosomes[24]. EGFRs in early endosomes are more tyrosine phosphorylated than those in late endosomes, indicating that EGFR might be partially dephosphorylated in an LR-dependent manner before accessing the late compartment; however, once localized, the EGFRs in endosomal LRs are relatively resistant to dephosphorylation[24]. Some of the kinase receptors that are internalized through caveolin-containing LRs have a high probability of being directed to the degradative pathway rather than having a function in signaling[70]. To this extent, the EGFR is no

exception: the EGF-engaged EGFRs that are localized in LRs are more tyrosine phosphorylated than EGFRs in whole endosomes. Endosomal LRs recruit highly Tyr-phosphorylated EGFR along with various signaling proteins, including Grb-2, Shc, and c-Src, indicating a likely role for this compartment in signal transduction[24]. According to Teis *et al*[25], targeting of the MP1-ERK1/2 complex to late endosomes during EGF-mediated ERK activation depends on the adaptor protein; further, p14-MP1-ERK1/2 complex formation is needed for EGF-induced ERK activation at later stages of this process. Thus, it seems plausible that intracellular LRs target the ubiquitinated PY-EGFR/Grb2/Shc complex to the cytoplasmic face of the late endosomes for specific activation of ERK1/2. If this theory holds true, the ubiquitinated EGFRs should be later sorted into the intraluminal vesicles of late endosomes before their degradation or may be recycled back to the PM. This hypothesis was in part confirmed by data from Balbis *et al*[24] and by Lai *et al*[26], thus placing the LRs among the key players in ensuring efficient EGF/EGFR function. The possibility that this LR-dependent mechanism can be exploited is valid, because some of the widely-used drugs can be employed for this purpose. For example, heparin can suppress LR-mediated signaling and ligand-independent EGF receptor activation[71]. The detailed role of LR-mediated EGF/EGFR function in liver diseases remains to be determined.

Angiotensin Ⅱ (AT) engages and initiates angiotensin AT_1 receptor (AT_1) signaling which is involved in cell growth and mitogenesis[72]. At least in C9 liver cells, Ang Ⅱ-stimulation involves LR-mediated activation of ERK1/2[73]. Furthermore, Yin *et al*[74] reported that in angiotensin-stimulated C9 cells the cholesterol-rich LR domains mediate the actions of early upstream signaling molecules such as Src kinases and intracellular Ca^{2+} and that LR resident caveolin-1 has a scaffolding role in this process. Angiotensin causes caveolin-1 phosphorylation that is in turn regulated by intracellular Ca^{2+} and Src, thus indicating reciprocal interactions between LRs, caveolin-1, Src kinases, and intracellular Ca^{2+} through the AT_1[74].

Insulin is an anabolic hormone with a role in carbohydrate and lipid metabolism and cell growth. The IR is a transmembrane tyrosine kinase that binds insulin with its two extracellular α-subunits and transmits signals *via* its two β-subunits that contain the tyrosine kinase domain; the receptor is activated by autophosphorylations. LRs ensure the initial steps of IR activation[24,75,76]. IR β subunits require palmitoylation for proper function[75]; however, regardless of its palmitoylation status, the unoccupied IR has low affinity to LRs[76]. In adipose or muscle tissue, IR needs to partner with caveolin-1 for function. In the liver, the autophosphorylation of the insulin engaged-IR still takes place in the LRs but can occur with[24] or without[76] caveolin participation. In the latter context, glycophospholipid clustering inhibits IR phosphorylation and IR is excluded from the LRs when the latter are more rigid[76]. These results suggest that LRs offer an additional level of control over IR function in the liver and support the hope that manipulation of LRs could modulate IR activity and thus liver metabolism.

LR role in bile formation/secretion: Bile production is one of the basic functions and an organ-specific signature of the liver. ABC transporters are located in the canalicular membrane of hepatocytes and are critical players in bile formation and detoxification. ABC is a family that includes P-glycoprotein (MDR1) for organic cations; MDR2 for phosphatidylcholine translocation; P-glycoprotein-related protein acting as bile salt export pump; and MRP2 (or cMOAT) for non-bile acid organic anions[51]. ABC transporters are located in LRs of the hepatocyte cellular membrane and mediate the transport of the majority of lipids secreted into the bile[77].

The canalicular PM is constantly exposed to bile acids, which act as detergents. Once secreted into bile canaliculi, bile salts extract phosphatidylcholine from the outer leaflet of the cellular membrane and incorporate it into mixed micelles. The bile preferentially extracts phosphatidylcholine; the latter constitutes only about 35% of canalicular phospholipids in the context of a relative abundance of sphingomyelin, thus the bile threatens the well-being of healthy cells. During evolution, a protective mechanism was developed in all bile-exposed cells to prevent self-solubilization with own bile by means of expressing a combination of distinct LRs that have unique lipid composition and protein content. Ismair *et al*[35] reported the existence of at least two such LR entities on the canalicular side of the hepatocyte PM. One of these is rich in caveolin-1, contains the majority of canalicular cholesterol and phospholipids, portions of the marker enzymes APN and DDPIV, and a large portion of all known ABC transporters including ABCG5, BSEP, MRP2, MDR2, and MDR1; this LR is Lubrol-soluble. In contrast, Triton-soluble LRs are associated with reggie-1/2 proteins and sphingomyelin; they contain a minor fraction of canalicular cholesterol, APN, DDPIV and MDR1/2 ABC transporters[35]. The exact purpose of such differential expression of ABC transporters in distinct LRs is not fully understood; furthermore, their distribution in LRs in steady-state *vs* activated state is largely unknown. However, several liver diseases are linked to ABC defects; for example, recessively inherited hepatobiliary phenotype is related to mutations in ABC transporter genes; defects in ABCB11 and MDR3 lead to familial intrahepatic cholestasis types 2 and 3, respectively; Dubin Johnson syndrome is associated with defects in MRP2 produced, and sitosterolemia with defects in ABCG5 or ABCG8[51]. It is also known that an altered secretory process of biliary cholesterol is associated with elevated hepatic cholesterol levels[78], thus suggesting the possibility that low membrane fluidity due to stabilization with cholesterol could provide an additional layer of control of ABC transporter function. It remains to be investigated whether the increased rigidity of LRs, which occurs when cholesterol content in the cell increases[15,16], may supplement the impairment of bile se-

cretion in real-life liver diseases in addition to functional impairment of ABC transporters *per se*.

LRs in KCs/monocytes

KCs are active immune players and participants in a wide variety of liver diseases. The role of the LR in KCs is currently unknown, in part due to the scarcity of primary KCs and difficulties with their isolation. However, taking into account that KCs are tissue macrophages (Mf), several key findings about the role of LR in other types of tissue Mf could be extrapolated to KCs.

Inflammation occurs in a diverse array of liver diseases and is highly dependent on Mf. In Mf, the key molecules involved in pathogen recognition from exogenous danger signals either reside (CD14) or are recruited [Toll-like receptor (TLR)2, TLR4] to the LRs upon ligand engagement[79,80]; the function of pathogen recognition is thus highly dependent on LRs and suggests that LR modulation may be a candidate for managing the macrophages. Indeed, exposure to acute alcohol renders Mf temporarily insensitive to TLR4/CD14 ligands in a LR-dependent manner[81,82]. Similar to alcohol, selective modulation of fat content in LRs by macrophage-specific ABC transporter A1 also dampens inflammation by reducing MyD88-dependent TLRs trafficking to LRs[83]. Furthermore, peritoneal macrophages from dyslipidemic mice are primed for more robust TLR responses, reflecting increased LRs and increased TLR4 expression[84]. In the same dyslipidemic mice, the Mf from the lung airspace, in which cholesterol is maintained as constant during dyslipidemia, have normal responses and normal composition of LRs[84]. It is important to note that LRs also drive the Mf response to endogenous danger signals, such as those coming from dead cells. In this context, necrotic but not apoptotic cells co-localize with LRs within engulfing Mf. Interestingly, necrotic cell-induced secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-1β by Mf is susceptible to LR destruction, suggesting a role for LRs in the signaling of necrosis-driven inflammatory response[85].

Related to the role of liver in the iron cycle, the ability of Mf to capture/store iron is highly dependent on the LRs. Expression of the iron exporter ferroportin at the PM of Mf is enhanced by iron loading and is decreased by hepcidin[86]. Macrophage ferroportin is preferentially located in caveolin/flotillin 1-enriched LRs; iron overload strongly increases the presence of ferroportin in the LRs, while LR destruction decreases hepcidin activity on macrophage ferroportin[86]. Collectively, these data support the idea that LRs participate in several key Mf functions that are key for liver homeostasis.

LRs in SCs

The SCs store retinol and are the main cellular source of collagen and other extracellular matrix substances in normal as well as fibrotic livers. The LR composition, and their distribution and function in primary SCs are largely unknown, mainly due to limited numbers of SCs isolated from the normal liver and extreme technical difficulties of their isolation from the fibrotic liver. Andrade *et al*[87] employed the murine hepatic SC line GRX, which expresses the myofibroblast phenotype at baseline and can be induced *in vitro* to display the fat-storing phenotype (lipocytes), to show that total ganglioside content and GM2 synthase activity were lower in myofibroblasts compared to lipocytes. Both SC phenotypes presented similar content of gangliosides GM2, GM1, and GD1a, as well as their precursor GM3. Sphingomyelin and all the gangliosides were expressed as doublets; their ratio is increased in retinol-induced lipocytes due to increased content of long-chain fatty acids[87]. Taken together, these results indicated that myofibroblasts and lipocytes can use distinct ceramide pools for sphingolipid synthesis. Differential ganglioside expression and presence of long-chain saturated fatty acids suggested that these components could participate in the formation of LRs with specific functions in the two phenotypes of GRX-SCs[87].

Anandamide (AEA) is an amide of arachidonic acid and ethanolamine with endogenous endocannabinoid function that engages cannabinoid and other yet unknown receptors, and exerts a variety of physiological and pharmacological effects in chronic liver diseases[87]. Yang *et al*[88] employed the hepatic SC line T6 and reported that moderate AEA amounts inhibited hepatic SC proliferation and high-dose AEA caused hepatic SC death; cell death was necrotic rather than apoptotic and occurred independently of cannabinoid receptors. More importantly, AEA-mediated death in hepatic SCs was dependent on the cholesterol content of the membrane LRs, the fatty acid composition of the membrane, and the function of PI3K/protein kinase B signaling pathway[87]. These data gave support to speculations that AEA may be a potential antifibrogenic drug in the treatment of liver fibrosis in a LR-dependent manner.

Sonic hedgehog (Shh) is an embryonic morphogen that is key in cell proliferation, differentiation, and morphological patterning during embryogenesis; the Shh signaling pathway also promotes maintenance of adult stem cells and is involved in tumorigenesis[89]. In the hepatic SC line HSC8B, Shh physically interacts with caveolin-1 within the LRs in the Golgi apparatus to form large protein complexes that are packaged as large punctuate structures (transport vesicles) and transported to the PM in an LR-dependent manner[90]. Collectively these data suggest that LR manipulation can have a significant impact on liver SCs.

LRs in endothelial cells

Liver sinusoidal endothelial cells (ECs) isolate and protect the hepatocytes from passing blood and play an important role in hepatic microcirculation. Microvascular exchange in the liver is governed by fenestrations in sinusoidal ECs and is key to proper liver function. One of the basic properties of liver endothelium is the fact that there is no basal lamina, thus allowing free passage of macromolecules up to medium-sized chylomicrons[91]. The fenestrae are surrounded by a dense ring of actin,

whereas the sieve plates are formed by microtubules; both number and size of fenestrae can be regulated by a variety of processes that will impact on hepatic function. For example, loss of fenestrae or sinusoidal capillarization occurs in alcoholic liver disease (ALD) and cirrhosis, respectively[91,92]. While the details in the liver are unknown, in other vascular systems the ECs are heavily governed by the functionality of their LRs. Disruption of LRs in ECs causes loss of cell viability and altered cell morphology, including loss of fenestration[93,94]. LRs govern the function of EGFR, multi-drug resistance P-glycoprotein, and adhesion molecules in ECs, and are critical to a sound EC-gated vascular barrier, at least at the blood/brain interface[95-98]. LRs also house the NADPH oxidase components gp91, p22phox, and p47phox; several pro-inflammatory cytokines, such as TNF-α, employ a LR-dependent mechanism to trigger oxidative stress and eNOS production in ECs[99].

LRs in cholangiocytes

Cholangiocytes outline the intrahepatic bile ducts; they are of epithelial origin, share their common hepatoblast progenitor with the hepatocytes and, similar to them, are polarized cells[100]. The cholangiocytes function as regulators of ductal bile secretion; in addition, they absorb and secrete water, organic anions, organic cations, lipids, and electrolytes[101]. Cholangiocytes interact with the immune cells and are potent cytokine producers, thus playing a role in liver immune responses[100,101]. Several research groups have recently reported that polarized primary rat cholangiocytes express LRs[102,103]. McWilliams identified that Shank2E, which is an ankyrin repeat-rich multidomain scaffolder, is localized in the LRs of the apical surface of the cholangiocytes[103]. LRs not only host but are also vital for the function of Shank proteins, which involves coordination of the targeted delivery of the proteins to the apical membrane in actin-containing cytoskeleton-dependent fashion[103]. These novel findings point to the role of LRs in cholangiocyte function.

LRs in non-resident liver immune cells (dendritic cells, natural killer cells, lymphocytes)

At any given time healthy liver accommodates a wide variety of non-resident immune cells, including dendritic cells (DCs), natural killer (NK) cells and lymphocytes; selective recruitment and retention of certain immune populations occurs during diverse liver diseases and these cells play a critical role in development and resolution of liver inflammation, remodeling and injury[2]. All immune cells have LRs as components of their cellular membranes; more importantly, LRs participate in some of the key functions of the immune cells.

DCs recognize, engulf, process and present antigens to other immune cells while producing a wide array of immune-modulating factors, including cytokines, chemokines, and metabolites, to control the immune responses. LRs provide the signaling platforms for a variety of DC receptors, including TLRs, major histocompatibility complex molecules, and co-stimulatory molecules; disruption of LRs significantly impairs the functional capacity of DCs[104-106]. More recently, Wang *et al*[107] reported that high-density lipoprotein (HDL) promotes reverse cholesterol transport and is protective against dyslipidemia and atherosclerosis. Further, they reported that HDL and apolipoprotein A-I promoted tolerance and inhibited immune responses by inhibiting the ability of antigen presenting cells, and DCs in particular, to stimulate T cells in a cholesterol-dependent manner[107]. HDL-induced cholesterol efflux from cells alters their LR structure, which in turn activates the TNF-α converting enzyme ADAM17-dependent processing of transmembrane substrates[108]. These findings suggest that LRs may play a role in HDL-induced DC impairment and further regulate the inflammatory processes; these authors also suggested that modulation of LRs in DCs could provide a desirable approach to inducing immune tolerance[108].

NK cells link innate and adaptive immunity *via* the production of cytokines and have the ability to kill/lyse infected non-malignant and tumor cells. Unlike other immune cells, NKs are activated and inhibited by separate sets of receptors which ensure their rapid initiation and prompt silencing to promote immune response without autoimmune reactions. LRs are implicated in both activating and limiting steps of NK function. Upon CD2 cross-linking or target cell binding, the NK-activating receptors aggregate in the LRs, which further leads to the formation of complexes of LAT with PI3K and PLC-γ1 which are essential for the NK lytic mechanisms[109]. Moreover, NK cell cytotoxicity was found to be closely related to total plasma cholesterol concentration in humans[110]. Further, colocalization of the IL-12 receptor and FcγRIIIa to LRs leads to activation of ERK and enhanced production of interferon-γ by NKs[111]. Engagement of inhibitory receptors by HLA class I on target cells blocks phosphorylation of 2B4 receptor which is found exclusively in LRs. CD94/NKG2A is an ITIM-containing inhibitory receptor expressed by NK cells that recognizes HLA-E; the engagement of this receptor prevents NK cell activation by disruption of the actin network and exclusion of LRs from the point of contact with its ligand. The latter structure is an inhibitory NK cell immunological synapse (iNKIS) and is key to NK function. Thus, targeted LR exclusion from the iNKIS is an active process that aims to maintain LRs outside the inhibitory synapse[112,113]. These data suggest that LRs are actively engaged in dousing the NK activity. However, the DCs and NK cells work closely together; in the reverse order of events, activation of resting NK cells by mature DCs is important at the initiation phases of immune responses; more importantly, DC/NK cross-talk is dependent on CX3CL1, intact cytoskeleton and LRs[114].

Lymphocytes are classified into T and B cells, and further differentiated in diverse subpopulations depending on their abilities to produce certain cytokines or antibodies, respectively.

In T-lymphocytes, LRs are implicated in signaling from the T-cell antigen receptor (TCR) and in localization and function of proteins recruited/activated downstream

from the receptor. Statins (inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase) block cholesterol biosynthesis and reduce the pathogenesis of classical T-cell-inducing damage in experimental autoimmune encephalomyelitis by interfering with leukocyte recruitment, transmigration through vascular barriers and their adhesion, and ultimately limiting T-cell activation, as reviewed by Weber *et al*[115]. Polyunsaturated fatty acids (PUFAs) intercalate and thus remodel LRs; treatment of TCR-stimulated T-lymphocytes with PUFAs leads to miss-localization of LR-anchored TCR adaptor LAT, reduction in LAT tyrosine phosphorylation and low IL-2 production[116]. PUFAs inhibit the formation of the immune synapse[117]; it remains to be determined if this is due to isolated effects of PUFA on LRs of T cells, on DCs, or on both. Exposure of T cells to sialidases, which are able to hydrolyze the non-reducing terminal sialic acid linkage, led to control of LR-associated glycosphingolipid content and influenced activation in T-lymphocytes. Furthermore, sialidase inhibitor oseltamivir (Tamiflu) down-regulated the expression of LR-resident GM1 on the surface of T-cells and reduced cytokine production in activated T-cells[118,119]. These results suggest that LR-dependent modulation of T cells has translational potential.

In B lymphocytes, B-cell depleting anti-CD20 antibodies, namely rituximab, induced translocation of CD20 to the membrane LR fraction and triggered cell apoptosis; this process was prevented by LR disruption[120,121]. While the detailed mechanisms of such LR-mediated cytotoxicity of malignant B cells is not well understood, it is known that in normal B cells, trafficking of internalized Ag/B-cell receptor (BCR) complexes to intracellular Ag-processing compartments is driven by ubiquitination of the cytoplasmic domain of the BCR. The ubiquitinated Ag/BCR complexes are formed *via* a signaling-dependent mechanism and restricted to PM LRs[122]; the signaling from BCR is initiated only when the LR localization is accomplished. At this stage, Bright, which is a B-cell-restricted factor, forms complexes with Bruton's tyrosine kinase (Btk) and its substrate transcription initiation factor-I (TFII-I), and acts to activate immunoglobulin heavy chain gene transcription in the nucleus[123]. In resting B cells, palmitoylated pool of Bright is diverted to LRs where it associates with signalosome components. After BCR ligation, Bright transiently interacts with sumoylation enzymes, blocks calcium flux and interferes with phosphorylation of Btk and TFII-I; Bright is then excluded from LRs as a Sumo-I-modified form. The remaining LR-associated Bright contributes to the signaling threshold of B cells: the sensitivity to BCR stimulation decreases as the levels of Bright increase[123]. Thus, LRs play a key role in governing B cell function.

IMPLICATIONS OF LRS IN LIVER DISEASES

Bacterial and viral diseases

Role of LRs in bacterial infections involving liver: Listeria monocytogenes (LM) causes severe liver disease in children, pregnant women and in immunocompromised individuals. LM proteins internalin and InIB bind E-cadherin (E-CAD) and HGFR, respectively; engagement of E-CAD and HGFR are sufficient for LM invasion into the host hepatocyte[124]. More importantly, intact host LRs are needed to complete the E-CAD/HGFR-mediated entry of LM[124,125].

Elevated intestinal permeability is implicated in the pathogenesis of a wide variety of diseases, including those of liver[126,127]. While the translocation of whole bacteria across the intact intestinal wall is unusual, the inflamed gut is permissive to bacterial-derived products, including endotoxin, which acts as TLR4 ligand and is considered a 2nd hit in the pathogenesis of several liver diseases, including non-alcoholic fatty liver disease and ALD[12,13,126,127]. In this context, it has been hypothesized that gut colonization plays a key role in the pathogenesis of liver diseases. *Campylobacter* species represent a risk factor for the development of inflammation in the GI tract *via* largely unknown mechanisms. Kalischuk *et al*[128] reported that *Campylobacter jejuni* (*C. jejuni*) induced translocation of commensal intestinal bacteria to the liver of infected mice. *In vitro Campylobacter*-induced internalization and translocation of *Escherichia coli* (*E. coli*) occurred *via* a transcellular pathway without increasing epithelial permeability; this process was blocked by cholesterol depletion and thus LR impairment[128]. Invasion-defective mutants and *Campylobacter*-conditioned cell culture medium also favored *E. coli* translocation, indicating that *C. jejuni* does not directly "shuttle" other bacteria into enterocytes. In *C. jejuni*-treated epithelial monolayers, translocating *E. coli* was associated with LRs and this phenomenon was blocked by cholesterol depletion[128]. However, translocation of commensals does not require the presence of active infection with pathogens at the intestinal epithelial border. Clark *et al*[129] reported that interferon (IFN)-γ influences the epithelium to mediate transcellular translocation of *E. coli* C25; this process required intact LRs. These data suggest that intestinal LRs are key to establishing leaky gut, which in turn plays a role in the pathogenesis of liver diseases; it remains to be identified whether liver infection with translocated commensal intestinal bacteria is mediated by the LRs in hepatocytes.

Role of LRs in viral infections of liver: (1) Hepatitis C virus (HCV). The lifecycle of HCV is dependent on the integrity of LRs. Using a subgenomic HCV replicon system, Aizaki *et al*[130] showed that HCV RNA synthesis occurs in LRs. HCV replication complex is highly dependent on the abundance of cholesterol and it is protected within LRs; only LRs that contain both non-structural proteins and viral RNA were capable of performing HCV RNA synthesis using the endogenous HCV RNA template, while depletion of cellular cholesterol selectively reduced HCV RNA replication[130]. However, the virus has preferences for specific types of LRs. Core protein, for example, does not colocalize with classical PM LR markers, such as

caveolin-1 and the B subunit of cholera toxin, suggesting that core protein is bound to cytoplasmic raft microdomains distinct from caveolin-based LRs[131]. Furthermore, while both the structural core and NS5A protein associate with membranes, they do not always colocalize in the LR. Finally, the ability of core protein to localize to the LRs does not require other elements of the HCV polyprotein[131]. These results suggest that the LR implications in HCV lifecycle are complex and require further investigation. Nevertheless, HCV takes over LR control in the host cells and leads to modification of host LR-resident proteome upon its replication[131]. Roughly 10% of proteins residing in LRs of HUH7 cells are modified by HCV presence[132]. Interestingly, the majority of the host proteins involved in HCV recognition/internalization/sensing[133] including CD81, claudins, TLR2, LDL-R, Scavenger Receptor type B, and DC-SIGN/L-SIGN reside in LRs[134-140]. It is thus plausible to foresee that LR modulation may have a place in anti-HCV therapy; the proof of this hypothesis is awaited; (2) Hepatitis B virus (HBV). Funk *et al*[141] identified that duck hepatitis B virus (DHBV) attaches predominantly to detergent-soluble LR domains on the PM, but that cholesterol depletion from host membranes and thus disruption of LRs does not affect DHBV infection. In contrast, depletion of cholesterol from the envelope of both DHBV and human HBV strongly reduces virus infectivity[141]. Cholesterol depletion increases the density of viral particles and leads to changes in the ultrastructural appearance of the virus envelope; the infectivity and density of viral particles were partially restored upon cholesterol replenishment. Binding and entry of cholesterol-deficient DHBV into hepatocytes were not significantly impaired, in contrast to their release from endosomes. The authors therefore concluded that viral, but not host cholesterol is required for endosomal escape of DHBV[141]. Bremer *et al*[142] did not confirm a role of LRs in viral binding, but identified that LRs are indispensable for the entry process of HBV and might be important for a later step in viral uptake, such as fusion in a yet-unknown compartment.

Cholesterol accumulation/storage diseases

Niemann-Pick type C disease (NPCD) is a lysosomal storage disorder that affects the neural system and viscera, including liver[143,144]. Defective trafficking of cholesterol, sphingolipids and fatty acids leading to endosomal cholesterol sequestration coupled to impaired cholesterol transport to PM and ER were identified as key in the pathogenesis of NPCD[145]. Sequestration of cholesterol/sphingolipid-rich LRs in the late endosomal compartment was also reported in NPCD[146,147]. Vainio *et al*[76] reported a significant perturbation of LR composition and LR-dependent signaling in NPCD livers. They also showed that the membrane of NPCD hepatocytes is stabilized with cholesterol, exhibits higher anisotropy and resides in a more ordered phase; i.e. it is less fluid and thus has higher ratio of raft/non-raft content, compared to normal hepatocytes. More importantly, these rigid LRs lead to increased expression and enrichment of IR in the membrane of NPCD hepatocytes. Further, IR redistribution was associated with its defective function, thus aggravating the metabolic syndrome in NPCD[76].

Alcohol-induced liver disease

Alcohol exposure is associated with a significant amount of ROS production, which leads to oxidative stress[148-156]. While the generation of alcohol-induced oxidative stress is well documented, the understanding of the effects of oxidative stress on different molecular systems is poor. Marquês *et al*[46] employed *in situ* AFM to show that low ethanol concentrations lead to a marked thinning of the fluid but not of the gel domains of the model membranes, due to water/bilayer interfacial tension variation and freezing point depression, induction of acyl chain disordering (including opening and looping), tilting, and interdigitation. These results suggested that ethanol influences the bilayer properties by altering the lateral organization of the membrane. Several groups have confirmed the pivotal role of membrane fluidity in ethanol-induced oxidative stress using both *in vitro* and *in vivo* models of alcohol exposure[150-156]; however, the role of LRs *per se* in this process is still under evaluated.

In primary rat hepatocytes, short-term alcohol exposure increases membrane fluidity and leads to ethanol-induced ROS generation, which interferes with mitochondria, ER, cytochrome P450, cytosolic free iron, and cytosolic enzymes such as xanthine oxidase or aldehyde oxidases[153,154,157]. More recently, it was appreciated that LRs play a pivotal role in ethanol-induced oxidative stress in hepatocytes[152]. Alcohol exposure modulates the LR composition of hepatocytes, causes spontaneous clustering of LRs due to elevation in membrane fluidity, and promotes the increase of low-molecular-weight iron intracellular content *via* the activation of a PI-PLC-dependent mechanism. Further, ethanol oxidizes the LR proteins, as indicated by the increased LR content of malondialdehyde-acetaldehyde (MDA) adduct, which is a secondary end product of the degradation of oxidized PUFAs that can diffuse from its production site to react with free amino groups in proteins and lipids. From the therapeutic point of view, thiourea and vitamin E pretreatment inhibited the alcohol-induced MDA adduct formation in LRs[152]. The effects of chronic alcohol exposure on LR function in hepatocytes are largely unknown.

Alcohol exposure affects the Shh signaling in an *in vivo* model of fetal alcohol syndrome in zebrafish[90]. Various cell function alterations due to changes in cell membrane composition in alcohol-induced fetal damage have also been described[15]. Mao *et al*[90] identified that alcohol does not significantly interrupt translation of Shh mRNA in ER or the trafficking of Shh from the ER to the Golgi apparatus in the SC line HSC8B. However, alcohol does prevent the entry of Shh into transport vesicles from Golgi to PM and specifically decreases the amount of caveolin-1/Shh complex found in LRs, causing cytoplasmic accumulation of Shh and leading to a deficiency of Shh

ligand secretion into the extracellular matrix[90]. These data indicate that SC-mediated tissue remodeling upon alcohol exposure involves LRs.

In macrophages, and presumably KCs, acute alcohol exposure dampens the inflammatory response to pathogens *via* TLRs in a LR-dependent manner and involves downregulation of MAPK activity, low nuclear factor κB activity and impaired TNF-α production[81,82,158]. Such profound inhibitory effects could be envisioned as a protective measure; the effects of chronic alcohol exposure on LR function in Mf are largely unknown.

RAFTS AS THERAPEUTIC TARGETS

Development of new treatment strategies using low-toxicity high-efficiency chemotherapeutic agents is a forefront priority in research. Several novel approaches to therapeutic agents are based on LRs. In drug discovery, a viable strategy to block viral entry and its replication may incorporate the use of natural dietary and plant-derived compounds that target LRs; this strategy is based on the affinity of these products to cholesterol, as proposed in a recent review by Verma regarding HIV[159]. The nanoparticle-based drug-delivery approach for delivery of lipophilic substances to the target cell PM acts *via* lipid mixing and subsequent intracellular trafficking through LR-dependent processes[160]; the advantage of the latter strategy is in its ability to deliver therapeutics specifically to selected cell types, thus limiting general toxicity.

Regulation of cell death is yet another area influenced by LR modulation. Cell death occurs at low rate in normal tissue and stimulates tissue regeneration as part of normal organ lifecycle; when the tissue is malignant the cell death is deliberately desired. LRs ensure the connection between extrinsic and intrinsic apoptotic pathways[161,162], thus become an attractive therapeutic target. The Fas death receptor (CD95 or APO-1) delivers apoptotic signals through binding to its cognate ligand, FasL (CD95L). However, because of severe liver toxicity due to the high presence of Fas in hepatocytes, the putative clinical antitumor action of FasL cannot be accomplished for extra-hepatic tumors; the utility of this approach for liver tumors is yet to be defined. Recent evidence for FasL-independent activation of Fas suggests that the death receptor can also be activated intracellularly, in the absence of its ligand. According to Mollinedo *et al*[161], Fas-mediated apoptosis involves translocation of Fas - and downstream signaling molecules - into LRs, a process that can be pharmacologically modulated. FasL-independent clustering of Fas in membrane LRs generates high local concentrations of death receptor, providing scaffolds for coupling adaptor and effector proteins involved in Fas-mediated apoptosis. Thus, LRs act as the linchpin from which a potent death signal is launched and have become a new promising anticancer target[161-164]. The utility of this therapeutic approach has been explored in B cell malignancy[120,121,163] and seems plausible for other diseases.

Sakamoto *et al*[165] reported that a secondary fungal metabolite, NA255, which targets LRs, also inhibits HCV replication and suggested that the inhibition of sphingolipid metabolism and thus LR modulation may provide a new therapeutic strategy for treatment of HCV infection. Hirata showed that serine palmitoyltransferase (SerPT) inhibitor had the ability to favor the transport of HCV RNA-dependent RNA polymerase NS5B to LRs, thus facilitating binding of sphingomyelin to NS5B[166]. Further, the authors identified that the anti-HCV effect of SerPT inhibitor could be translated to *in vivo* systems using humanized chimeric mice: SerPT inhibitor led to a rapid decline in serum HCV-RNA of about 1-2 log within 8 d. Furthermore, combination therapy of SerPT inhibitor and PEG-IFN achieved about 3 log reduction in serum HCV-RNA[166]. Several research groups have reported that lipid metabolism could be a target in anti-HCV therapy[165-167]. Amemiya *et al*[167] used combination treatment with myriocin, a sphingomyelin synthesis inhibitor, and IFN, a pleiotropic cytokine, or myriocin and simvastatin, an inhibitor of cholesterol biosynthesis with pleiotropic effects owed to inhibition of prenylation, to show synergistically attenuated HCV replication. They also identified impaired replication of HCV-1b replicon and of JFH-1 strain of genotype 2a infectious HCV RNA in Huh7/Rep-Feo cells, upon treatment with either myriocin-based combination[167]. While encouraging, these data do not fully translate into human systems: O'Leary *et al*[168] recently reported that statins alone do not exhibit antiviral activity against HCV at conventional doses.

CONCLUSION

In conclusion, we have compiled the published evidence that LRs are present in all liver cells; more importantly, data suggest that LRs play a role in liver health and diseases. The LR area of liver physiology and pathology is understudied. In order to facilitate exploration of this area future improvements are needed, the foremost of which are listed below: (1) Better markers and better methodologies for LR research; (2) New drugs for LR modulation; and (3) Experimental data-supported evidence of the feasibility and outcomes of LR modulation in liver health and disease.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2536

World J Gastroenterol 2011 May 28; 17(20): 2536-2542
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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microRNAs: Fad or future of liver disease

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Supported by Grants from NIH (DK38825, HLB; AA014891, LWS) and by institutional funds from CMC
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Received: January 5, 2011 Revised: March 23, 2011
Accepted: March 30, 2011
Published online: May 28, 2011

Abstract

microRNAs (miRs) are small non-coding RNAs that regulate both mRNA and protein expression of target genes, which results in alterations in mRNA stability or translation inhibition. miRs influence at least one third of all human transcripts and are known regulators of various important cellular growth and differentiation factors. miRs have recently emerged as key regulatory molecules in chronic liver disease. This review details recent contributions to the field of miRs that influence liver development and the broad spectrum of disease, from non-alcoholic fatty liver disease to fibrosis/cirrhosis, with particular emphasis on hepatic stellate cells and potential use of miRs as therapeutic tools.

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Key words: Liver; Fibrosis; microRNA; mRNA; Hepatic stellate cells

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Lakner AM, Bonkovsky HL, Schrum LW. microRNAs: Fad or future of liver disease. *World J Gastroenterol* 2011; 17(20): 2536-2542 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2536.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2536

INTRODUCTION

Post-transcriptional regulation of gene expression involves numerous modifications to the mRNA, including alterations at both 5' and 3' ends in addition to splicing of the transcripts. The 3' untranslated regions (UTRs) of mRNAs contain key stability elements that are subject to various regulatory proteins, as well as microRNA (miR) binding sites. miRs are small non-coding RNAs that alter gene expression through perfect complementarity of the miR to the target sequence, which results in mRNA instability and subsequent endonucleolytic cleavage or alternate 5' modifications[1]. Imperfect base pairing with the 3' UTR of target mRNA results in gene translation inhibition[2]. Research focusing on miR modes of action has primarily examined interactions with the 3' UTR of target genes; however, recent studies have expanded our knowledge and have demonstrated that miRs can interact with the 5' UTR as well as interact with DNA methylation machinery and affect chromatin status[3]. Since their discovery in 1993, miRs have been described in all multicellular organisms

and are associated with a vast breadth of biological functions including proliferation, cellular differentiation and immunity, as well as tissue remodeling and various human disease states, notably cancer[4]. Although much remains to be learned, miRs have already been shown to influence the expression of many mRNAs and proteins that are important in liver homeostasis in health and disease (Table 1). Emerging roles for miRs in liver development and disease are discussed in this review, as well as their potential for clinical application.

miRs IN LIVER DEVELOPMENT AND DISEASE

Liver development

miRs, while currently being exploited to understand disease pathologies, have been less appreciated in the role of functional gene repression during cellular growth and liver development. miRs, specifically let 7 and lin4, control larval development transition in *Caenorhabditis elegans*, as well as apoptotic pathways and growth in *Drosophila*[5]. Recent studies have elegantly demonstrated that miRs regulate differentiation of human cells and development of whole organ systems, which shows tissue-specific expression. miR 122 accounts for approximately 70% of the total liver miR population; however, specific roles for this miR outside of disease-associated functions [e.g. hepatitis C virus (HCV) and hepatocellular carcinoma (HCC)] are less clear, especially with regard to normal liver development. Xu *et al*[6] have shown that miR 122 is strongly upregulated during liver embryonic development with expression correlative to liver enriched transcription factors (LETFs) C/EBPα, HNF1α and HNF3 isoforms, which are all necessary for proper hepatocyte development and function. Additionally, the transcriptional repressor CUTL1, which is known to promote proliferation and suppress differentiation, is gradually repressed by miR 122 during development. *In vitro* miR 122 knockdown experiments have shown restoration of CUTL1 expression and of downstream target genes, which emphasizes that, during development, miR 122, as an effector molecule of LETFs, regulates a delicate balance between differentiation and proliferation[6]. Quantitative analysis of miRs in fetal *vs* adult livers has demonstrated that miR expression levels are higher in the fetal period and expression levels during this time are dynamic[5]. Additionally, recent studies have demonstrated that 162 miRs exhibit tissue-specific distribution in mice[7]. Endoderm-derived liver, jejunum and pancreas display differential miR enrichment, with miRs 122, 21, 101b, 107, 192 and 221 among those with the highest reads per million concentrations. Additionally, mapping of transcriptional start sites has demonstrated that divergent sites are used by miRs in endoderm-derived liver tissue as opposed to stem cells. Maternal influences on miR expression have also been identified and may be affected by diet. For example, studies by Zhang *et al*[8] have shown that, as a result of maternal high-fat diet, expression of miR let 7c is decreased in offspring, along with 23 other miRs including 122 and 194. let 7c was identified by Tzur *et al*[9] as being highly expressed in adult compared to embryonic liver, and that profibrotic transforming growth factor-β receptor 1 is a target of this miR. The miR 30 family has recently been shown to be crucial for liver development, because it is upregulated during later stages that influence known regulators of hepatic function, including epidermal growth factor receptor. GW182, a crucial component in miR-mediated gene repression, is also a target of this miR family, which indicates a more global role for miRs in earlier stages of epigenetic silencing *via* the RNA-induced silencing complex[10]. Functional analyses in zebrafish larvae have demonstrated that biliary morphogenesis is under the regulation of miR 30a, as knockdown studies have resulted in defective bile duct excretion[11]. The liver, as a vital multifunctional organ, must maintain proper epigenetic programming to ensure xenobiotic and bile metabolism, and vitamin and glucose storage among a plethora of other tasks, which emphasizes the importance of proper cellular differentiation and development.

Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation without significant alcohol consumption or other underlying etiology[12]. NAFLD incidence is rising as a result of the increased population of obese individuals worldwide, with severity of the disease ranging from steatosis/steatohepatitis (NASH) to cirrhosis. Although most patients with NAFLD are asymptomatic, clinical manifestations of the disease are well described and include hyperechoic appearance of the liver on ultrasound, dyslipidemia, systemic arterial hypertension, and insulin resistance. Although disease pathogenesis is not completely understood, recently, a role for miRs in NAFLD has been established[13]. Lipid droplet accumulation in hepatocytes as a marker of steatosis is associated with various liver diseases including NAFLD. Recent high-content screening studies have shown that 11 miRs are able to alter lipid droplet formation/retention[14]. Specifically, miR 181d decreased droplets by approximately 60%, which subsequently reduced cellular triglycerides and cholesterol. Peroxisome proliferator-activated receptor (PPAR)α, a major contributor to NAFLD pathogenesis, has recently been identified as a target of miR 10b, which has been shown to regulate steatosis level in L02 cells (steatotic hepatocyte model)[15]. Additionally, post-transcriptional regulation of PPARα by miR 10b is maintained by a single binding site, as demonstrated by mutation analyses. NASH can progress to end-stage cirrhosis in approximately 15% of patients, with the mechanism that underlies disease progression being undefined. Recent studies in humans have demonstrated that miR expression profiles are altered in NASH. Unsurprisingly, miR 122 is significantly underexpressed (63%) in NASH subjects compared to those without the metabolic syndrome[16]. Overexpression of miR 122 in HepG2 cells results in a significant decrease in sterol regulatory element binding proteins (SREBP1-c, SREBP2), FAS and 3-hydroxy-3-methyl-glutaryl-CoA reductase; all of which are key lipogenic genes in human NASH. Lesser known

Table 1 Differentially expressed microRNAs in liver development and disease

Liver status	Upregulated miRs	Downregulated miRs	Ref.
Development	122, 92a, 483, 486-5p, 30 family	22, let 7 family, 199a, 21	[6,21]
NASH/NAFLD	34a, 146b, 200a, 224, 222, 10b, 22, 33, 31, 29c	122, 181d, 132, 150, 28-3p, 511, 517a, 671-3p, 99b, 433	[14-16]
HCV	122, let 7f, 155, 146, 296, 351, 128, 296, 141	196, 15a, 15b, 17, 106a, 106b, 181a, 29a, 29b, 93, 310, 30a, 30c, 24, 221, 222	[24,26,27]
ALD	705, 1224, 212	182, 183, 199a-3p, 199, NSC/NPC: 9, 21, 153, 335	[18,34]
Fibrosis	125-5p, 199b, 221, 302c, 223, 34c, 24	29a, 29b, 29c, 30b, 30c, 183, 96, 877, 341, 193, 132	[40,42]
Regeneration	21, 130a, 181b, 20a, 20b	378, 689, 7	[48,49]

NSC: Neural stem cell; NPC: Neural progenitor cell; miRs: microRNAs; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

miRs 34a and 146b are significantly overexpressed (99 and 80%, respectively) in NASH. In a smaller sample study (n = 12/group), seven miRs have been found to be differentially expressed, including 132, 150, 197 and 99; the latter two are significantly associated with pericellular fibrosis[17]. It is of particular importance to note that previous studies have shown that miR signatures change in accordance with disease stage and source of hepatic injury (ethanol or high fat diet)[18,19]. These studies emphasize the importance of avoiding generalization when examining miRs as potential biomarkers, because miR expression patterns of end-stage disease may be similar but expression profiles that lead to organ dysfunction appear to be divergent.

Viral infection

Along with NASH, HCV is one of the most common global causes of hepatic fibrosis and cirrhosis. According to the World Health Organization, over 180 million people are infected with the single-stranded RNA virus, with > 70% of that population at risk for developing cirrhosis[20]. Pegylated interferons in conjunction with ribavirin have improved treatment of chronic hepatitis C; however, a large percentage of the infected population remains unresponsive to available regimens, which emphasizes the need to better understand the mechanisms of cellular infection, for development of novel drug therapies. Numerous studies have emphasized a role for miR 122 in several aspects of hepatic function and disease, including HCV replication[21]. Plasma levels of miR 122 are elevated in hepatitis B virus (HBV)- and HCV-infected patients, as well as in models of alcohol and drug-induced liver damage reinforcing a role for miRs as biomarkers[22]. However, recent studies by Ura *et al*[23] have shown that miR expression patterns are divergent in HBV- and HCV-infected livers. Additionally, elegant studies by Hou *et al*[24] have demonstrated that miR 196 directly acts on the 3'UTR of Bach1, the transcriptional repressor of heme oxygenase 1, a critical cytoprotective enzyme. This miR also inhibits HCV expression (both genotype 1b and 2a) in human hepatocytes *in vitro*, which shows clinical promise. Accordingly, parallel miR and mRNA expression profiling studies in genotype-1b-expressing cells have shown numerous anti-correlated miR/mRNA pairs affected by the presence HCV[25]. Among the noted anti-correlated pairs that have been detected in a human cell line that expresses the HCV Con1 replicon, miR 130a/b is correlated with decreased PPARγ expression, which is a known regulator of the quiescent hepatic stellate cell (HSC) profile. Additional miR/mRNA profiling in HCV-infected hepatoma cells has shown a total of 108 differentially expressed miRs, and among this pool, miRs 24, 149, 638 and 1182 are predicted to control HCV entry, replication and viral propagation[26]. Scagnolari *et al*[27] have examined miR expression in peripheral blood mononuclear cells from healthy and chronic-HCV-infected patients and have found that miRs 1, 30, 128, 196 and 296 are differentially expressed and that treatment with interferon (IFN)α induces all aforementioned miRs. Although limited by small sample size, baseline levels of miRs do not appear to differ significantly between responders and non-responders; however, expression of miRs 128 and 196 appears higher in the responsive population. Additionally, Pedersen *et al*[28] have demonstrated this same set of miRs, which possess sequence-predicted targets within HCV genomic RNA, and are induced by IFNβ stimulation. The tumor suppressor gene DLC-1 (deleted in liver cancer-1) is also a target of miR repression[29]. Levels of DLC-1 are decreased in HCV-infected cells, which are correlated with the increase in miR 141. Depletion of this miR inhibits viral replication, while addition of exogenous miR 141 increases replication rates, which indicates that miR regulation of tumor suppressor genes can influence HCV infection and play a role in HCC development (for a comprehensive review of miRs in HCC see[30,31]).

Alcoholic liver disease

Alcoholic liver disease (ALD) is a major healthcare burden worldwide and a leading cause of liver-related mortality. It is well known that ethanol consumption alters methylation status and other epigenetic factors, including histone tail signatures, signal transduction pathways and transcription factor expression. Ethanol metabolism affects chromatin condensation, which results from direct DNA methylation or histone protein alterations (acetylation, methylation or phosphorylation)[32]. Additionally, DNA methylation is connected to direct interference by small RNAs[33]. Therefore, it is plausible that, as a result of epigenetic remodeling, ethanol exposure can also alter miR expression. Ethanol has been shown to repress miRs 9, 21, 153 and 335 in neural stem cells and neural progenitor cells that alter cellular development and maturation[34]. Additionally, miR 21 influences PTEN/PI3K signaling, which ultimately contributes to the fibrotic response, and both miRs 9 and 335 are predicted (by TargetScan) to bind to targets

that influence the progression of ALD[35]. Previous studies have highlighted the importance of miRs in the gut-liver axis and have shown that ethanol exposure alters miR profiles in liver cells[36]. Specifically, endotoxemia and direct ethanol exposure modulate hepatic miRs 182, 183, 705, 1224 and 199a-3p[37]. Symptomatic of ALD, hepatic microcirculation is disrupted when increased portal pressure arises as a result of altered endothelin-1 (ET-1) expression, with subsequent increases in NADPH oxidase and hypoxia inducible factor-1α[38]. Recent studies have shown that miR 199 is involved in ET-1 expression in rat liver sinusoidal endothelial cells and human endothelial cells, by functioning as a negative regulator of ET-1 transcription and thus vascular tone in ALD[39]. For still unknown reasons, only 20%-30% of chronic heavy alcohol users develop hepatic fibrosis. Future studies of miRs involved in ALD may allow us to uncover additional factors that contribute to the disparity that exists in disease development.

Liver fibrosis

Commonalities of miR expression can also be seen among fibrotic disorders of different organ systems, including cardiac and renal fibrosis, with an overarching effect of the miR 29 family in regulation of effector cellular differentiation or in translation of extracellular matrix (ECM) components[35]. Additionally, miRs involved with translation of profibrotic transforming growth factor (TGF)β/SMAD signaling are shared amongst pathologies. Roderburg *et al*[40] have examined miR expression profiles in a CCl_4 rodent model of hepatic fibrosis, and microarray analyses have revealed 31 differentially expressed miRs; 10 of which are overexpressed in fibrotic tissue, including miRs 125-p, 199b, 221 and 302c. Marked downregulation has been observed in a pool of 21 miRs; specifically, miR 29 family members were significantly reduced. Additional experiments in an alternate model of fibrosis (bile duct ligation; BDL) have confirmed that miRs 29b/c are significantly reduced compared to sham controls. Downregulation of miRs 29a/b/c could also be observed in explanted liver samples with patients denoted by a Desmet fibrosis score of 2-4. Low plasma levels of miR 29a correlate with advanced stage liver fibrosis in human patients. The idea that low miR levels in the tissue correlate with low miR in the circulating plasma is in opposition to recent hypotheses that an inverse correlation between tissue and plasma miR concentrations exists as a byproduct of vesicles that release miRs[41]. Hepatic fibrosis is influenced by several epigenetic factors that control the wound-healing response. Recent studies by Mann *et al*[42] have shown that miR 132 is significantly decreased in fibrotic livers as demonstrated in two different models (BDL, CCl_4), and this downregulation influences HSC activation. Collectively, these studies have emphasized that, during development of fibrosis, there are important changes in miR expression that regulate wound-healing transcripts; however, effects exerted by miRs appear to act in concert with other epigenetic factors to direct disease progression.

miRs IN HSCs

HSCs are the main effector cell population in hepatic fibrosis as the primary source of type I collagen deposition following injury. Injury from several sources, including viral infection, obesity and alcohol consumption, causes activation of HSCs, in which quiescent lipid-rich cells transdifferentiate into fully activated myofibroblasts. The activated form of the HSC secretes profibrogenic mediators, including TGFβ, and generates ECM components including fibrillar collagens, fibronectin and laminin, which exacerbates the wound-healing process[38]. Recent studies have shown that miRs are involved in HSC transdifferentiation (Figure 1). Specifically, miRs 150, 187, 194 and 207 are significantly downregulated in HSCs isolated from BDL animals compared to sham controls, whereas let 7 family members are significantly upregulated[43]. Overexpression of miRs 150 and 194 in human HSCs (LX-2) results in proliferation inhibition as well as decreases in type I collagen and smooth muscle alpha actin (αSMA), which are hallmarks of HSC activation. Specific action of these two miRs includes inhibition of c-Myb and Ras-related C3 botulinum toxin substrate 1 (Rac-1), which both contribute to development and progression of fibrosis. Additional studies have examined differential expression in quiescent (day 2) and activated (day 14) rat HSCs, and have shown 12 upregulated and nine downregulated miRs, of which, 15b, 16, 122, 138, 140 and 143 have been validated[44,45]. Gene ontology has revealed specific linkages between the miR 15/16 family and anti-apoptotic pathways that are important in HSC activation. Administration of miR mimics induced Bcl-2 inhibition and subsequent apoptosis in the activated HSCs, an important aspect of potential therapeutic targeting. Additional studies by Guo *et al*[44] have further demonstrated that lentiviral delivery of miR 16 greatly reduces cyclin D1 levels in addition to inhibiting proliferation and increasing apoptosis in activated HSCs. More recent studies by Ogawa *et al*[46] have shown

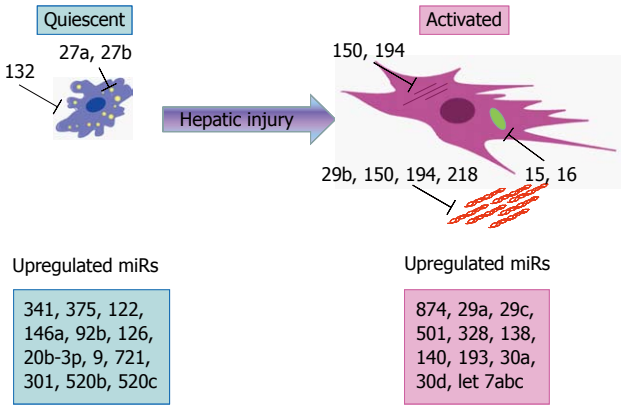

Figure 1 microRNAs involved in hepatic stellate cell transdifferentiation. Functional manipulation studies utilizing mimics and/or antagomirs have demonstrated that the miRs depicted in the above schematic regulate key genes/functions in hepatic stellate cells (HSCs). (Quiescent HSC: yellow circles represent cytoplasmic lipid droplets; activated HSC: purple lines indicate cytoskeletal protein smooth muscle alpha actin; green oval represents Bcl-2; red fibrils represent collagen). Additional profiling studies have shown upregulation of several microRNAs (miRs) in both phenotypes, some of which are already associated with hepatic disease (boxes contain a small fraction of published miRs). References[38-43,47] were used to generate the contents of this figure with design software BioDraw Ultra 12.0.

that TGFβ1 or IFNα stimulation validate *in silico* analyses of miR-pro-collagen [Colα1 (Ⅰ)] mRNA binding sites. miRs 29b, 143 and 218 demonstrate the highest degree of homology to the Colα1 (Ⅰ) 3'UTR and have been further analyzed. miR 29b directly bound to the 3'UTR suppresses type Ⅰ collagen at the mRNA and protein levels. Potential antifibrotic benefit to miR 29b has also been validated *in vivo* using a murine CCl_4 model of injury (see Liver fibrosis section). *In vitro* studies conducted by Roderburg *et al*[40] have also shown that overexpression of miR-29b in murine HSCs results in decreased collagen expression and that lipopolysaccharide and nuclear factor (NF)κB are involved in downregulation of this miR. The process of HSC activation includes the methyl CpG binding protein MeCP2 and constituents of the polycomb repressive complex. More recently, miR 132 has been shown to activate this epigenetic pathway because its downregulation, observed in fibrotic livers, permits translation of MeCP2, which is subsequently recruited to the 5' end of PPARγ, and through altered methylation patterns, it confers suppression of the quiescent profile[42]. Previously studies by Ji *et al*[47] showed miRs 27a and b are upregulated during activation and directly repress RXRalpha. Inactivation of these miRs in vitro showed partial reversion of the cell to a quiescent phenotype with restoration of lipid droplet accumulation. Improved understanding of the regulation of HSC activation by miRs will undoubtedly advance our knowledge of many liver etiologies.

miRs IN LIVER REGENERATION

As a result of hepatic injury, hepatocytes are capable of undergoing multiple divisions, and in the case of partial (2/3) hepatectomy (PH), liver mass is restored within 7 d[48]. Liver regeneration is a complex process that involves intricate cytokine and growth factor signaling influencing cell cycle progression. Recent studies have begun to examine the role of miRs in this process, to uncover underlying mechanisms that may be manipulated to ensure rapid tissue repair. miR-deficient mice (DGCR8 inactivated) that have undergone PH are clearly delayed in cell cycle progression; specifically G1 to S phase transition[48]. Examination of differentially expressed miRs in wild-type mice has revealed marked induction of miR 21 at 18 h post-PH, and downregulation of miR 378. These studies also have shown that miR 21 targets cell cycle inhibitor B cell translocation gene 2 (*Btg2*), which prevents DNA synthesis in hepatocytes, whereas miR 378 inhibits ornithine decarboxylase (*Odc1*), a promoter of DNA synthesis. Studies by Castro *et al*[49] have confirmed that miR 21 expression is increased following PH, and can be modulated by ursodeoxycholic acid (UDCA), a pro-survival agent in hepatic regeneration. miR profiles from rats that have undergone 70% PH have shown that miR 21 levels are highest at 24, 36 and 72 h post-hepatectomy. Inhibition of this miR reduces hepatocyte proliferation and increases levels of lactate dehydrogenase. UDCA treatments increase miR 21 as well as several others, including 143 and 151, which points towards a mechanism of action of the compound. As previously noted, miR 21 has been shown to repress PTEN signaling, the negative repressor of the AKT pathway that is necessary for hepatic regeneration. Additional experiments with miR 21 have shown that it is upregulated in early stages of regeneration and its overexpression inhibits NF-κB signaling[50]. Although the miR signature following 50% PH is slightly distinct from published data on 2/3 PH, miR 21 as well as miRs 22a, 26a, 30b and members of the let 7 family, which are already known to play a role in hepatic tissue development, are also differentially expressed[51]. Although key regulatory factors that govern organ regeneration are mostly unknown, recent studies in zebrafish have shown that suppression of miRs 133 and 203 is required for appendage regeneration; however, this process is mediated by stem cells in contrast to fully differentiated hepatocytes that are necessary to regenerate mammalian liver tissue. Enhanced knowledge of miRs that regulate hepatocyte proliferation following PH may also allow restoration of this function in diseased parenchymal cells[48].

CLINICAL APPLICATION OF miRs

Currently, there are no established FDA-approved treatments for hepatic fibrosis. Outside of surgical resection, full organ transplantation is an option for certain disease states; however, the current list of patients in need of transplant far exceeds the annual donor rate. As is always the case, the clinical applications of new discoveries, such as the existence and manifold roles of miRs in liver physiology and pathophysiology, lag behind the laboratory-based discoveries summarized above. Thus, currently, profiles of miRs and creation of miRs in various liver diseases, and the use of alterations in these as prognostic indicators or for categorization of liver diseases, have not been introduced into routine clinical practice. Nevertheless, it seems likely that, within the next 3-5 years, some centers and physicians will begin to use miR profiling to help categorize patients at higher or lower risk of adverse outcomes, especially from acute liver diseases, such as drug- or toxin-induced injury or fulminant liver failure of any cause. Another likely application will be the profiling of miR expression in hepatic tumors as an aid to prognosis, and perhaps, to help guide therapeutic decisions. However, it seems unlikely that such profiling will become generally available for widespread clinical application within the next 10 years, because many more studies with large numbers of subjects will be needed, with demonstration that such specialized profiling offers clear advantages in improving outcomes at reasonable and justifiable incremental costs.

What seems more likely is that therapy directed at influencing the hepatic levels of selected miRs such as miR 122, already shown to play an important role in affecting levels of serum cholesterol and levels of the HCV, both in cell culture[52,53] and chimpanzee models[54], will find a place in clinical therapeutics. Indeed, phase 2 clinical studies of locked nucleic acids that downregulate miR 122 expression are already in progress, and results of these are awaited with great interest. Similarly, therapeutic alteration of miR 196[24] and, in future, other miRs seems likely also to be pursued. However, we must also keep in mind

the high costs and lengthy duration usually needed for approvals of all new drugs. Also, the unwanted side effects of such treatments may limit their clinical application.

CONCLUSION

Thus, at this juncture, miRs are clearly more than just the latest fad. They are important and fundamental modulators of mRNA and protein expression. In future, purposeful modulation of these levels and effects will, more likely than not, become important in the therapeutic armamentarium of hepatic and other diseases. Although the clinicopathological and prognostic value of miRs remains highly anticipated, further exploration into systemic effects of miR modulation, both endogenously and exogenously, is needed, as well as a deeper understanding of miR biogenesis in both the development and progression of liver diseases.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2543

World J Gastroenterol 2011 May 28; 17(20): 2543-2551
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Hepatic stellate cells and innate immunity in alcoholic liver disease

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Author contributions: Suh YG and Jeong WI contributed equally to the writing of the manuscript.

Supported by A grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, South Korea (A090183)

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Received: January 7, 2011 Revised: February 25, 2011
Accepted: March 4, 2011
Published online: May 28, 2011

Abstract

Constant alcohol consumption is a major cause of chronic liver disease, and there has been a growing concern regarding the increased mortality rates worldwide. Alcoholic liver diseases (ALDs) range from mild to more severe conditions, such as steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. The liver is enriched with innate immune cells (e.g. natural killer cells and Kupffer cells) and hepatic stellate cells (HSCs), and interestingly, emerging evidence suggests that innate immunity contributes to the development of ALDs (e.g. steatohepatitis and liver fibrosis). Indeed, HSCs play a crucial role in alcoholic steatosis *via* production of endocannabinoid and retinol metabolites. This review describes the roles of the innate immunity and HSCs in the pathogenesis of ALDs, and suggests therapeutic targets and strategies to assist in the reduction of ALD.

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Key words: Alcoholic liver disease; Hepatic stellate cell; Natural killer cell; Kupffer cell; Endocannabinoid; Steatosis; Steatohepatitis; Fibrosis

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Suh YG, Jeong WI. Hepatic stellate cells and innate immunity in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2543-2551 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2543.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2543

INTRODUCTION

Alcoholic liver disease (ALD) caused by chronic alcohol consumption shows increased mortality rates worldwide[1,2]. As an adverse risk factor of alcohol abuse, ALD includes a broad spectrum of liver diseases, ranging from steatosis (fatty liver), steatohepatitis, fibrosis, and cirrhosis to hepatocellular carcinoma[3,4]. Generally, steatosis is considered to be a mild or reversible condition, whereas steatohepatitis is a pathogenic condition, which has the potential to progress into more severe diseases, such as liver fibrosis/cirrhosis, insulin resistance, and metabolic syndrome in rodents and humans[5-7]. For the past decade, evidence has suggested that the innate immune cells of liver and hepatic stellate cells (HSCs) play crucial roles in ALD. For example, previous studies demonstrated that alcoholic liver steatosis was induced by HSC-derived endocannabinoid and its hepatic CB1 receptor, and alcoholic liver fibrosis was accelerated due to abrogated antifibrotic effects of natural killer (NK) cells/interferon-γ (IFN-γ) against activated HSCs *via* the upregulation of transforming growth factor-β (TGF-β) and suppressor of cytokine

signaling 1 (SOCS1)[8,9]. However, the molecular and cellular mechanisms underlying ALD remain controversial[4,6,10]. Therefore, in the present review, we briefly describe the innate immunity of liver and HSCs, summarize the roles of these in ALD (with particular emphasis on alcoholic liver steatosis, steatohepatitis and liver fibrosis), and provide better strategies for the prevention and treatment of ALD.

INNATE IMMUNITY AND HSC IN LIVER

The innate immune system is the first line of defense against pathogenic microbes and other dangerous insults, such as tissue injury, stress, and foreign bodies[11]. It consists of three sub-barriers: physical (e.g. mucous membrane and skin), chemical (e.g. secreted enzymes for antimicrobial activity and stomach HCL), and cellular barriers (e.g. humoral factors, phagocytic cells, lymphocytic cells, *etc*), which immediately respond to the pathogens entering the body. Most body defense cells have pattern recognition receptors (PRRs) that recognize the overall molecular patterns of pathogens, known as pathogen associated molecular patterns. The examples of PRRs are toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors, and the retinoic acid-induced gene I-like helicases[12].

When extraneous molecules enter the human body, they have to be processed by the liver, either by metabolism or detoxification. Therefore, the liver is considered as a barrier against pathogens, toxins, and nutrients absorbed from the gut *via* the portal circulation system. Consequently, the liver is enriched in innate immune system including humoral factors (e.g. complement and interferon), phagocytic cells (e.g. Kupffer cells and neutrophils), and lymphocytes [e.g. NK cells, natural killer T (NKT) cells and T cell receptor γδ T cells][11,13-15]. In a healthy liver, the principal phagocytic cells, the Kupffer cells, representing 20% of the non-parenchymal cells (NPC), assist in the clearance of wastes *via* phagocytosis in the body[15,16]. However, when the liver is injured, Kupffer cells elicit immune and inflammatory responses (e.g. hepatitis, fibrosis, and regeneration) by producing several mediators, including tumor necrosis factor-α (TNF-α), TGF-β, interleukin-6 (IL-6), and reactive oxygen species (ROS)[17-19]. Among these, TGF-β plays a crucial role in the transdifferentiation of quiescent HSCs into fibrogenic activated HSCs, *via* the suppression of their degradation and the stimulation of the production of extracellular matrix (ECM), especially in collagen fibers[19-21]. In a healthy liver, liver lymphocytes constitute about 25% of the NPC. Mouse liver lymphocytes contain 5%-10% NK cells and 30%-40% NKT cells, whereas rat and human liver lymphocytes consist of approximately 30%-50% NK cells and 5%-10% NKT cells[11,13,15,16]. These distributions of NK and NKT cells are quite abundant compared with those in peripheral blood, which contains 2% of NKT cells and 13% of NK cells[13]. Previously, NK/NKT cells were regarded to assume a crucial role in mediating the immune responses against tumor and microbial pathogens. However, recent studies have suggested that they contribute significantly to liver injury, regeneration, and fibrosis[22-25].

More interestingly, there are enigmatic cells in the liver that were previously called Ito cells or sinusoidal fat-storing cells, but are now standardized as HSCs[21]. HSCs comprise up to 30% of NPC in the liver and are located in specialized spaces called Disse, between hepatocytes and sinusoidal endothelial cells. In addition, quiescent HSCs store retinol (vitamin A) lipid droplets and regulate retinoid homeostasis in healthy livers. However, they become activated and transformed into myofibroblastic cells that have special features with retinol (vitamin A) loss and enhanced collagen expression when liver injuries occur[19,21,26]. For several decades, activated HSCs have been considered to be major cells that induce liver fibrosis *via* the production of ECM and inflammatory mediators (e.g. TGF-β) in humans and rodents[19-21]. However, recent studies have suggested that the novel roles of HSCs are closely associated with other diseases, such as alcoholic liver steatosis and immune responses, by producing endocannabinoids and presenting antigen molecules, respectively[8,27,28]. Moreover, HSCs can directly interact with immune cells, such as NK cells, NKT cells and T cells, *via* the expression of retinoic acid early inducible-1 (RAE1), CD1d, and major histocompatibility complex (MHC) Ⅰ and Ⅱ[22,28,29]. During HSC activation, they metabolize the retinols into retinaldehyde (retinal) *via* alcohol dehydrogenase (ADH), and the retinal is further metabolized into retinoic acid (RA) *via* retinaldehyde dehydrogenase (Raldh)[3,29]. Surprisingly, activated HSCs express an NK cell activating ligand known as RAE1; however, RAE1 expression is absent in quiescent HSCs. This suggests that the activation processes of HSCs are necessary for the expression of a NK cell activated ligand, RAE1. Furthermore, several TLRs have also been identified in HSCs[30]. Taken together, HSCs might be important not only in liver fibrosis, but also in other liver diseases related to immune responses.

ALCOHOLIC LIVER STEATOSIS BY INNATE IMMUNITY AND HSCS

Alcoholic liver steatosis has long been considered as a mild condition; however, increasing evidence suggests that it is a potentially pathologic state, which progresses into a more severe condition in the presence of other cofactors, such as the sustained consumption of alcohol, viral hepatitis, diabetes, and drug abuse[31,32]. It is believed that fat accumulation in the hepatocytes is a result of an imbalanced fat metabolism, such as decreased mitochondrial lipid oxidation and enhanced synthesis of triglycerides. Several underlying mechanisms of these processes indicate that it might be related to an increased NADH/NAD+ ratio[33,34], increased sterol regulatory element-binding protein-1 (SREBP-1) activity[35,36], decreased peroxisome proliferator-activated receptor-α activity[37,38], and decreased AMP-activated protein kinase (AMPK) activity[8,36].

Moreover, recent studies have suggested the involvement of innate immune cells, particularly Kupffer cells,

in alcoholic liver steatosis[39,40]. Generally, alcohol intake increases gut permeabilization, which allows an increased uptake of endotoxin/lipopolysaccharide (LPS) in portal circulation[18]. Kupffer cells are then activated in response to LPS *via* TLR4 signaling cascade, leading to the production of several types of pro-inflammatory mediators such as TNF-α, IL-1, IL-6, and ROS[3,4,39]. Of these mediators, the increased expression of TNF-α and enhanced activity of its receptor (TNF-α R1) have been observed in alcoholic liver steatosis in mice[39-42]. In addition, it has been reported that TNF-α has the potential to increase mRNA expression of SREBP-1c, a potent transcription factor of fat synthesis, in the liver of mice and to stimulate the maturation of SREBP-1 in human hepatocytes[43,44]. Furthermore, a recent report demonstrated that alcohol-mediated infiltration of macrophages decreased the amount of adiponectin (known as anti-steatosis peptide hormone) production of adipocytes, leading to alcoholic liver steatosis[45]. Therefore, Kupffer cells/macrophages might contribute to the development of alcoholic liver steatosis *via* the upregulation of the SREBP1 activity in hepatocytes and the downregulation of the production of adiponectin in adipocytes. In contrast, IL-6 produced by Kupffer cells/macrophages is a positive regulator in protecting against alcoholic liver steatosis *via* activation of signal transducer and activator of transcription (STAT)3, consequently inhibiting of SREBP1 gene expression in hepatocytes[46-48].

Endocannabinoids, endogenous cannabinoids, are lipid mediators that interact with cannabinoid receptors (CB1 and CB2) to produce effects similar to those of marijuana[49]. There are the two main endocannabinoids, arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). Recently, an intriguing report suggested that alcoholic liver steatosis is mediated mainly through HSC-derived endocannabinoid and its hepatocytic receptor[8]. The study suggested that chronic alcohol consumption stimulated HSC to produce 2-AG, and the interaction with the CB1 receptor upregulated the expression of lipogenic genes SREPB1c and fatty acid synthase but downregulated the activities of AMPK and carnitine palmitoyltransferase 1. Consequently fat is accumulated in the hepatocyte. More recently, a related study reported that the increased expression of CB1 receptors on hepatocytes because of alcohol consumption was mediated by RA acting *via* a RA receptor (RAR)-γ[27]. This study also showed that 2-AG treatment in mouse hepatocytes increased the production of RA by Raldh1, the catalytic enzyme of retinaldehyde into RA. RA then binds with RAR-γ, increasing the expression of CB1 receptor mRNA and protein, and consequently exacerbating the alcohol-mediated fat accumulation *via* enhanced endocannabinoid and lipogenic signaling pathways[27]. Reports stating that alcohol consumption simultaneously elevated the expression of RAR and the production of retinol metabolites, including RA, in mouse and rat liver, supported these findings[50-52]. Moreover, hepatocytes and HSCs are major sources of retinoids, including retinol and RA, in the body[26,53]. In contrast to the CB1 receptors, the association of CB2 receptors with the development of hepatic steatosis has not yet been studied in depth. One study showed that the expression of CB2 receptors was increased in the livers of patients with non-alcoholic fatty liver disease[54]. In an animal model, however, feeding of high-fat diet for 15 wk induced severe fatty liver in wild-type mice, but not in hepatic CB2 knockout mice[55]. The involvement of endocannabinoid, RA, and their receptors has been integrated in Figure 1.

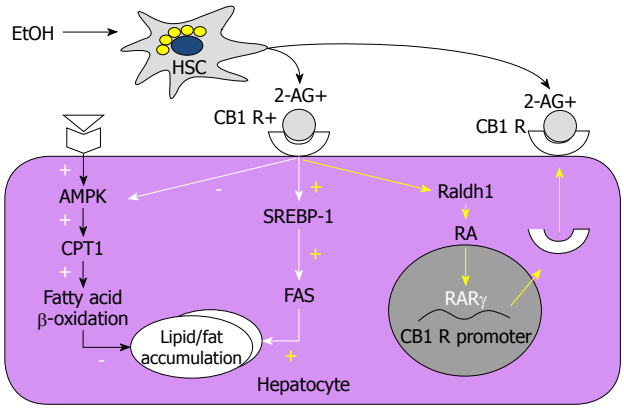

Figure 1 Regulatory mechanisms of the hepatic lipogenesis and CB1 receptor expression *via* hepatic stellate cell-derived endocannabinoids/CB1 receptors and retinoic acid/retinoic acid receptor-γ in hepatocytes, respectively. CB1 R: CB1 receptor; AMPK: AMP-activated protein kinase; HSC: Hepatic stellate cell; 2-AG: 2-arachidonoylglycerol; SREBP-1: Sterol regulatory element-binding protein-1; FAS: Fatty acid synthase; RA: Retinoic acid; RAR: Retinoic acid receptor.

Interestingly, in contrast with previous reports that endocannabinoids activated HSCs to induce liver fibrosis and alcoholic liver steatosis[8,56], Siegmund *et al* reported that HSCs' sensitivity to anandamide (AEA)-induced cell death was because of low expression of fatty acid amide hydrolase and that 2-AG also induced apoptotic death of HSCs *via* ROS induction[57-59]. These data indicated that endocannabinoids might play negative roles in liver fibrosis. Therefore, the functions of endocannabinoids to HSCs are still unclear and need to be studied further.

ALCOHOLIC STEATOHEPATITIS BY INNATE IMMUNITY AND HSCS

Alcoholic steatohepatitis has a mixed status with fat accumulation and inflammation in the liver, which has the potential to progress into more severe pathologic states such as alcoholic liver fibrosis, cirrhosis, and hepatocellular carcinoma. In response to alcohol uptake, many hepatic cells participate in the pathogenesis of alcoholic steatohepatitis. However, as described above, mainly Kupffer cells and HSCs initiate and maintain hepatic inflammation and steatosis[4,8,60-63]. Considering their specific location at the interface between the portal and systemic circulation, Kupffer cells are the central players in orchestrating the immune response against endotoxin (LPS) *via* TLR4 signaling pathways[62,64]. TLR4 initiates two main pathways, and when TLR4 binds LPS, TIR domain-containing adaptor protein and myeloid differentiation factor 88 (MyD88) are recruited, resulting in the early-phase activation of nu-

clear factor-κB (NF-κB). The activation of NF-κB leads to the production of pro-inflammatory cytokines, including TNF-α, IL-6, and monocyte chemotatic protein-1 (MCP-1). Meanwhile, TIR-domain containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule activate interferon regulatory factor 3 (IRF3), leading to the production of type I IFN and late activation of NF-κB[62,65]. Recent studies reported that alcohol-mediated liver injury and inflammation were primarily induced by in a TLR4-dependent, but MyD88-independent, manner in NPCs (Kupffer cells and macrophages), whereas IRF3 activation in parenchymal cells (hepatocytes) rendered protective effects to ALD[66,67]. In addition, the importance of gut-derived endotoxin/LPS in ALD was suggested by experiments where animals were treated with either antibiotics or lactobacilli to remove or reduce the gut microflora provided protection from the features of ALD[68]. Among pro-inflammatory cytokines, TNF-α primarily contributes to the development of ALD, and its levels are increased in patients with alcoholic steatohepatitis[39] and in the liver of alcohol-fed animals[40,69]. Moreover, Kupffer cells secrete other important cytokines, including IL-8, IL-12, and IFNs, which contribute to the intrahepatic recruitment and activation of granulocytes that are characteristically found in severe ALD, and influence immune system polarization[70]. Interestingly, TLR4 is expressed not only on innate immune cells, such as Kupffer cells and recruited macrophages, but also on hepatocytes, sinusoidal endothelial cells, and HSCs in the liver[30].

In addition to LPS, oxidative stress-mediated cellular responses also play an important role in activations of innate immune cells and HSCs. Furthermore, Kupffer cells represent a major source of ROS in response to chronic alcohol exposure[71,72]. One important ROS is the superoxide ion, which is mainly generated by the enzyme complex NADPH oxidase. Underlining the important role of ROS in mediating ethanol damage, treatment with antioxidants and deletion of the p47phox subunit of NADPH oxidase in ethanol-fed animals reduced oxidative stress, activation of NF-κB, and TNF-α release in Kupffer cells, thus preventing liver injury[71,73]. Moreover, NADPH oxidase induces TLR2 and TLR4 expression in human monocytic cells[74], and direct interaction of NADPH oxidase isozyme 4 with TLR4 is involved in LPS-mediated ROS generation and NF-κB activation in neutrophils[75].

Besides Kupffer cells, HSCs also contribute to alcoholic steatohepatitis by producing endocannabinoids and releasing proinflammatory cytokines and chemokines, such as TNF-α, IL-6, MCP-1, and macrophage inflammatory protein-2[63,76-78]. Moreover, Kupffer cells activated by alcohol stimulate the proliferation and activation of HSCs *via* IL-6 and ROS-dependent mechanisms in a co-culturing system[17,79]. Furthermore, retinol metabolites of HSCs activate latent TGF-β, leading to suppression of apoptosis of HSCs[80-82]. Recently, an intriguing review provided novel roles for HSCs in liver immunology, where HSCs, depending on their activation status, can produce several mediators, including TGF-β, IL-6, and RA, which are important components in naïve T cell differentiation into regulatory T cells (Treg cells) or IL-17 producing T cells (Th-17 cells)[83]. Based on this review, it can be hypothesized that HSCs regulate hepatic inflammation *via* modulation of T cell differentiation into Treg or Th-17 cell under certain circumstances. However, this remains an unclear proposition; therefore, further studies on the role of HSCs in hepatic inflammatory diseases, including alcoholic steatohepatitis and viral hepatitis, are necessary.

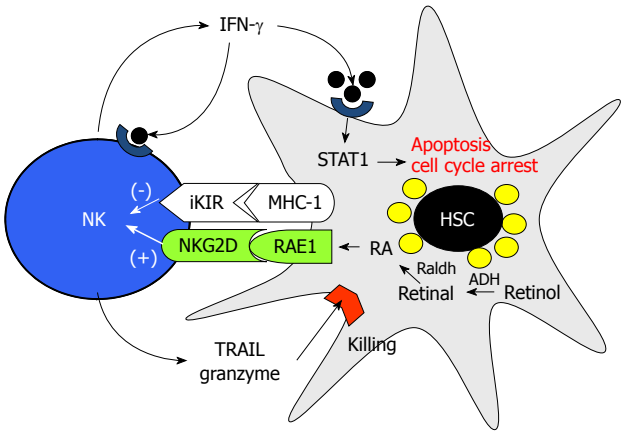

Figure 2 Mechanism of natural killer cell cytotoxicity against activated hepatic stellate cells. STAT: Signal transducer and activator of transcription; IFN: Interferon; NK: Natural killer; HSC: Hepatic stellate cell; MHC: Major histocompatibility complex; RAE1: Retinoic acid early inducible-1; RA: Retinoic acid; ADH: Alcohol dehydrogenase.

ALCOHOLIC LIVER FIBROSIS BY INNATE IMMUNITY AND HSCS

Chronic alcohol drinking is one of major causes of liver fibrosis, which is characterized by the excessive accumulation of ECM components because an imbalanced ECM degradation and production[6]. However, only 10%-40% of heavy drinkers develop alcoholic liver fibrosis[1,3]. Although the underlying mechanisms of alcoholic liver fibrosis are not yet completely understood, several suggestions have been made in the literature. First, acetaldehyde and ROS generated by hepatic alcohol metabolism activate the production of collagen and TGF-β1 in HSCs through a paracrine mechanism[84,85]. Secondly, hepatocyte apoptotic bodies induced by alcohol are phagocytosed in Kupffer cells and HSCs, resulting in the production of TGF-β1 and subsequently activating HSCs[86,87]. Thirdly, alcohol-mediated activation of Kupffer cells, such as LPS/TLR4 signaling, also activates HSCs *via* release of cytokines, chemokines, and ROS[17,63,88]. Moreover, TLR4/MyD88 signaling in HSCs enhances TGF-β signaling, inducing liver fibrosis *via* down-regulation of a transmembrane TGF-β receptor inhibitor, Bambi[89]. Furthermore, it is reported that NADPH oxidase–mediated ROS production contributes to liver fibrosis[90]. However, recent studies have inferred another possibility - that chronic alcohol consumption predisposes NK/NKT cells to decrease in function, which accelerates the development of liver fibrosis[9,91].

Originally, as we depicted in Figure 2, NK cells have

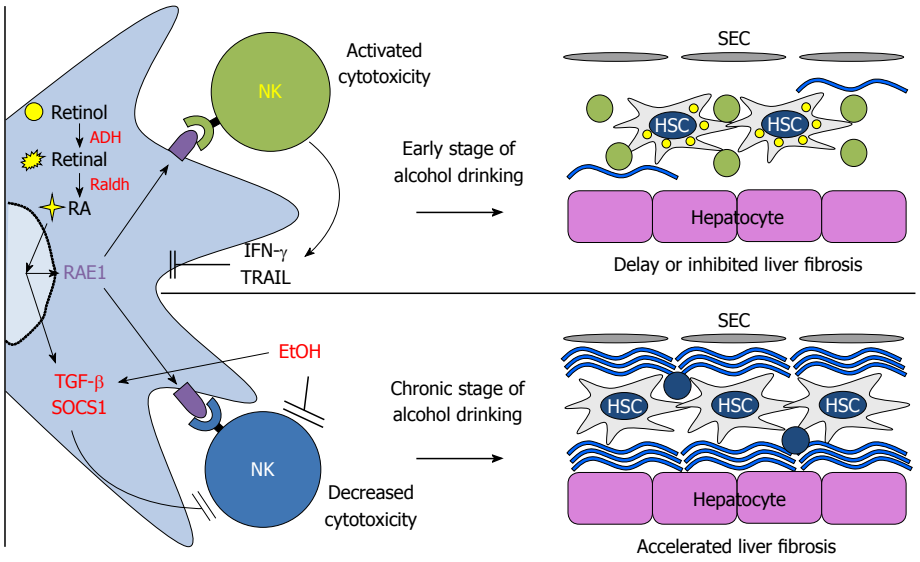

Figure 3 A model for chronic alcohol acceleration of liver fibrosis *via* inhibition of natural killer cell killing against hepatic stellate cells and suppressor of cytokine signaling 1 suppression of interferon-γ signaling in hepatic stellate cells. SEC: Sinusoidal endothelial cell; ADH: Alcohol dehydrogenase; HSC: Hepatic stellate cell; RA: Retinoic acid; RAE1: Retinoic acid early inducible-1; IFN: Interferon; NK: Natural killer; TGF: Transforming growth factor; SOCS1: Suppressor of cytokine signaling 1.

anti-fibrotic effects *via* several mechanisms. First, NK cells can directly kill activated HSCs by NKG2D- and TNF-related apoptosis, dependent on the induction TRAIL ligand, whereas NK cells cannot induce apoptosis of quiescent HSCs[24,92]. This is because early activated HSCs express NK cell-activating ligand RAE-1, which is an activating ligand of NKG2D on NK cells, by RA and TRAIL receptors, but they express decreased MHC- I , an NK cell-inhibitory ligand[29,92]. Second, NK cells can suppress liver fibrosis *via* production of IFN-γ, which can induce HSC cell cycle arrest and apoptosis in a STAT1-dependant manner and induce autocrine activation of NK cells[93,94]. Similar to NK cells, NKT cells (invariant NKT cells) can also suppress HSC activation *via* direct killing and IFN-γ production; however, the anti-fibrotic effects of NKT cells are beneficial only at the onset stage of liver fibrosis because of iNKT depletion tolerance[22]. In contrast, strong activation of iNKT cells by a single injection of α-galactosylceramide adversely enhanced liver fibrosis *via* highly increased IFN-γ-mediated hepatocyte apoptosis[22]. However, in alcoholic liver fibrosis, it is now accepted that chronic alcohol consumption accelerates liver fibrosis because of the suppressed activity of NK cells (as shown in patients and mice)[9,91,95]. In patients with alcoholic liver cirrhosis, the number and cytolytic activity of peripheral blood NK cells were significantly decreased compared to those of patients without liver disease[95]. In parallel with this report, decreased numbers and cytotoxicity of liver NK cells against HSCs and tumor cells were observed in chronically alcohol-fed mice[9,91]. In addition, direct IFN-γ treatment failed to increase activities of NK cells and to suppress activated HSCs in chronically alcohol-fed mice, showing no beneficial effects of IFN-γ in alcoholic liver fibrosis[9]. These results are possibly due to increased expression and production of TGF-β and SOCS1 by monocytes and activated HSCs[9,96]. We have integrated these findings in Figure 3, and in the case of NKT cells, they seem to contribute to alcoholic liver injury because the activation of NKT cells accelerate alcoholic liver injury while NKT deficiency delays the process[97,98]. Nevertheless, reports on the effects of alcohol on NK/NKT cell functions are still controversial. Therefore, further studies of the effect of alcohol on NK/NKT functions are necessary.

Although the underlying mechanisms of liver fibrosis are not clear, alcohol consumption in patients with hepatitis C virus (HCV) infection may accelerate the process. This is because HCV triggers dysfunction and apoptosis of lymphocytes, such as T cells, NK cells, and NKT cells, *via* NADPH oxidase-derived oxygen radicals, which might be enhanced by alcohol-mediated apoptosis of hepatocyte and ROS production, and subsequently accelerating liver fibrosis[99,100]. In addition, HCV core and nonstructural proteins either induce TLR4 expression in hepatocytes and B cells, leading to enhanced production of IFN-β and IL-6, or enhance the secretion of TGF-β1 and the expressions of procollagen α(I) or α-smooth muscle actin in human-activated HSCs and LX-2 cells[101,102]. Therefore, all these factors and findings may be promoting the effect of alcohol on liver fibrosis in patients with HCV infection.

TREATMENT STRATEGY FOR ALD

In alcoholic patients, the best therapeutic is to reduce ethanol intake significantly, subsequently avoiding further liver injury[1]. However, abstinence is very difficult to achieve. The alternative option is liver transplantation, but donors are relatively scarce[2]. For these reasons, many studies have been performed to determine targets or strategies for treating ALD. Regarding the critical role of TNF-α and ROS in animal models with ALD, several

drugs have been developed and are currently available for clinical trial. To suppress the inflammatory responses, phosphodiesterase inhibitor (Pentoxifylline) and corticosteroid therapies were also administered and resulted in reductions of TNF-α, IL-8, and soluble and membranous forms of intracellular adhesion molecule 1 in patients with ALD, *via* inhibition of activator protein 1 and NF-κB[103-106]. Even though treatments with antioxidants have shown inhibitory effects on alcohol-mediated oxidative stress in animal models, studies of treatment with antioxidants (S-adenosylmethionine, vitamin E, and silymarin, the active element in milk thistle) had no beneficial effects in either patients with alcoholic hepatitis or those with alcoholic cirrhosis[107,108]. In addition, other treatments, such as antifibrotics (colchicines) and nutritional therapies, have been tried, but the effects were minimal. Based on this discrepancy between animal studies and clinical trials, therapeutic strategies should be reconstituted to overcome ALD. For example, treatments for the amelioration of ALD should be targeted simultaneously to HSCs and innate immune cells (e.g. Kupffer cells and NK cells), because these cells can produce endocannabinoid (e.g. 2-AG), inflammatory mediators (e.g. TNF-α, ROS), pro-fibrotic cytokines (e.g. TGF-β), and negative regulators against NK cells (e.g. TGF-β, SOCS1) concurrently in response to chronic alcohol consumption. Thus, we need novel orchestrated strategies, which are capable of enhancing NK cell cytotoxicity while simultaneously suppressing the activation of HSCs and Kupffer cells.

CONCLUSION

The present review summarized the pathogenesis of ALD, in which NK cells, Kupffer cells and HSCs are highly involved. Alcohol-mediated activation of Kupffer cells appears to be required for the development of alcoholic steatohepatitis *via* LPS-TLR4 signaling pathways. In addition, alcohol-induced paracrine activation of HSC-derived endocannabinoid in hepatocytes might be a major factor in the induction of alcoholic steatosis. Furthermore, both Kupffer cells and HSCs play important roles in alcoholic liver fibrosis *via* the suppression of the antifibrotic effects of NK cells. Therefore, the interactions among them should be simultaneously considered when developing therapeutics for ALD. For example, even though Kupffer cells are appropriately suppressed by a certain drug, alcohol-activated HSCs still might enhance the accumulation of fat in the liver, leading to lipotoxicity, which in turn generates oxidative stress and inflammation, subsequently restoring steatohepatitis. Besides, functions of NK cells are abrogated or suppressed by alcohol-induced ROS and high levels of TGF-β in the liver. Thus, additional antioxidant and neutralizing TGF-β1 antibody treatment may have beneficial effects in slowing down ALD. Conclusively, further studies to elucidate the roles of innate immunity and HSCs might aid in the development of novel therapeutic targets for the treatment of ALD.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2552

World J Gastroenterol 2011 May 28; 17(20): 2552-2557
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Role of MGST1 in reactive intermediate-induced injury

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Received: March 17, 2011 Revised: April 15, 2011
Accepted: April 22, 2011
Published online: May 28, 2011

Abstract

Microsomal glutathione transferase (MGST1, EC 2.5.1.18) is a membrane bound glutathione transferase extensively studied for its ability to detoxify reactive intermediates, including metabolic electrophile intermediates and lipophilic hydroperoxides through its glutathione dependent transferase and peroxidase activities. It is expressed in high amounts in the liver, located both in the endoplasmic reticulum and the inner and outer mitochondrial membranes. This enzyme is activated by oxidative stress. Binding of GSH and modification of cysteine 49 (the oxidative stress sensor) has been shown to increase activation and induce conformational changes in the enzyme. These changes have either been shown to enhance the protective effect ascribed to this enzyme or have been shown to contribute to cell death through mitochondrial permeability transition pore formation. The purpose of this review is to elucidate how one enzyme found in two places in the cell subjected to the same conditions of oxidative stress could both help protect against and contribute to reactive oxygen species-induced liver injury.

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Key words: Microsomal glutathione transferase 1; Oxidative stress; Mitochondrial permeability transition; Glutathione; Liver injury

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Schaffert CS. Role of MGST1 in reactive intermediate-induced injury. *World J Gastroenterol* 2011; 17(20): 2552-2557 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2552.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2552

INTRODUCTION

Tissue damage due to excessive production of reactive intermediates (reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxides, free radicals) has been shown to be a major part of various pathologies including atherosclerosis, diabetes, cancer, ischemia-reperfusion injury, aging, and liver injury from both alcoholic and non-alcoholic origins[1-3]. Organisms have evolved many defense mechanisms as protection from these reactive intermediates. These include, but aren't limited to, various enzymes such as superoxide dismutase, Se-dependent glutathione peroxidase, catalase, glutaredoxins, peroxiredoxins, and glutathione transferases (GSTs)[4]. GSTs bind and conjugate electrophiles to GSH to, in effect, neutralize them, and protect the cells. Some GSTs also have glutathione peroxidase activities. While many GSTs are cytosolic, other GSTs exist that are integral membrane proteins. They are members of the membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) family, which include enzymes that synthesize prostaglandins and leukotrienes. Microsomal glutathione transferase 1 (MGST1) is a member of this family and is the subject of the following review.

MGST1 STRUCTURE AND FUNCTION

MGST1 is an approximately 17 kDa membrane bound glutathione s-transferase[5]. It is found in the highest

quantity in the liver; however it is also present in other tissues in smaller amounts. It is found primarily in the microsomes (3% of microsomal protein) and the inner and outer mitochondrial membranes (5% of mitochondrial membrane protein) of cells, where the majority of reactive intermediates are produced[6]. It exists in membranes as a homotrimer which can bind three molecules of GSH. However, only one molecule is modified to a thiolate anion, and this molecule binding site has a much higher affinity than the other two sites[7-10].

MGST1 has two enzymatic activities; one as a GST, and the other as a glutathione dependent peroxidase (GPX)[11]. The function of MGST1 in both organelles is thought to be the neutralization of lipid peroxides and conjugation of other reactive intermediates to glutathione. Based on these two enzymatic activities, MGST1 has been shown to have three substrate binding sites[12]. One site binds GSH on the cytosolic face of each monomer. A second site binds electrophilic substances in a hydrophobic pocket, also on the cytosolic face. This site may partially overlap with the glutathione thiolate anion binding site. This site contains cys49 (the only cysteine per monomer) which acts as an oxidative and chemical stress sensor[13]. Because the binding pocket for electrophiles is hydrophobic, MGST1 is uniquely suited among the GSTs to detoxify more hydrophobic reactive intermediates, as often occurs during drug metabolism. The third site is the phospholipid/fatty acid binding site that may bind these hydrophobic lipid peroxides through an opening in the tertiary structure of the trimer, rather than binding them through the cytosolic face. This site also overlaps the GSH binding site, but is separate from the electrophilic site[12].

The majority of research has been performed on MGST1 purified from liver microsomes. These studies have identified that MGST1 exists as a trimeric complex where three monomers interact to give the active enzyme[10,14]. Modification of the thiol group of cys49 induces a conformational change and increased activation, and at increasing protein concentration, the trimers aggregate into higher molecular weight complexes that have even further increased activity as measured by GST assay[15]. Multiple studies have also shown that the MGST1 trimer is activated by various chemical treatments that appear to modify the cys49. These modifications include alkylation, formation of disulfide bonds (either mixed disulfides with glutathione, or protein dimer formation), or sulfenic acid modification[16-20]. An additional mechanism of activation is proteolysis at lys41[21]. In fact, a liver enzyme, hepsin, has been implicated in this mode of activation[22]. It is postulated that proteolysis and cysteine modification induce a conformational change that induces the GSH that is bound to be converted to the thiolate anion more readily than what occurs under basal levels.

PROTECTIVE EFFECTS OF MGST1

A variety of reactive intermediates can induce activation of MGST1 *in vitro* and *in vivo*[16-20,23,24], but the physiological function of MGST1 is still unclear. It as also been noted that transfection of cytochrome P450 2E1 (CYP2E1), a significant generator of ROS, in a hepatoma cell line (HepG2) induced increased expression and activation of catalase, GSTα (cytosolic GST) and MGST1[25]. Incubation of these cells with increasing concentrations of H_2O_2 showed that they had better viability and were better able to remove the H_2O_2, when compared with vector controls. The CYP2E1 transfected cells also showed higher viability compared to controls when incubated with 4-HNE (a lipid peroxidation product) or the free radical generators, menadione or antimycin A. Resistance to ROS-induced injury in this cell line was attributed to the increased expression of the previously mentioned antioxidant response proteins; although it is unclear if similar effects would be observed if only MGST1 were overexpressed.

To help specifically determine the role of MGST1 in protection against oxidative stress, a series of studies was performed using a breast cancer cell line, MCF7, stably transfected either with the sense strand of MGST1 or its anti-sense negative control. Transfection yielded a clone that expressed MGST1 at 0.2-0.5 μg/mg total cellular protein. This level is approximately a tenth of what is expressed in the liver, but is comparable to other extrahepatic tissue expression levels. This cell line was originally designed to determine the contribution of MGST1 to anticancer drug resistance[26], but was used in subsequent studies to test the ability of MGST1 to protect the cells from reactive intermediates[27,28]. Treatment of the MGST1 transfected cells with either lipophilic hydroperoxide (cumene hydroperoxide, CuOOH), or hydrophilic H_2O_2, revealed that they were more resistant to cell death (by MTT, LDH, and colony forming efficiency assays) than the cells transfected with the antisense vector. CuOOH is a substrate for MGST1 and is a target for its GPX activity, while H_2O_2 is not a direct substrate. So MGST1 in this system has both direct and indirect effects on hydroperoxide induced toxicity. Measurement of hydroperoxide levels in these cell lines revealed a significant drop only in the MGST1 containing cell line. These results are consistent with transient MGST1 transfection studies by Maeda *et al*[29].

Subsequent studies revealed that not only did MGST1 lower hydroperoxide levels, it lowered lipid peroxidation[27]. Treatment of MGST1 transfected cells with CuOOH and tert-Butyl hydroperoxide (BuOOH) resulted in fewer lipid peroxidation products than in antisense controls. Notably, in the absence of any treatments, the MGST1 transfected cells exhibited less lipid peroxidation than their control counterparts. Vitamin E added to the MGST1 transfected cells mediated protection against cytotoxicity induced by the hydroperoxides, but was ineffective in contributing to the MGST1 mediated suppression of the cytotoxic effects of 4-HNE (lipid peroxidation product) or cisplatin. This suggests that these cytotoxic agents may be neutralized by the GST activity of MGST1 rather than its GPX activity. This is consistent with the previous findings that MGST1 neutralizes 4-HNE through its GST activity[30]. The study by Johansson *et al*[27] also examined the effect of hydroperox-

ides on mitochondrial function in the control and MGST1 transfected cells. Excessive ROS has been shown to induce mitochondrial dysfunction, MPT and cell death[3,31] and MGST1 makes up a sizable amount of the mitochondrial outer membrane protein, possibly as a defense mechanism against lipid peroxidation and reactive oxygen species generated by normal mitochondrial function[27]. This study examined mitochondrial respiration in the control and MGST1 transfected cells after treatment with CuOOH[27]. They found that in control cells, treatment with CuOOH suppressed both phosphorylating respiration and uncoupled respiration, while overexpression of MGST1 protected against the CuOOH induced suppression. The effect of MGST1 on mitochondrial Ca++ loading and release was also examined after CuOOH treatment, since high levels of Ca++, especially under conditions of oxidative stress, can damage the mitochondria, inducing mitochondrial permeability transition (MPT). MPT is identified as a key event in some forms of apoptosis and in necrosis[31]. CuOOH treatment lowered the amount of Ca++ required to induce MPT in the control cells. However, MGST1 overexpression had a protective effect on the mitochondria, as an increased amount of Ca++ was required to induce MPT in these cells after CuOOH treatment. These authors attribute this to the mitochondrial population of MGST1 (hereafter, referred to as mtMGST1), but it cannot be ruled out that the microsomal population of MGST1 is also actively detoxifying the CuOOH, since these experiments were performed in plasma membrane permeabilized cells. Further studies need to be performed on mitochondria from these cells to discern the role of mtMGST1 concerning oxidative stress induced mitochondrial dysfunction. In addition, while overexpression of MGST1 has protective effects in this study, the levels in the MGST1 transfected cells are only one tenth of what occurs normally in the liver. Therefore this cell line may not be appropriate for studying the deleterious effects of mtMGST1 that contribute to oxidative stress induced liver injury as described below.

DELETERIOUS EFFECTS OF MGST1

Until recently, examination of MGST1 has focused on the microsomal population. From these studies, it has been suggested that MGST1 plays an essential role in the protection of cells from reactive intermediates[13,25,27,28]. Up to this point, virtually nothing was known about the mitochondrial population of MGST1 concerning its activation and function. Mitochondria are a significant producer of ROS, and oxidative stress has been shown to deplete mitochondrial glutathione and induce both MPT and the release of cytochrome C[3,32-34]. This mitochondrial dysfunction leads to apoptosis and necrosis. Since mtMGST1 is localized in the inner and outer mitochondrial membranes, it is most likely activated under these conditions of oxidative stress, similar to the microsomal population. In fact, early animal studies showed that administration of galactosamine, carbon tetrachloride, or a combination of galactosamine and lipopolysaccharide (GalN/LPS) induced liver injury that was characterized by damaged mitochondria, depleted mitochondrial GSH, increased ROS and lipid peroxidation and apoptosis, and increased mtMGST1 activity[35-37]. Preliminary studies indicated that mtMGST1 was activated by ROS, so using the GalN/LPS animal model of liver injury, Lee *et al*[32] set out to examine specifically the activation and function of mtMGST1. GalN/LPS induced activation of mtMGST1, which was reduced by dithiothreitol. Immunoblotting of mitochondrial extracts showed the formation of S-S linked mtMGST1 dimers, as well as glutathionylated mtMGST1 (mixed S-S with GSH). In contrast, mitochondrial extracts from control rat livers showed no modified mtMGST1. This is consistent with previous studies which showed that modification of cys49 in purified MGST1 by disulfide and mixed disulfide formation induced activation[17,23]. In addition, mitochondria from the GalN/LPS treated rats exhibited cytochrome C release, indicative of MPT and mitochondrial dysfunction[32]. Incubation of mitochondria with anti-MGST1 antibodies inhibited the cytochrome C release, suggesting the involvement of activated mtMGST1 in ROS-induced mitochondrial dysfunction and MPT pore formation.

Complementary *in vitro* studies using mitochondria from control rats treated with diamide (a thiol oxidizing agent) or diamide with GSH showed that treatment induced dimerization or glutathionylation of mtMGST1 through cys49. These treatments increased mtMGST1 activity, and induced mitochondrial swelling and cytochrome C release similar to the GalN/LPS treatment mentioned above[32]. Addition of cyclosporin A (CsA) or bongkrekic acid (BKA) (two inhibitors of MPT pore formation) inhibited mtMGST1 activation, mitochondrial swelling and cytochrome C release. Based on the *in vivo* and *in vitro* studies, the authors concluded that activation of mtMGST1 through modification of cys49 contributed to MPT pore formation and mitochondrial dysfunction.

Subsequent studies delved further into the role of mtMGST1 activation and MPT pore formation. Hossain *et al*[38] investigated the effect of the ROS generator gallic acid (GA), MPT inhibitors and GST inhibitors on activation of mtMGST1 and MPT. Incubation of rat liver mitochondria with GA induced significant activation of mtMGST1, which was blocked by antioxidant enzymes and singlet oxygen quenchers. Activation of mtMGST1 was also inhibited by GST inhibitors and CsA. However, when mitochondrial swelling was examined, GST inhibitors inhibited the swelling induced by GA, whereas addition of MPT inhibitors was largely ineffective. This suggested that GA induced non-classical or unregulated MPT, and that mtMGST1 activation was involved. Previous studies have indicated that protein misfolding induces aggregation and pore formation in a Ca++ independent manner that is insensitive to CsA. In fact, there is evidence that triterpenoids like GA interact with mitochondrial proteins, resulting in oxidation of their thiol residues and leading to formation of higher molecular weight aggregates, which then form a CsA insensitive MPT pore[39-42]. It was noted that GA treatment induced thiol oxidation (sulfenic acid) of cys49 of

mtMGST1, instead of disulfide formation as observed previously with diamide and diamide + GSH[32]. In addition, treatment of mitochondria with GA induced formation of high molecular weight protein aggregates containing mtMGST1, consistent with previous studies showing protein aggregation is involved in the non-classical MPT[40,41,43]. It is also noteworthy that examination of the inner and outer membrane populations of mtMGST1 showed that only the outer membrane population was affected by GA. Based on these results, the authors concluded that oxidation of the outer membrane mtMGST1 population by GA leads to its activation, protein aggregation and induction of non-classical MPT, suggesting a novel function for mtMGST1 involving mitochondrial dysfunction and cell death.

Further studies examined the contributions of the inner and outer membrane populations of mtMGST1 to MPT[44]. Using rat liver mitochondria under basal conditions, this group examined the effect of both MPT inhibitors and GST inhibitors on mtMGST1 activation. They observed that in the absence of nonionic detergent, MPT inhibitors CsA, BKA, ADP and ATP were all effective in decreasing basal mtMGST1 activity. However, addition of detergent ameliorated this effect. Incubation of mitochondria with GST inhibitors decreased mtMGST1 activity regardless of addition of detergent. These results indicated that while the GST inhibitors directly affected the mtMGST1 activity, the MPT inhibitors affected mtMGST1 activity indirectly, possibly by affecting interaction of mtMGST1 with other proteins like cyclophilin D (CypD) and the adenine nucleotide translocator (ANT), which are known to be involved in MPT. Further studies using inner mitochondrial and outer mitochondrial membrane fractions showed that MPT inhibitors were effective in reducing mtMGST1 activity only in the inner membrane component, and this inhibition was lost after addition of detergent. Subsequent experiments with cytosolic GST and microsomal MGST1 showed that the MPT inhibitors had no effect on their activity, further suggesting that the contribution of inner membrane mtMGST1 to oxidative stress-induced MPT resides in its ability to interact with specific proteins involved in regulating MPT (CypD and ANT). This may be due to activation induced (cys49 modification) conformational changes in the protein, that allow interaction with other MPT regulating proteins. Collectively, the studies using mtMGST have shown that it is activated by oxidant-induced modification of cys49, which induces a conformational change. This path to activation is similar to what has been observed for the microsomal population of MGST1. However, activation of mtMGST1 by reactive intermediates induces protein aggregation, MPT and mitochondrial dysfunction, either through non-classical or classical pathways which results in apoptosis and necrosis. Interestingly, it is unclear if activated mtMGST1 is able to actually detoxify reactive intermediates while contributing to MPT. It is also unclear if ROS-induced depletion of mitochondrial GSH could induce or contribute to mtMGST1 participation in mitochondrial dysfunction.

ETHANOL-INDUCED CHANGES IN MGST1

It has been previously shown that chronic alcoholism induces liver oxidative stress that is in great part produced by the mitochondria[3]. Ethanol-induced production of ROS is detrimental to the mitochondria, leading to glutathione depletion and mitochondrial dysfunction, including MPT and cytochrome C release. Initial studies have found that MGST1 from the livers of ethanol-fed rats is modified by a product of ethanol metabolism, the malondialdehyde-acetaldehyde (MAA) adduct[45-47]. Examination of both the mitochondrial and microsomal fractions from ethanol-fed rat livers and their pair-fed controls show that while ethanol feeding induces activation of both microsomal MGST1 and mtMGST1, the mitochondrial population has a higher activity, and is more highly modified with MAA[45]. These results suggest that the mtMGST1 may be involved in ethanol-induced mitochondrial dysfunction. Studies are in progress to further characterize both the ethanol-induced modification and the contribution of modified MGST1 to ethanol-induced liver injury.

CONCLUSION

This review focuses on the function of MGST1 in situations of oxidative stress. Extensive studies on this protein have identified its monomeric and trimeric structures, its three substrate binding sites, its ability to be activated by oxidation or alkylation of the lone cysteine (cys49) in each subunit, and its ability to detoxify reactive intermediates by either its GST activity or its GPX activity. Additional studies have also indicated that activation of the enzyme alters its conformation. Therefore, this enzyme has been implicated as part of a complex method of defense by cells in response to reactive intermediates to protect them from cell death. However, these studies focused only on the microsomal population of this enzyme. Recent studies examining mtMGST1 indicate that this population is activated by oxidative stress in a manner similar to that reported for the microsomal enzyme. mtMGST1 also undergoes cys49 modification, leading to increased GST activity and activation-induced conformational changes. However, in the mitochondria, oxidant-induced mtMGST1 activation induces or increases its association with other mitochondrial proteins, resulting in MPT pore formation, cytochrome C release and induction of apoptotic and/or necrotic pathways. From these collective studies it is apparent, depending on the context and MGST1 population involved, that MGST1 activation can either exhibit a protective or toxic effect on the liver (and possibly other tissues). However, much more work is necessary to evaluate not only the individual contributions of these two populations to oxidative stress-induced liver injury, but also how the effects of the activation of these two populations combine to help determine cell survival.

ACKNOWLEDGMENTS

The author would like to thank Michael J Duryee, MS, for his editorial assistance.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2558

World J Gastroenterol 2011 May 28; 17(20): 2558-2562
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Proteasome inhibitor treatment in alcoholic liver disease

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Received: January 6, 2011 Revised: February 2, 2011
Accepted: February 9, 2011
Published online: May 28, 2011

Abstract

Oxidative stress, generated by chronic ethanol consumption, is a major cause of hepatotoxicity and liver injury. Increased production of oxygen-derived free radicals due to ethanol metabolism by CYP2E1 is principally located in the cytoplasm and in the mitochondria, which does not only injure liver cells, but also other vital organs, such as the heart and the brain. Therefore, there is a need for better treatment to enhance the antioxidant response elements. To date, there is no established treatment to attenuate high levels of oxidative stress in the liver of alcoholic patients. To block this oxidative stress, proteasome inhibitor treatment has been found to significantly enhance the antioxidant response elements of hepatocytes exposed to ethanol. Recent studies have shown in an experimental model of alcoholic liver disease that proteasome inhibitor treatment at low dose has cytoprotective effects against ethanol-induced oxidative stress and liver steatosis. The beneficial effects of proteasome inhibitor treatment against oxidative stress occurred because antioxidant response elements (glutathione peroxidase 2, superoxide dismutase 2, glutathione synthetase, glutathione reductase, and GCLC) were up-regulated when rats fed alcohol were treated with a low dose of PS-341 (Bortezomib, Velcade®). This is an important finding because proteasome inhibitor treatment up-regulated reactive oxygen species removal and glutathione recycling enzymes, while ethanol feeding alone down-regulated these antioxidant elements. For the first time, it was shown that proteasome inhibition by a highly specific and reversible inhibitor is different from the chronic ethanol feeding-induced proteasome inhibition. As previously shown by our group, chronic ethanol feeding causes a complex dysfunction in the ubiquitin proteasome pathway, which affects the proteasome system, as well as the ubiquitination system. The beneficial effects of proteasome inhibitor treatment in alcoholic liver disease are related to proteasome inhibitor reversibility and the rebound of proteasome activity 72 h post PS-341 administration.

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Key words: Alcoholic liver disease; Glutathione; Oxidative stress; Proteasome inhibitor treatment; Steatosis

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Bardag-Gorce F. Proteasome inhibitor treatment in alcoholic liver disease. *World J Gastroenterol* 2011; 17(20): 2558-2562 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2558.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2558

INTRODUCTION

Excessive alcohol consumption is recognized worldwide as a leading cause of disease, disability, and death[1]. Alcoholic liver disease (ALD) is a collective term for the pathophysiological changes caused by chronic alcohol consumption. These changes include oxidative stress generation, liver steatosis and inflammatory response fibrosis, and cirrhosis.

The marked generation of oxidative stress associated with ethanol metabolism is one of the main liver injuries caused by chronic alcohol consumption. Oxidative stress causes dysfunctions in several cellular mechanisms, such as DNA repair and antioxidant systems. CYP2E1, which is up-regulated to metabolize ethanol[2], in mitochondria[3], and activated Kupffer cells[4], generates free radicals through the oxidation of NADPH to NADP+, which induces hepatocyte necrosis and apoptosis[5]. In addition, high levels of reactive oxygen species (ROS) promote lipid peroxidation and end-products formation, such as malondialdehyde and 4-hydroxynonenal. These aldehydes are highly interactive and form adducts by binding covalently to cellular proteins, thus forming antigenic adducts, which cause inflammation[6,7].

Oxidant stress can be counterbalanced by the hepatocyte antioxidant defense, which induces both enzymatic and non-enzymatic mechanisms. One of the major mechanisms by which cells protect themselves against oxidative stress is the up-regulation of a wide range of antioxidant genes. Among the intracellular antioxidant molecules, reduced glutathione (GSH) is the most abundant intracellular non-protein thiol in cells. Glutathione is the first level of cellular antioxidative response, and is important for a variety of biological functions, including protection of cells from oxidative damage by free radicals, detoxification of xenobiotics, and membrane transport. By keeping the cellular environment in a reduced state, GSH is responsible for the removal of potentially toxic electrophiles and metals, thereby protecting cells from toxic oxygen products[8]. Furthermore, GSH exhibits a large panel of actions in controlling gene expression, apoptosis mechanisms, and membrane transport[9]. Therefore, cells tightly regulate the synthesis, utilization, and export of GSH. L-S,R-buthionine sulfoximine (BSO), a potent specific inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis, has been used to deplete intracellular GSH and reverse drug resistance in tumor cells[10], showing that GSH is a chemoresistance factor in cancer cells. The second level of cellular antioxidative response is the gene expression up-regulation of antioxidative enzymes. Among the enzymatic antioxidant defenses are: (1) glutathione synthetase (GSS) and superoxide dismutases (SOD), which dismutates O_2 into H_2O_2 and O_2; (2) catalase, which removes H_2O_2, generating H_2O and O_2; and (3) glutathione peroxidase (GPX) and glutathione reductase (GSR), which, using the cofactor NADPH, decompose H_2O_2, while reducing glutathione[11]. Alcohol has been shown to deplete GSH levels, particularly in the mitochondria. Mitochondria are usually characterized by high levels of GSH needed to eliminate the ROS generated during respiratory chain activity[12], and can not synthesize GSH, but import it from the cytosol using a carrier protein embedded in the membrane surrounding the mitochondria. Alcohol has been reported to interfere with the function of this carrier protein, thereby leading to the depletion of mitochondrial GSH[13].

CURRENT TREATMENT FOR ALD

Several pharmaco-therapeutic studies have been undertaken to cure alcoholic hepatitis. The best known are the treatments that block tumor necrosis factor α and reduce inflammation (pentoxifylline, infliximab, etanercept)[14-16]. However, these treatments are associated with an increase of infections and death.

The antioxidant therapy included Vitamin E supplementation. However, the outcome of clinical trials did not show any improvement in patients with alcoholic hepatitis[17]. In addition, it has been shown that alcohol not only increases the production of ROS, but it also inhibits the antioxidants defense. It has also been shown that antioxidant therapy alone, or in combination with corticosteroids, did not improve 6-mo survival in severe alcoholic hepatitis[18]. The drug most widely used for alcoholism today is Disulfiram. Disulfiram, an inhibitor of aldehyde dehydrogenase, prevents acetaldehyde metabolism, and causes immediate and severe negative reactions to alcohol intake[19]. Recently, it has been used as an antitumor drug because it has the characteristic of a proteasome inhibitor[20,21]. New drugs and new treatments are thus needed since currently available treatments are not adequate. The latest studies using Disulfiram point to the potential of proteasome inhibitor treatment for ALD.

PROTEASOME INHIBITOR TREATMENT FOR ALD

Proteasome controls the degradation of cellular proteins and is closely implicated in signal transduction, development and cell cycle progression[22], antigen processing and immune response[23], and inflammation[24]. Proteasome inhibition has already proved to be a novel and promising strategy for the treatment of cancer[25-27]. Specifically, PS-341 (Bortezomib, Velcade®), a boronic acid dipeptide with selective activity as a proteasome inhibitor, has demonstrated clinical efficacy in patients with multiple myeloma[28], and has been approved by the U.S. Food and Drug Administration[29]. It is now under evaluation for its activity in a variety of other hematologic and solid malignancies[30-33]. Proteasome is considered an antioxidant defense in the cell due to its activity of removing damaged and oxidized proteins. Numerous reports have demonstrated that proteasome inhibitors cause an accumulation of oxidatively damaged proteins, indicating that a large majority of oxidatively damaged proteins, both in the cytosol and the nucleus of mammalian cells, are removed by the 20S proteasome[34,35]. However, it is also important to mention that proteasome inhibition is also an antioxidative defense, as it leads to an up-regulation in the gene expression of antioxidative enzymes. Although it is now well established that impairment of the ubiquitin proteasome pathway is implicated in the pathogenesis of ALD, a growing body of evidence shows that proteasome inhibitors provide protection against oxidative stress in the brain and in the heart[36-39].

Ethanol ingestion appears to have diverse effects on 26S proteasome activity, and no significant effects on the 20S proteasome[2]. The 26S proteasome activities are significantly decreased in the liver of rats fed ethanol[2,40-43]. This ethanol-induced proteasome pathway dysfunction is different from the proteasome inhibition obtained by using the proteasome inhibitor PS-341[44]. Microarray analysis studies have shown that the gene expression of antioxidative enzymes was not increased in the liver of rats fed ethanol chronically, when compared to that of rats given proteasome inhibitor PS-341[45].

Moreover, chronic ethanol exposure has been shown to deplete GSH levels, particularly in the mitochondria, which are usually characterized by high levels of GSH needed to eliminate the ROS generated during respiratory chain activity[13,46,47], while proteasome inhibition by PS-341 activates the gene expression of GSH recycling enzymes[48]. These authors showed a significant increase in the gene expression of antioxidative enzymes, such as glutathione reductase (GSR), glutathione synthetase (GSS), glutathione peroxidase 2 (GPX2), and superoxide dismutase 2 (SOD2), when rats were treated with the proteasome inhibitor PS-341. Exposure to a non-toxic low dose of proteasome inhibitor induced an increase in the antioxidative defense, thus suppressing ROS production, and therefore protecting against oxidative stress-induced hepatotoxicity due to chronic ethanol feeding. The beneficial effects of proteasome inhibition are not only related to the up-regulation of antioxidative enzyme gene expression[49], but also to the up-regulation of heat shock proteins[50], which is believed to prevent protein misfolding and the formation of protein aggregates. Thus, it is now postulated that these cytoprotective qualities obtained by the inhibition of proteasome at non-toxic doses might be beneficial in the treatment of hepatocyte injury associated with ALD. In addition, PS-341 is a highly specific and reversible proteasome inhibitor that produces a recovery and even a rebound of proteasome activity to higher levels 48 to 72 h post-treatment[48]. At the same time that proteasome inhibitor treatment up-regulated the antioxidant response elements, it down-regulated SREBP1-c[51] and the lipogenic enzymes gene expression, thus significantly decreasing steatosis in the liver of rats fed ethanol chronically[51]. It has also been found that IκB was significantly stabilized by proteasome inhibitor treatment in the liver of rats fed ethanol for 1 mo, which reflected a significant decrease in nNuclear factor (NF)-κB activation and a decrease in the expression of inflammatory genes regulated by NF-κB[52].

Chronic ethanol exposure increases the production of pro-inflammatory cytokines and disrupts immune defenses, increasing susceptibility to and the severity of infections. Proteasome inhibitor treatment can modulate the chronic ethanol-induced impairment of immune response and its consequences on host defense against microbial pathogens and tissue injury. These discoveries strongly indicate that proteasome inhibitor treatment has great potential in alcoholic liver disease therapy.

CONCLUSION

PS-341 is currently used in humans as an antitumor drug[26], and numerous studies have shown that it also represents a potential drug treatment for alcoholic liver disease. Proteasome inhibitors are a promising treatment to reduce ROS production, to reduce liver steatosis, and to reduce the production of pro-inflammatory cytokines caused by chronic ethanol feeding.

ACKNOWLEDGMENTS

The author thanks Emmanuel Gorce for typing and editing the manuscript.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2563

World J Gastroenterol 2011 May 28; 17(20): 2563-2571
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

siRNA targeting Livin decreases tumor in a xenograft model for colon cancer

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Received: October 6, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: May 28, 2011

Abstract

AIM: To evaluate the effect of silencing *Livin* gene expression with siRNA to apoptosis and proliferation in a colon cancer cell line.

METHODS: To investigate the anticancer effect of silencing *Livin* gene expression, we established an siRNA transfected cell line using the HCT116 colon cancer cell line. After confirming the successful transfection, MTT assay, flow cytometry and annexin V staining were employed to evaluate the antiapoptotic effect. To confirm the *in vivo* effect of Livin-siRNA, different doses of Livin-siRNA were injected into xenografted tumors in BALB/c nude mice model.

RESULTS: Livin expression was dramatically decreased after siRNA transfection, especially at 25 μmol/L of siRNA, but this suppression was not dose-dependent. The cell count at 18 h after transfection was significantly reduced as compared with controls ($P < 0.01$), but tended not to decrease proportionally depending on transfected dose or time. MTT assay revealed that silencing the *Livin* gene suppressed cellular proliferation at 18 h after transfection ($P = 0.04$); however, the inhibitory effect disappeared thereafter. Also, there was no significant difference in cellular proliferation depending on siRNA dose. The rate of apoptosis also increased with silencing of the *Livin* gene. *In vivo*, the tumor size significantly decreased after Livin-siRNA injection at 20 μmol/L concentration ($P = 0.03$). There were no significant body weight changes of mice after siRNA injection. Histologic examination revealed no significant toxic reaction in kidney, liver and brain of mice.

CONCLUSION: siRNA-mediated downregulation of Livin expression can induce apoptosis in colon cancer in vitro and *in vivo*, which suggests the possibility of new cancer therapeutics using siRNA.

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Key words: siRNA; Livin; Inhibitor of apoptosis; Colon cancer

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Oh BY, Lee RA, Kim KH. siRNA targeting Livin decreases tumor in a xenograft model for colon cancer. *World J Gastroenterol* 2011; 17(20): 2563-2571 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2563.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2563

INTRODUCTION

Colon cancer is one of the most common malignancies worldwide and has shown increased incidence, especially in developing countries[1]. Until recently, a major treatment strategy has been surgical resection that shows good outcome over other treatments. With the activation of a screen-

ing program, early cancer, less than stage I, is curable with only surgical treatment. But the treatment results for metastatic disease are still unsatisfactory with surgery alone. In these cases, clinicians consider other treatment options such as chemotherapeutic agents and targeted agents. Recently, development of techniques in manipulation of nucleic acids makes gene therapy possible to adopt in cancer therapy.

RNA interference (RNAi) is a fundamental protective process in eukaryotic cells including invertebrates and vertebrates, which is able to block harmful signal by targeting complementary mRNA and cleaving thereof[2]. Small interfering RNA (siRNA) is fragments from double-stranded RNA by the enzyme Dicer[3-5]. The natural role of RNAi is supposed to be a defense mechanism against some viral infection or deleterious genomic instability. This special mechanism has been of great interest recently as siRNA targeting small genes are easily manufactured and applied to major clinical problems such as cancer, asthma, inflammatory disease and infection[6]. This methodology is becoming a powerful tool for new drug development in such areas and is replacing the techniques of using antisense oligonucleotide and ribozymes[7,8].

Livin, a 280 amino acid protein and a member of the mammalian type of inhibitor of apoptosis (IAP), are well-conserved proteins across the species. It has a single baculovirus IAP repeat (BIR) domain and a COOH-terminal RING domain and is localized predominantly in the nucleus and a filamentous pattern of cytoplasm[9]. The Livin gene spans 46 kb and is located on chromosome 20 at band q13[10]. The major function of Livin is an inhibition of apoptosis by binding to caspase 3, caspase 7 and caspase 9 and also in inhibition of proteolytic processes of caspase 9[9,11]. Its overexpression protects cells from various proapoptotic stimuli.

The expression of Livin, similar to expression of Survivin, is rarely detected in normal adult tissues but exists abundantly in cancerous tissues and transformed cells[9]. The overexpression of Livin protein has been reported in colorectal cancer, leukemia, hepatocellular carcinoma, and melanoma[12]. Recently, major attention has been focused on the IAP family, especially Survivin and Livin, as they are easy to handle in the genetic field due to their small size. Moreover, the lack of expression in normal adult tissues of Livin is a very attractive characteristic in cancer therapy[9].

In this study, we used siRNA targeting to Livin transfection into the HCT116 colon cancer cell line to confirm the antitumor effect and blockade of Livin gene and performed an additional *in vivo* study using BALB/c nude mice xenografts to determine the direct tumor regression effect of Livin silencing with siRNA. These results suggested that Livin is an effective target for colorectal cancer treatment using siRNA technique.

MATERIALS AND METHODS

Materials

Cell culture-The HCT116 colon cancer cell line, purchased from Korean cell line bank, was grown in RPMI1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc.), penicillin (100 U/mL) and streptomycin (100 g/mL). Cells were maintained at 37℃ in a humidified atmosphere of 50 mL/L CO_2.

RT-PCR procedure

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA). The method for extracting total RNA was according to the manufacturer's protocol. RNA samples were quantified at 260 nm using spectrophotometry. One μg of total RNA was reacted for 15 min at 42℃ using the Promega's Reverse Transcription System (Promega Corp., Madison, WI), reacted for 5 min at 95℃, and cDNA was obtained. For polymerase chain reaction (PCR), cDNA (3 μL) was obtained by reverse transcription, 0.1 μmol primers, 1.25 μL GoTaq DNA polymerase, 0.2 mmol/L deoxynucleotide triphosphate (dNTP), 1 Go Taq reaction buffer and each primer was added, and DNA was amplified by performing PCR with primer of Livin; forward, 5-GTCAGTTCCT-GCTCCGGTCAA-3; reverse, 5-GGGCACTTTCAGACT-GGACCTC-3. Electrophoresis of PCR products was performed on 1.5% agarose gel and examined by staining with ethidium bromide. By measuring the brightness of bands using a densitometer, their amount was quantified using the value of β-actin as the standard value.

siRNA design and preparation

Synthetic 21-nt RNAs were purchased from Dharmacon Research (Lafayette, CO; in deprotected, desalted, and annealed form.). We used 4 primers for silencing the Livin gene to improve the blocking efficiency. The target sequences of Livin for production of siRNA were 5'-GGAGAGA-GGTCCAGTCTGA-3', 5'-GGAAGAACCGGAAGAC-GCA-3', 5'-GCTCTGAGGAGTTGCGTCT-3' and 5'-GCTCTGAGGAGTTGCGTCTTT-3'. The nonspecific control siRNA duplex was also purchased from Dharmacon Research.

Briefly, centrifuge tubes containing siRNA to ensure that the siRNA pellet was collected at the bottom of the tube. Resuspended siRNA to a convenient stock concentration with 1X siRNA buffer (Dharmacon, Inc., Lafayette, CO). After placing the solution on a shaker for 30 min at room temperature, the concentration of siRNA was verified using UV spectrophotometry at 260 nm.

HCT116 cells were diluted in fresh media without antibiotics and transferred to six-well plates (1×10^5 cells/well) 24 h before transfection and maintained at 37℃ in a humidified atmosphere of 50 mL/L. HCT116 cells grown to a confluence of 40%-50% were transfected with siRNA using Lipofectamine 2000 (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's recommendations. To detect the transfection efficiency, the cells were analyzed by FACSan (Becton Dickson, San Jose, CA) 6 h after transfection with FITC-labeled siRNA. After transfection, the cells were incubated and then used to various analyses.

MTT assay

Cell viability was examined by routine 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

HCT116 cells (1×10^6) were planted in 96-well plates with RPMI1640 in a final volume of 500 μL. On the following day, cells were treated with increasing concentration of siRNA and cultured for 48 h, then cell proliferation was assessed by MTT assay. Following incubation at 37℃ for 3 h, the reaction was stopped by the addition of 150 μL DMSO. After the crystal dissolved, the absorbency of the samples was determined at 492 nm.

Cell counting

HCT116 cells were planted in 24-well plates with RPMI 1640. Cells were treated with increasing concentration of siRNA and cultured for 48 h, then cells were collected by trypsinization, mixed in PBS and trypan blue added. Cells were counted using a microscope counting chamber.

Flow cytometric analysis

At 24 h after transfection, HCT116 cells on 60-mm tissue culture plate were treated with 1 × trypsin-EDTA (Invitrogen, Carlsbad, CA) for 5 min at 37℃; cells were then removed from the plate by gentle scraping, dispersed into cold PBS and washed twice. The cells were resuspended in 1 × binding buffer at a concentration of approximately 1×10^6 cells/mL. Five μL of Annexin V- FITC and 10 μL of PI were added to each cell suspension and incubated at room temperature for 10 min in the dark. The cells were subjected to fluorescence estimation using a FACS Calibur flow cytometer. Uninoculated cells stained with Annexin V-FITC and PI were used to determine background levels of apoptosis.

Xenograft model

All animal experiments in this report were performed with approval of the Institutional Animal Care & Use Committee in Ewha Womans University, School of Medicine. The 4-wk-old male BALB/c nude mice were grown in a sanitary room and adjusted for 1 wk before the start of the experiments. Viable HCT116 colon cancer cells (2.0×10^6 in PBS/100 μL) were injected subcutaneously into the right flank of the nude mice. Three weeks after tumor cell inoculation with confirmation of successful maturation of tumors, mice were divided randomly into four groups (ten mice per group) and were treated weekly for 4 wk by way of center-intratumoral direct injection at different doses (10, 20, 50 μmol/L) of siRNA manufactured with atelocollagen to achieve effective delivery. To prepare the siRNA/atelocollagen complex, equal volumes of atelocollagen (Atelo-Gene® Systemic Use; Koken, Tokyo, Japan. 0.1% in PBS at pH 7.4) and siRNA solution were combined and mixed by rotation for 20 min at 4℃. In the control group, pure PBS was injected using the same method. The tumors were monitored with a caliper every week over a 4 wk period after siRNA transfection. Tumor volume for each mouse was determined (in cubic millimeter) by measuring in two directions and was calculated as tumor volume = length × $(\text{width})^2/2$. Also, body weight of each mouse was monitored and recorded. Five weeks after the last siRNA injection, all mice were sacrificed according to the animal experimental guidelines and the xenografted tumors were excised and prepared with a routine pathological procedure. Tumor sections were deparaffinized and subjected to Hematoxylin and Eosin staining in the usual manner.

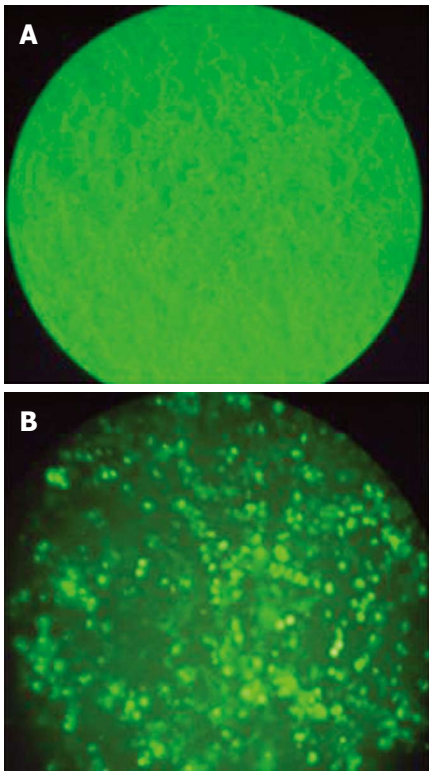

Figure 1 Transfection efficiency. Successful transfection of small interfering RNA was detected with fluorescence staining in HCT116 cell line. A: HCT116 cell culture; B: HCT116 cell with transfection.

Statistical analysis

Each experiment was repeated three times or more. Bands from RT-PCR were quantified with Quantity One software (Bio-Rad, Hercules, CA). mRNA levels were calculated by referring them to the amount of b-actin. The difference between means was performed with analysis of variance. All statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL). Statistical significance was considered when the *P* value was less than 0.05.

RESULTS

Transfection of siRNA-Livin to HCT 116 cell line

To identify whether Livin expression was affected by siRNA, we transfected siRNA at different concentrations (25, 50, 100, 150 and 200 μmol/L) to HCT 116 cell line, and then detected Livin density at 18, 24 and 30 h after transfection by RT-PCR.

Successful transfection of siRNA was detected with fluorescence staining (Figure 1) and expression of Livin gene was compared to β-actin. Control HCT 116 cells expressed Livin very well and this expression was dramatically decreased after siRNA transfection. But this effect was attenuated with time and Livin expression was re-

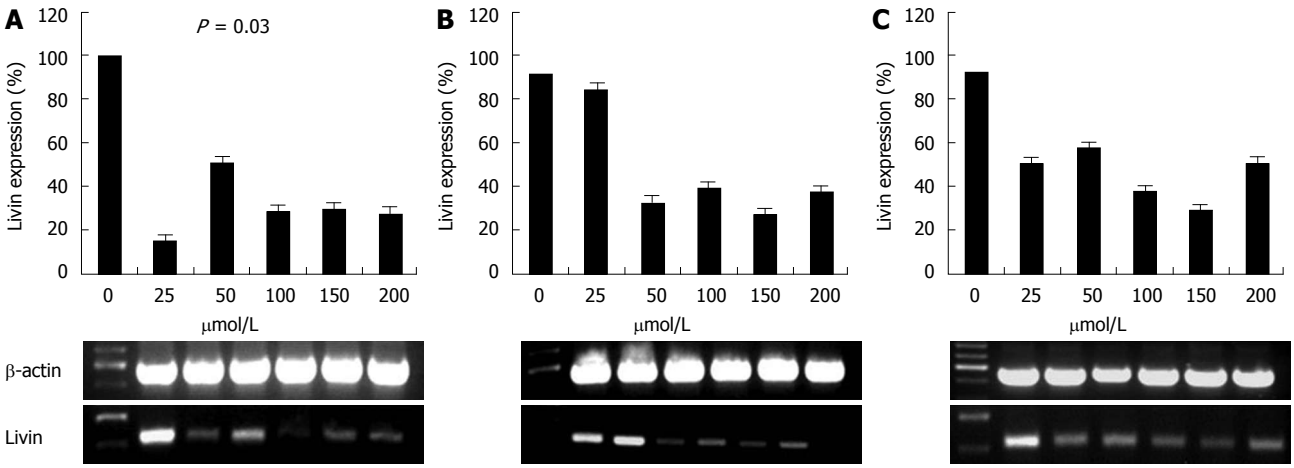

Figure 2 Change of Livin expression after small interfering RNA transfection. This figure shows Livin expression (%) in HCT116 cells at 18, 24 and 30 h post-transfection with 25, 50, 100, 150 and 200 μmol/L of siRNA. Livin expression was compared with β-actin by Western blotting. A: Livin expression at 18 h post-transfection. It was effectively suppressed by 25 μmol/L of siRNA, but this suppression was not dose-dependent; B: Livin expression at 24 h post-transfection; C: Livin expression at 30 h post-transfection; It was retrieved 30 h after transfection.

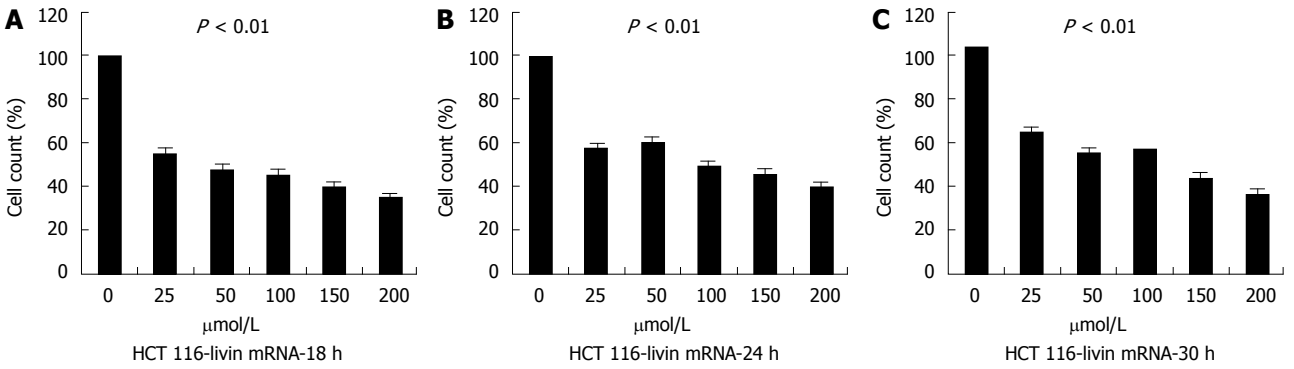

Figure 3 Cell counting after small interfering RNA transfection in HCT116 cell line. We estimated cell count (%) of each groups in HCT116 cells after Livin silencing with 25, 50, 100, 150 and 200 μmol/L of siRNA. The cell count at 18 h after transfection was the most effectively reduced, but this effect was not time-dependent or dose-dependent. A: Cell count at 18 h post-transfection was significantly reduced as compared with control. ($P < 0.01$); B: Cell count at 24 h post-transfection; C: Cell count at 30 h post-transfection.

trieved 30 h after transfection. Livin expression was most effectively suppressed at 25 μmol/L of siRNA, but this suppression was also not dose-dependent (Figure 2).

Reduction of viable cell numbers with siRNA to Livin

First, we estimated cell counts of each group to evaluate tumor cell growth after Livin silencing. We analyzed cultured cells grown in three dishes per group at one time, and repeated this study three times. As seen in Figure 3, the cell count at 18 h after transfection was significantly reduced as compared with control ($P < 0.01$), but tended not to decrease proportionally depending on transfected dose or relapsing time.

Suppression of cellular proliferation by SiRNA

To quantify the cellular viability, MTT assay was performed. The MTT assay showed that, compared with controls, the proliferation of cells transfected with siRNA was remarkably inhibited at 18 h after transfection ($P = 0.04$), but the inhibitory effect disappeared thereafter, which indicated that the number of viable cells began to increase. There was no significant difference in cellular proliferation depending on siRNA dose (Figure 4).

Induction of apoptosis by SiRNA

Flow cytometric analysis after Annexin V staining was performed to investigate whether Livin-silencing induces apoptosis resulting in reduction of cell count or suppression of cellular proliferation as per the above findings. As a result, the apoptotic rate was increased continuously since 50 μmol/L of siRNA was treated. The apoptotic rate in control was 3.91%, the apoptotic rate of cells transfected with siRNA 50 μmol/L was 30.32%. The apoptotic rates of cells transfected with siRNA 100 μmol/L, 150 μmol/L and 200 μmol/L increased to 32.88%, 36.91% and 45.08%, respectively (Figure 5). The apoptotic rate was increased dose-dependently, but the necrotic portion was also increased. So, it was thought low dose siRNA was more effective in the clinical setting.

Change of tumor volume in vivo after siRNA treatment

Next, we designed a xenograft model to identify the po-

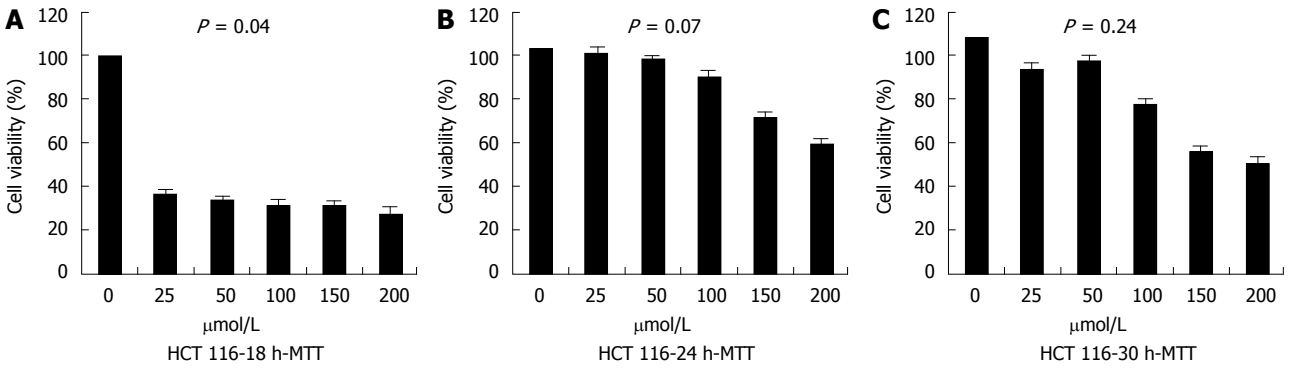

Figure 4 MTT assay after small interfering RNA transfection in HCT116 cell line. A: The proliferation of cells transfected with siRNA was remarkably inhibited at 18 h post-transfection ($P = 0.04$); B and C: Cellular proliferation at 24 and 30 h post-transfection. The inhibitory effect shown A disappeared, cellular proliferation was similar with control.

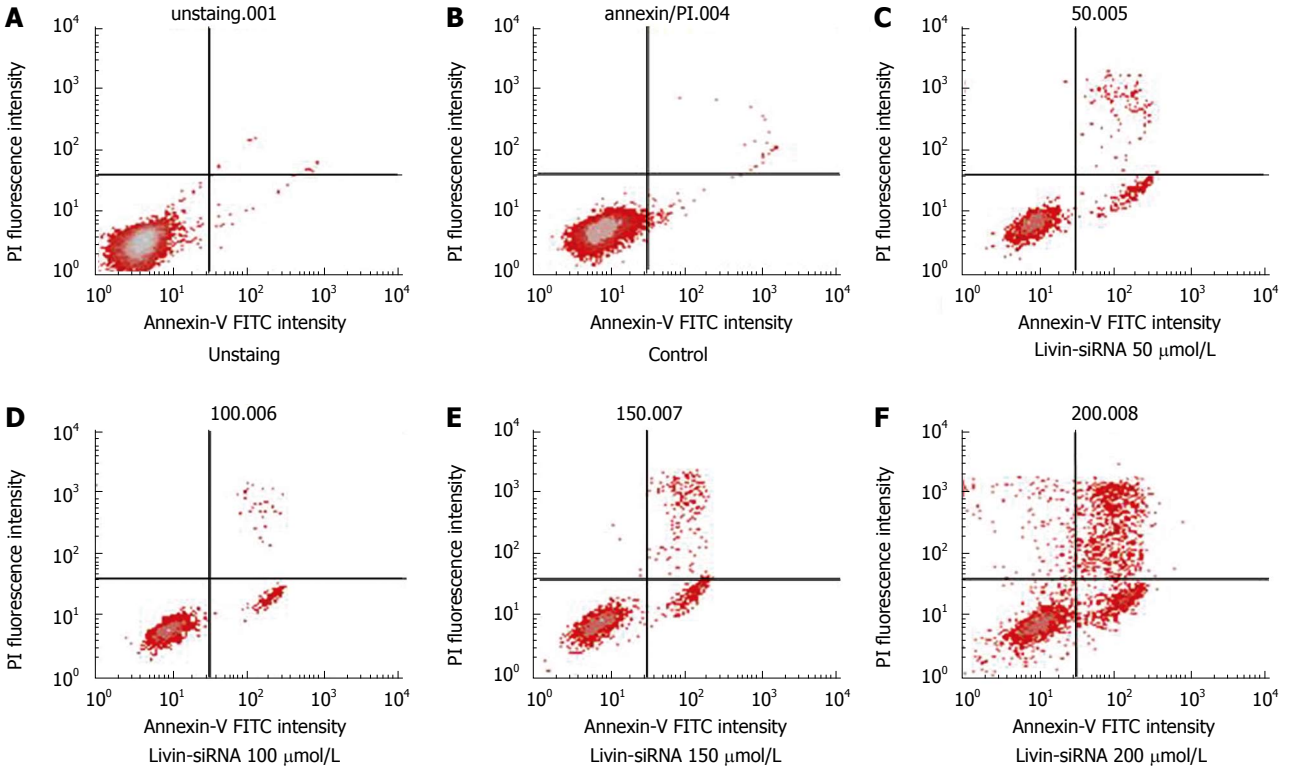

Figure 5 Results of Annexin V staining after small interfering RNA transfection in HCT116 cell line. The apoptotic portion (right lower quadrant) was increased continuously after siRNA transfection, but the necrotic portion (right upper quadrant) was also increased. A: The apoptotic rate of staining was 0.26%; B: The apoptotic rate of control was 3.91%; C: The apoptotic rate of cells transfected with siRNA 50 μmol/L was 30.32%; D: The apoptotic rate of cells transfected with siRNA 100 μmol/L was 32.88%; E: The apoptotic rate of cells transfected with siRNA 150 μmol/L was 36.91%; F: The apoptotic rate of cells transfected with siRNA 200 μmol/L was 45.08%.

tential *in vivo* effects of siRNA on inhibition of the proliferation of colon cancer cells. Viable HCT 116 colon cancer cells were injected subcutaneously into the right flank of the nude mice.

In the first series of experiments, the inoculated mice were divided randomly into four groups of ten mice each and were treated with different doses of siRNA (control, 10, 20 and 50 μmol/L) weekly, for 4 wk. Over a 4 wk period after siRNA injection, the tumor volumes were estimated every week. As seen in Figure 6, the rates of tumor growth significantly decreased in higher than 20 μmol/L transfected groups with siRNA as compared with the control group 2 wk following treatment ($P = 0.03$). However, there was no difference in antitumor effect between 20 μmol/L and 50 μmol/L in the transfected group.

In the first study series, we detected that siRNA 20 μmol/L was the most effective dose for reduction of tumor growth in xenograft models. Thereafter, in the second series of experiments, we divided the inoculated mice randomly into two groups (control group and siRNA 20 μmol/L treated group) of ten mice per group. The mice of the siRNA 20 μmol/L treated group were injected with siRNA daily for 4 wk, and then the tumor volumes were measured every week during that period. The result was similar with the previous experiment; the mean volume of tumors decreased in transfected groups with siRNA

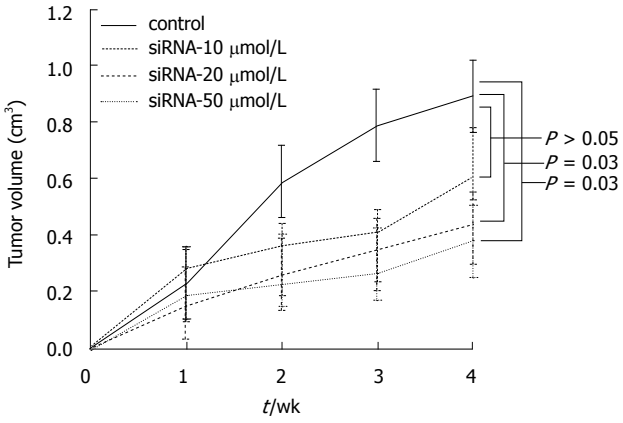

Figure 6 Tumor volume after small interfering RNA intratumoral injection *in vivo*. The inoculated mice were divided into four groups and were treated with different doses of siRNA (10, 20 and 50 μmol/L) weekly for 4 wk; tumor volumes were checked every week. In higher than 20 μmol/L siRNA, the tumor volumes were significantly decreased as compared with control group from 2 wk after transfection (P = 0.03).

20 μmol/L as compared with control groups (Figure 7).

To evaluate the toxic effect of siRNA *in vivo*, the body weights of mice were taken during the experimental period. The body weight of mice in all groups slightly increased over the entire experimental period; there were no significant body weight changes after siRNA injection compared to the control group. Histologic examination was performed with tissues taken from sacrificed mice after experiment completion. It revealed no significant toxic reaction in kidney, liver and brain of siRNA treated mice compared with the control group (data not shown).

Figure 7 Tumor xenograft in nude mice. The inoculated mice were divided into two groups (control and siRNA-20 μmol/L treated group), and then tumor volume was measured in the the same manner as described in Figure 6. A: Control group; B: siRNA-20 μmol/L treated group. The tumor volume was dramatically decreased as compared with control.

DISCUSSION

Eight human IAP molecules have been reported to date; NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7), and IAP-like protein 2 (BIRC8)[12]. They are categorized as IAP by the presence of a baculovirus IAP repeat (BIR) protein domain, which forms a zinc-fold, a critical motif for regulating apoptosis. Many in this family also harbor a COOH-terminal RING finger domain. The BIR domain constitutes the zinc-fold, which is the important motif in delivering the anti-apoptotic signals[9]. It has been proposed that endogenous Livin has a minor direct effect on caspase activity whereas its anti-apoptotic effect could be ascribed to its antagonizing activity on the XIAP-Smac/DIABLO interaction and has been regarded as counteraction molecules against apoptosis through the blocking the terminal effectors such as caspases, thus far[12]. That the IAPs are highly conserved from yeast to mammals suggests the importance of this family in maintain life[11]. There are numerous reports on overexpression of the IAP family in cancer tissues to normal counterparts. The role of Survivin and Livin, in particular, in carcinogenesis has been well confirmed to date.

Livin was reported in 2001 as a novel IAP family protein[9]. The overactivity of Livin was regarded as a predictor of poor prognosis in cancer patients and a resistance factor to chemotherapeutic agents or radioactive drugs in several cancers[13,14]. The overexpression of Livin mRNA has been found in some tumors including melanoma, breast, cervical, colon and prostate cancers, as well in leukemia, in lymphoma and in hepatoma cell lines[12]. Moreover, researchers have shown that only overexpression of Livin-α isoform is correlated with high risk of relapse in bladder cancer[10].

In the previous study, Livin gene was expressed well in the stools of colon cancer patients as opposed to the healthy control group. This finding suggests that the exfoliated cancer cells from the gastrointestinal tumors were contained in the patients' stool and overexpressed tumor related genes such as Livin were detected from the stool. Based on this result, we chose Livin gene as a treatment target in this experiments.

In recent years, numerous genetic engineering techniques were introduced in drug development markets worldwide such as antisense oligonucleotides, aptamer (single stranded nucleic acids), use of ribozymes, dominant negative mutant construction and siRNA. We focused on siRNA technology because of its target sequence-specific degradation ability and relatively simple application procedure.

In higher organisms, defense mechanisms are more complex but long dsRNAs should not be used as an experimental tool to trigger RNAi in mammalian cells. Long dsRNAs are processed by the endonuclease Dicer

into short 21 bp duplexes with siRNA instead of direct suppression[15]. RNA interference is a post-transcriptional regulation process which exploits a complex pathway that regulates gene expression and includes machinery for sequence specific mRNA degradation[8,16].

But there are still some limitations to overcome in applying siRNA in clinical fields. The greatest interference is the short half-life of the delivered siRNA *in vivo* due to renal clearance. Another is a normally existing RNase activity that can degrade siRNA administered artificially. The next key to successful *in vivo* application of siRNA is the delivery system. Transfering the siRNA into target tissues and into the cell cytoplasm is not easily achieved and a most delicate process is required to get the therapeutic potential. Usual methods to achieve maximum therapeutic effects are lipid or protein carriers, liposomes, antibody promoter fusions, cyclodextrin nanoparticles, fusogenic peptides, aptamers, biodegradable polylactide copolymers and polymers[17].

There are several reports on siRNA targeting Livin treatments for cancer in the literature. Crnkovic-Mertens *et al*[18] first reported the induction effects of apoptosis with Livin targeted-siRNA in a HeLa cell line. Their results suggested that the possibility of intracellular interference with Livin gene expression sensitized human tumor cells to apoptosis. Recently, Wang *et al*[19] investigated the apoptotic susceptibility of the SGC-7901 gastric cancer cell by shRNA-mediated silencing of the Livin gene. When Livin gene was silenced, the reproductive activity of the gastric cancer cells was significantly lower than the control groups ($P < 0.05$). The study also showed that IC50 of 5-FU and cisplatin on gastric cancer cells treated by shRNA decreased and the cells were more susceptible to proapoptotic stimuli (5-FU and cisplatin) ($P < 0.01$). In another study[20], it was reported that expression of Livin was downregulated by siRNA in dose- and time-dependent manners, and silencing Livin promoted apoptosis in malignant melanoma LiBr cells. They also demonstrated that silencing Livin by siRNA leads to cell cycle arrest at the G0/G1 phase, reducing the rate of DNA synthesis or resulting in apoptosis. With these promising results, a combination treatment of Livin-targeting siRNA with proapoptotic drugs appears to be more effective in clinical perspectives.

In this study, we confirmed that silencing of Livin gene expression with siRNA was well documented in HCT116 colon cancer cells associated with an increased apoptotic response. In preliminary experiments, we tested the expression of Livin immediately, 2, 6, 12 and 24 h after treatment of siRNA (data not shown). The decrease of Livin expression was notable from 2 h after treatment but the effect was not much different before 24 h, so we extended the time to 30 h after treatment of siRNA. Testing dosage was arranged from 25 to 200 μmol/L, but the silencing effect of siRNA was not proportional in order of concentration. It was possible that a very small amount of siRNA could create the action to achieve the silencing of Livin gene in the cell line. Unfortunately, the silencing effect weakened from 24 h after treatment, which means duration of siRNA to Livin is very short and time limited. Another consideration is the proliferation of the cells not influenced by siRNA affected the total expression of the Livin with time. On the other, the cell count decreased 45% with 25 μmol/L siRNA at 18 h after treatment and lasted for 30 h and cellular proliferation counted by MTT assay revealed the same results with cell counting. Proliferation was more influenced by siRNA treatment in the early phase compared to the decrease of total cell count numbers. With this phenomenon apoptosis increased 24 h after treatment with dose dependent manner. To integrate the full results, silencing of Livin in colon cancer cell was easily achieved with siRNA treatment into the induction of apoptosis from minimal dosage only in *in vitro* environment.

We used the mouse xenograft model to identify the antitumor effect of siRNA to Livin *in vivo*. We selected the direct intratumoral injection method to verify the direct effect on tumors. In the clinical setting, it is very difficult to use the infected cells of siRNA before confirmation of drug safety regulation, so direct implication to the tumor itself would be more readily applicable to the human body. Because of the limitation of uptake of siRNA, we adopted atelocollagen in this experiment to obtain maximal therapeutic effects. Atelocollagen is derived from type I collagen of calf dermis by pepsin digestion. A telopeptide that is an amino acid sequence of N- and C-terminal of the collagen contains the most antigenicity. Atelocollagen has limited antigenicity because of a lack of telopeptides, so it is more easily applicable to drug delivery processes. It is well confirmed that atelocollagen-siRNA complexes are taken up into cells to achieve a silencing effects. Also, atelocollagen-siRNA complexes are resistant to nucleases, which is another important advantage in siRNA application *in vivo*[8]. Several reports were found in the database revealing siRNA effects with atelocollagen complex. Atelocollagen is one of the more useful DDS available in clinical settings. Kawata *et al*[21] reported PLK-1 (polo-like kinases-1) siRNA with atelocollagen in hepatic metastatic cells from lung cancer. Takeshita *et al*[22] demonstrated the evidence of tumor targeted delivery of systemically administered siRNA with atelocollagen, injected siRNA intracardiac to luciferase and luciferase-expressing bone-metastatic tumor models. In another study, a therapeutic effect *via* atelocollagen-mediated systemic administration of siRNA targeting Bcl-xL into pregrown PC-3 xenografts was reported[17].

A number of studies have reported antitumor effects of siRNA *in vivo*. Verma *et al*[23] demonstrated that siRNA was effective in reducing growth of HCT116 cells in nude mice. These mice were injected with HCT116 cells intraperitoneally and then were treated with siRNA directed against β-catenin, the regulator of cellular proliferation in colon cancer. There was a significant decrease in the tumor size of cells transfected with siRNA directed against β-catenin[23]. In another study, it was found that transfected HCT116 colon cancer cells with siRNA against Survivin, a type of IAP family such as Livin, significantly decreased in size as compared with controls in xenograft models[24]. In a recent study, it was

Figure 8 Hemorrhagic necrosis in small interfering RNA injection site. The inoculated mice were injected with siRNA ***via*** the intratumoral route; there was local hemorrhagic necrosis in the direct injection site of siRNA.

observed that siRNA against Survivin remarkably induced apoptosis in SW480 cell as a consequence of the inhibition of Survivin and led to significant inhibition of tumor growth *in vivo*[1].

In our xenograft model, the rates of tumor growth were significantly decreased in higher than 20 μmol/L transfected groups with siRNA as compared with control groups. This antitumor effect tended to be greater at a higher dose of siRNA but the differences between each group were not statistically significant. So it would be expected that a low dose of siRNA showed a satisfactory effect on tumor suppression. In addition to the antitumor effect, toxic effects of treatment should be considered. Some therapeutics have been associated with toxic side effects such as weight loss and other organ injury. These systemic toxicities may be related with higher morbidity and lower response rate, resulting in poorer survival. Thus, if there are systemic toxicities caused by drugs, no matter how effective, they should not be used. In this study, measured body weights of mice had no differences, and there were no significant pathologic findings on tissues withdrawn from siRNA-treated group. For these reasons, it could be thought that siRNA targeting Livin can be used in colon cancer therapy without systemic toxic effects *in vivo*. In our present study, another interesting finding was detected despite an effective reduction of tumor size with siRNA. Local hemorrhagic necrosis was seen in the intratumoral injection site of siRNA with syringe, this finding was not identified in other studies (Figure 8). If there are local side effects such as this, even though there is no evidence of systemic toxicity of siRNA, we should carefully determine administration routes to develop antitumor treatments using siRNA.

In conclusion, siRNA-mediated downregulation of Livin expression can induce apoptosis in colon cancer *in vitro* and *in vivo*, which implies new potential cancer therapeutics using siRNA in colon cancer.

COMMENTS

Background

Colon cancer is one of the most common malignancies and has increased in incidence in recent years. Several treatment modalities have been used, especially in metastatic diseases. Several studies have been reported to implicate gene therapy for metastatic colon cancer. Livin, a type of inhibitor of apoptosis, protects cells from various proapoptotic stimuli. So Livin is an effective target for colorectal cancer treatment.

Research frontiers

The overexpression of Livin has been reported in colorectal cancer. However, the direct tumor regression effect of Livin silencing has not been unequivocally addressed. In this study, the authors investigate the effect of silencing *Livin* gene expression with siRNA to apoptosis and proliferation in colon cancer cell line.

Innovations and breakthroughs

There are several reports on siRNA targeting Livin treatments for cancer. But these studies were nearly all performed *in vitro* and there were few studies about colorectal cancer. In this study, the authors confirmed that silencing of Livin gene expression with siRNA was well documented *in vitro and in vivo*, especially in colorectal cancer.

Applications

siRNA-mediated downregulation of Livin expression can induce apoptosis in colon cancer *in vitro* and *in vivo*, which may imply new potential cancer therapeutics using siRNA in colorectal cancer.

Terminology

Livin is a 280 amino acid-protein and a member of the mammalian type of inhibitor of apoptosis. It inhibits apoptosis by binding to caspase 3, caspase 7 and caspase 9. Its overexpression protects cells from various proapoptotic stimuli, and is shown in numerous cancers. RNA interference is a fundamental protective process in eukaryotic cells, which is able to block harmful signals by targeting complementary mRNA and cleaving it. The role of RNAi is supposed to be a defense mechanism against deleterious genomic instability.

Peer review

The experiments are properly performed, and findings support their conclusion.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2572

World J Gastroenterol 2011 May 28; 17(20): 2572-2579
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Differential protein expression during colonic adaptation in ultra-short bowel rats

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Supported by A Grant from the Natural Science Foundation of Guangdong Province, China, No. 07005961
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Received: December 31, 2010 Revised: April 13, 2011
Accepted: April 20, 2011
Published online: May 28, 2011

Abstract

AIM: To investigate the proteins involved in colonic adaptation and molecular mechanisms of colonic adaptation in rats with ultra-short bowel syndrome (USBS).

METHODS: Sprague Dawley rats were randomly assigned to three groups: USBS group (10 rats) undergoing an approximately 90%-95% small bowel resection; sham-operation group (10 rats) undergoing small bowel transaction and anastomosis; and control group (ten normal rats). Colon morphology and differential protein expression was analyzed after rats were given post-surgical enteral nutrition for 21 d. Protein expression in the colonic mucosa was analyzed by two-dimensional electrophoresis (2-DE) in all groups. Differential protein spots were detected by ImageMaster 2D Platinum software and were further analyzed with matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight-mass spectrometric (MALDI-TOF/TOF-MS) analysis.

RESULTS: The colonic mucosal thickness significantly increased in the USBS group compared with the control group (302.1 ± 16.9 μm *vs* 273.7 ± 16.0 μm, $P < 0.05$). There was no statistically significant difference between the sham-operation group and control group ($P > 0.05$). The height of colon plica markedly improved in USBS group compared with the control group (998.4 ± 81.2 μm *vs* 883.4 ± 39.0 μm, $P < 0.05$). There was no statistically significant difference between the sham-operation and control groups ($P > 0.05$). A total of 141 differential protein spots were found in the USBS group. Forty-nine of these spots were down-regulated while 92 protein spots were up-regulated by over 2-folds. There were 133 differential protein spots in USBS group. Thirty of these spots were down-regulated and 103 were up-regulated. There were 47 common differential protein spots among the three groups, including 17 down-regulated protein spots and 30 up-regulated spots. Among 47 differential spots, eight up-regulated proteins were identified by MALDI-TOF/TOF-MS. These proteins were previously reported to be involved in sugar and fat metabolism, protein synthesis and oxidation reduction, which are associated with colonic adaption.

CONCLUSION: Eight proteins found in this study play important roles in colonic compensation and are associated with sugar and fat metabolism, protein synthesis, and molecular chaperoning

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Key words: Ultra-short bowel syndrome; Enteral nutrition; Colon adaptation; Proteomics

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INTRODUCTION

Short bowel syndrome (SBS) is a chronic malabsorptive syndrome resulting from extensive small bowel resections[1]. Residual small bowel less than 30 cm from proximal end or distal end is termed ultra-short bowel syndrome (USBS)[1]. It occurs when the bowl is less than 200 cm *in situ*[2]. The estimated population prevalence is approximately one per million[3]. In adults, SBS usually results from resection of unviable intestine secondary to vascular insufficiency, Crohn's disease, malignancy or radiation[3]. In children with congenital intestinal anomalies, such as gastroschisis or atresia, necrotizing enterocolitis leads to insufficient intestinal length[4]. Malabsorption of macronutrients and micronutrients may predominate as a clinical manifestation, whereas other patients may struggle to maintain fluid and electrolytes homeostasis[5]. These patients may become dependent on parenteral nutrition (PN) support to maintain their energy balance. A study showed that the colon could undergo adaptation like the small intestine[6]. Epithelial hyperplasia occurred 24-48 h after small intestinal resection in experimental models[7-9]. Animal models showed that adaptation process is stimulated by enteral nutrition for enterocyte reproduction, resulting in the release of trophic factors[10]. Our previous studies have also found changes in morphology and ultrastructure of rat colon and adaptation of absorption functions[11]. However, the proteins and molecular mechanisms associated with the colonic adaptation are still unknown. In this study, we used a proteomics approach to identify the proteins associated with colonic adaptation in USBS rats, and discussed the molecular roles of the identified proteins in colonic adaptation.

MATERIALS AND METHODS

Laboratory animals grouping

Thirty healthy male Sprague Dawley rats (aged 3-4 mo, weighing 250-300 g) were randomly assigned to three groups (10 rats in each group): USBS group undergoing an approximately 90%-95% small bowel resection; sham-operation group undergoing small bowel transaction and anastomosis; and normal control group.

USBS model and postoperative feeding

On the day of operation, animals were fasted from solid food, but with unrestricted access to water. Animals in the USBS group were anesthetized with chloral hydrate (0.3 mL/100 g) for surgical resection of the small bowel. The entire small intestine was resected from 1 cm distal to the Treitz ligament to 1 cm proximal to the ileocecal valve. End-to-end anastomosis of the jejunum to the ileum was performed.

After recovery from anesthesia, rats were fed Peptisorb (Holland Nutricia). The nutrient fluid had an osmotic pressure of 410 mosm/L and a concentration of 25.2%. The nutrient mix could provide 413.82 J of energy per 100 mL. The fluid contained carbohydrates and 85% protein made up the short-chain hydrolytic lactalbumin, including 50% medium-chain triglycerides and 50% long-chain triglycerides. Both the USBS group and sham-operation group were fed with 12.6% Peptisorb on days 1-3 after surgery. Intake was increased to 16.8% on days 4-6 and to 25.2% from day 7 after surgery to the end of the experiment on day 21. Rats were fed 80 mL nutrient mixture daily using a special bottle. Diphenoxylate (0.75 mg/kg daily) was added to the feeding solution to reduce diarrhea.

Morphologic observation

All rats were anesthetized using chloral hydrate (0.3 mL/100 g) prior to a terminal surgical procedure. A 0.5 cm colon was resected from near the ileocecal valve and HE stained in paraffin. The thickness of tunica mucosa coli and the height of plica were measured with an automatic image analysis. Five non-serial sections were measured from each specimen. Average value and standard deviation (SD) were calculated.

Extraction of colonic mucosa

A 1-cm segment of colon was removed from near the ileocecal valve and incised longitudinally after enteral nutrition for 21 d. After being repeatedly washed with double-distilled water, the colon was quickly scraped with glass slides in an ice bath to collect intestinal mucosa. The intestinal mucosa were stored at -80℃ or used for immediate schizolysis to extract protein.

Protein extraction and quantification

Total mucosa was obtained by mixing colonic mucosa from rats in each group. The combined intestinal mucosa was then triturated in liquid nitrogen and lyzed in ice-cold lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% (w/v) CHAPS, 1 mmol/L DTT, 10 mmol/L PMSF and protease inhibitor cocktail). The cellular lysate was centrifuged at 13 200 r/mim for 30 min. The supernatant was collected and stored at -80℃. Protein concentration was determined using the Bradford assay, as previously described (REF).

Two-dimensional electrophoresis (2-DE) analysis

Protein from intestinal mucosa (150 μg) was applied by 2-DE analysis. 2-DE was performed as previously described. Briefly, one-dimensional isoelectric focusing (IEF) was performed on 13-cm gel strips, pH 3-10 NL (Amersham Ettan IPGPhor IEF system). The following IEF protocol was used at 20℃: rehydration at 30 V for 16 h; 500 V for 1 h; 1000 V for 1 h; and 8000 V for 64000 Vh.

After IEF, the strips were incubated for 15 min with 10 mL equilibration solution A [2% sodium dodecyl sulfate (SDS), 50 mmol/L Tris-HCl pH 8.8, 6 Murea, 30% v/v glycerol, 0.002% bromophenol blue, and 100 mg dithiothreitol (DTT)]. The strips were further equilibrated for 15 min with equilibration solution B, which was identical to solution A except that 250 mg iodoacetamide replaced the DTT. Samples were then transferred onto 12.5% SDS-PAGE for 2-D separation. Proteins in 2-DE gels were stained with silver. Images were scanned using Image-scanner. Each sample was analyzed three times.

Gel image analysis

The protein spots and their differential expressions were analyzed using ImageMaster 2D Platinum software. USBS group gel was compared with the other two groups. Two groups of differentially acquired protein spots were compared to find common differential protein spots. Protein spots achieving a > 2-fold increase in spot intensity and observed in three replicate gels from three independent experiments were scored and subjected to matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight-mass spectrometric (MALDI-TOF/TOF-MS) analysis.

In-gel digestion and protein identification by mass spectrometry

The differential protein spots were in-gel digested, with minor modifications. In brief, the differential protein spots were destained using 15 mmol/L $K_4Fe(CN)_6$ and 50 mmol/L sodium thiosulfate. Spots were digested with trypsin at 37℃ for 16 h. Peptide mixtures were extracted from the gel spots. The extracted peptide mixtures were dried using the SpeedVac Centrifuge.

The peptide mixtures were analyzed on an ABI 4800 plus MALDI-TOF/TOF-MS (ABI, CA). Each spectrum was produced by accumulating the data from 500 consecutive laser shots in the mass range of 900-3500 Da. Seven maximum precursor ions with a signal-to-noise ratio > 50 were chosen for tandem mass spectrometry (MS-MS). The obtained MS and MS-MS data were processed by GPS Explorer software (V3.6). Proteins were identified by the MASCOT search engine (V2.1) in the IPI mouse database based on these MS and MS/MS spectra. Proteins were considered a match if the error for peptide mass was 100 ppm or lower and the mass accuracy was 0.2 Da or lower. Scores > 59 were considered statistically significant ($P < 0.05$).

Class of differentially expressed proteins

Using information from the European Bioinformatics Institute (http://www.ebi.ac.uk), the differential proteins were classified based on function. The relationship between protein expression and biological function in the USBS group was further analyzed, as described below.

Bioinformatics analysis of protein-protein interaction network

The protein-protein interaction network was analyzed by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) system. The following sets of STRING programs were employed: organism, required confidence (score), interactions, and additional nodes shown as "homo sapiens," "low confidence (0.150)," "no more than 10 interactions," and "0." For other parameters, default settings were used.

Statistical analysis

One-way analysis of variance and the Student-Newman-Keuls test were performed using SPSS 13.0 statistical software. Data were expressed as mean ± SD. P values less than 0.05 were considered statistically significant.

RESULTS

Colonic plica and thickness increased in USBS rats

Among USBS rats, the survival rate was over 90%. Only one rat died of intestinal obstruction 6 d after operation. Rats were observed with dull coats and severe hair loss, especially within the first two weeks after the operation. Rats gradually regained a dense, shiny coat. Rats experienced severe diarrhea, rectal eversion, perineal eczema and infections, and perianal and tail skin ulcers forming ringtail disease.

The colonic mucosal thickness was 302.1 ± 16.9, 276.6 ± 19.1 and 273.7 ± 16.0 μm in the USBS group, sham-operation group and control group, respectively. The colonic mucosal thickness was significantly higher in the USBS group than in other two groups ($F = 7.46$, $P = 0.003$). The colonic mucosal thickness was not significantly different between the sham-operation and control groups ($P = 0.717$). The height of colon plica of the three groups was 998.4 ± 81.2 μm, 893.7 ± 20.2 μm, and 883.4 ± 39.0 μm, respectively. The height of colon plica markedly improved in the USBS group compared with the control group ($F = 13.96$, $P < 0.001$). There were no significant differences in plica height between sham-operation and control groups ($P = 0.665$, Figure 1).

Proteome profile alterations in ultra-short intestine mucosa

To identify the proteins involved in colonic adaptation, total proteins isolated from colonic mucosa of the three groups were separated on a 2-D gel (pH 3-10 NL), respectively. The gels were visualized by silver staining (Figure 2). In total, approximately 700 protein spots were detected in each of the silver-stained gels using ImageMaster software. Differential spots were scored when there was a > 2-fold change in spot intensity in three replicate gels from three independent experiments. The analysis revealed 141 differential protein spots, including 49 increased spots in USBS group and 92 decreased spots in the control group; and 133 differential protein spots, including 103 increased spots in USBS group and 30 decreased spots in the sham-operation groups. Among these differential protein spots, 47 differential protein spots were frequently observed in all three groups, including 30 increased spots and 17 decreased spots.

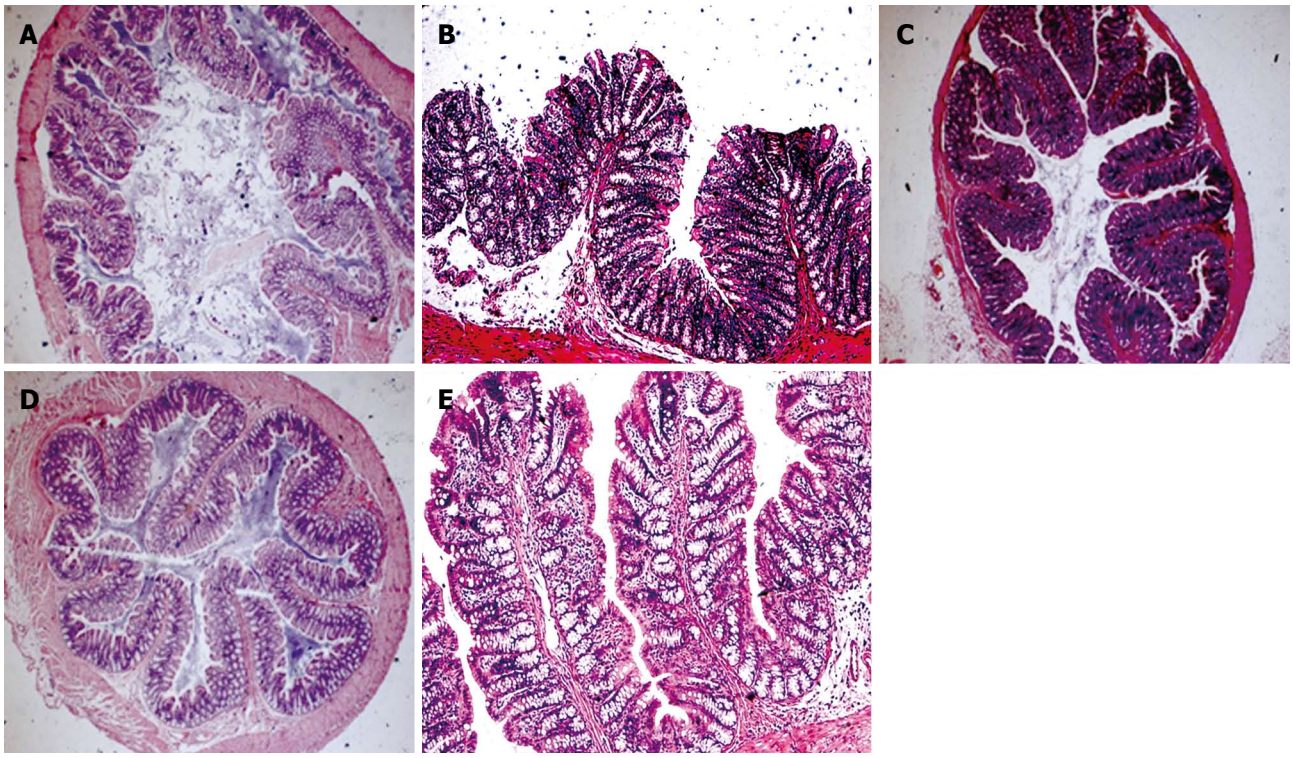

Figure 1 Change in colonic mucosal thickness and height of colon plica in ultra-short bowel syndrome, sham-operation, and control rats. A: Normal group, 25 ×; B: Normal group, 50 ×; C: Sham group, 25 ×; D: Ultra-short bowel syndrome group, (USBS) 25 ×; E: USBS group, 50 ×.

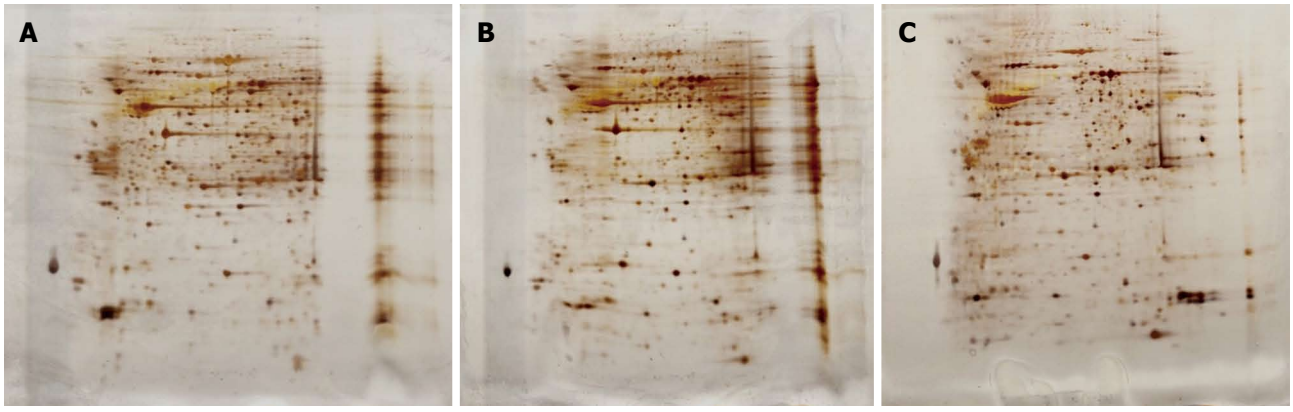

Figure 2 Image of 2-DE gels for proteins extracted from the colon mucosa of ultra-short bowel syndrome, sham-operation, and control rats. Representative results from three independent experiments are shown. A: Normal group; B: Sham group; C: Ultra-short bowel syndrome group.

Identification of proteins associated with colonic adaptation

Protein spots were subjected to in-gel digestion with trypsin. Protein identities were determined with MS and MS-MS by searching the IPI mouse database using the MASCOT program. This allowed us to identify eight proteins from 43 differential spots, including protein disulfide-isomerase A3, phosphoglycerate kinase 1, pyruvate kinase isoforms M1/M2, alcohol dehydrogenase class-3, mitochondrial ribosomal proteins, pancreatic triacylglycerol lipase, Hnrph1, and Lambda-crystallin homolog. These proteins are listed in Table 1, along with their IPI accession number, molecular weight, isoelectric point, scores and fold changes among the groups.

Functional classification of differential proteins

Eight differential proteins were preliminarily classified by functions using the European Bioinformatics Institute Bioinformatics Database (http://www.ebi.ac.uk). Protein disulfide-isomerase A3 is associated with molecular chaperoning. Phosphoglycerate kinase 1 and pyruvate kinase 3 isoforms M1/M2 are associated with glucose metabolism. Pancreatic triacylglycerol lipase is associated with fat metabolism. Lambda-crystallin homolog and alcohol dehydrogenase class-3 are associated with oxidation and reduction. Hnrph1 protein, and mitochondrial ribosomal protein L12 are associated with protein synthesis.

Table 1 Differential proteins identified by mass spectrometry

No.	Spot No.	Protein name	Accession No.	Protein MW/PI	Protein score	FD (24 h)
1	528	Phosphoglycerate kinase 1	IPI00231426	44510 /8.02	243	1000000
2	725	Pancreatic triacylglycerol lipase	IPI00198916	51407/36.31	60	1000000
3	946	Hnrph1 protein	IPI00650124	20567/3 5.2	172	1000000
4	331	Protein disulfide-isomerase A3	IPI00324741	57043 /5.88	391	2.4 ± 0.2
5	599	Pyruvate kinase isoforms M1/M2	IPI00231929	57938.9/6.63	67	2.0 ± 0.5
6	603	Alcohol dehydrogenase class-3	IPI00568787	39550.2/7.45	70	2.2 ± 0.3
7	746	Lambda-crystallin homolog	IPI00213610	35318/5.94	222	2.1 ± 0.4
8	1033	Mitochondrial ribosomal protein L12	IPI00203773	29422/99.7	122	2.3 ± 0.2

Protein names and accession numbers are from the International Protein Index rat database. MW: Molecular weight; PI: Isoelectric point; FD: The average fold change among; USBS group: Sham-operation group and control group.

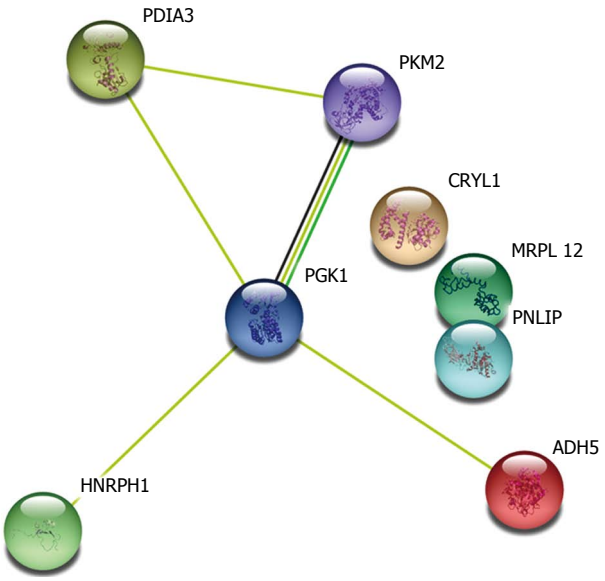

Figure 3 Protein-protein interaction networks involved in colonic adaptation predicted by the STRING program.

Protein-protein interaction networks involved in colonic adaptation

STRING is a system for mapping protein-protein interaction networks. We used the STRING system to construct the protein-protein interaction network of the differential proteins associated with colonic adaptation. Five (phosphoglycerate kinase 1, Hnrph1, protein disulfide-isomerase A3, pyruvate kinase 3 isoforms M1/M2, and alcohol dehyogenase 5) of the eight identified proteins were directly involved in the protein-protein interactions (Figure 3). Notably, phosphoglycerate kinase 1 was found to be a signal node in the network, suggesting that phosphoglycerate kinase 1 may be crucially involved in colonic adaptation. Previous studies have shown that the five proteins played an important role in energy metabolism in cells. It is well known that quickly-grown cells frequently exhibit increases in glycolytic metabolic pathway for adenosine triphosphate (ATP) generation to meet their energetic needs. Therefore, over-expression of these five proteins resulted in increased ATP production, rapid growth of colonic musocal cells and thickness of colonic musocal thickness.

DISCUSSION

Experiments have demonstrated that the colon can compensate after the extensive removal of the small intestine, with thickening of the colonic mucosa and heightening of the colon plica[1,5]. Epithelial hyperplasia was found 24-48 h after small intestinal resection[7-9]. Adaption process is stimulated by enteral nutrition by providing energy for enterocyte reproduction, stimulating the release of trophic factors[10]. Our previous experiments have shown that nutritional absorption of the colon can be increased by adaptation, and microvilli became more abundant, longer and thicker[11]. The colon may adapt to the absorption of a large volume of solutes[12-14]. Absorptive cell hyperplasia of colonic mucosa was found in the USBS rats assessed under electron microscope. These results showed that the colonic absorption area was greatly increased in the USBS rats. There were less apoptosis, less goblet cells and more absorptive epithelial cells in the USBS rats under transmission electron microscope. Adjacent cell membranes merged tightly and formed a rugged structure. There was an increase in cell connections and connection complexes in USBS rats. The junctions, bridge corpuscles, endoplasmic reticulum, Golgi apparatus, and mitochondrion were all increased. This resulted in improvement in the absorptive functions of water, xylose and 15N-glycine[15].

In this study, the entire small intestines of rats were removed to ensure maximum colonic adaptation. We tried to determine how the colon could compensate when the small intestine was lost. We found that USBS rats could survive using colonic compensation after all small intestines were lost, since rats were fed only with enteral nutrition and had a postoperative survival rate over 90%. The length, diameter and height of the mucosal fold in the mucosal thickness of the colon were increased in the USBS rats compared with the control group.

The colon can undergo adaptations in USBS patients and rats. Some materials have been shown to improve the adaptation. In recent years, the more studies of intestinal compensation have focused on compensatory mechanisms, adsorptive function, and cellular hyperplasia of short bowel syndrome (SBS) remnant intestines. Understanding of the mechanisms and ultimate treatment of the

disease will eventually be reflected in protein expression levels and changes in post-translational modifications. We used a proteomics approach to observe differential protein expressions to fully understand the molecular mechanisms of colon adaptation in an attempt to help develop drugs that can promote colon compensation.

The term proteome was first proposed by Mac Wilkins and Keith Williams in 1994[16] to describe the complete set of proteins that are expressed and modified by an entire genome or cell. The intracellular and dynamic changes in protein composition, expression levels and modification states should be analyzed to observe protein-protein interactions and relationship with the disease, revealing the protein functions and activities important in the disease development.

In the diseases of the digestive system, proteomics have not been as widely used to study the USBS as gastric cancer, esophageal cancer and liver cancer[17-20]. A number of other growth and trophic factors have been implicated in animal models including enteroglucagon, epidermal growth factor, glutamine, growth hormone, chole-cystokinin, gastrin, neurotensin, leptin and insulin-like growth factors[21-23]. Fat-stimulated glucagon-like peptide-Ⅱ may lead to hyperplasia[24,25]. In this study, 47 protein spots were found to be expressed nonrepetitively, including 30 upregulations and 17 downregulations. Their functions were reported to be associated with metabolism of sugar, protein and fat, oxidation-reduction and molecular chaperones. This implied that multiple proteins might affect the metabolism pathway involved in increasing absorptive functions and compensation.

Among these up-regulated proteins, protein disulfide-isomerase A3 is a special multifunctional protein rich in the endocytoplasmic reticulum. It has a strong non-specific peptide binding ability, and acts as both an enzyme and a chaperone[26]. As an enzyme, protein disulfide-isomerase A3 can assist in protein folding by catalyzing covalent bond changes that directly affect protein folding and functional conformations.

As a chaperone, protein disulfide-isomerase A3 may have acquired a new function during evolution to increase catalytic efficiency and assist in protein folding. It was up-regulated in carcinoma of the large intestine[27]. Ryu *et al*[28] found that protein disulfide-isomerase A3 was over-expressed when gastric cancer and paraneoplastic mucosal biological markers were studied with proteomics. This study indirectly demonstrated that protein disulfide-isomerase A3 is associated with hyperplasia of mucosa. Our previous experiments demonstrated that the endoplasmic reticulum in USBS rat colon was rich in protein disulfide-isomerase A3, suggesting that it was closely related to the adaptation of tunica mucosal coli cells and compensation of the colon.

Another up-regulated protein is pyruvate kinase, which participates in the last step of glycolysis, catalyzing phosphoenolpyruvate and adenosine diphosphate (ADP) into pyruvate and adenosine triphosphate (ATP). Pyruvate can generate ATP by zymolysis or by entering tricarboxylic acid (TCA) circulation. In animals, pyruvate kinase may serve as a gene, promoter, mRNA splicer, and signal transducer with polyadenylic acid. In this study, the pyruvate kinase was up-regulated in ultra-short intestinal mucosa. This implies that pentose phosphate shunts may be improved by pyruvate kinase. The excessive ribose-5-phosphate and one carbon unit produced by protein decomposition synthesize purine nucleosidase, improving the hyperplasia of tunica mucosa coli cells.

In this study, phosphoglycerate kinase 1 was richly expressed. Phosphoglycerate kinase 1 is the key enzyme of glycolysis and is also an essential enzyme for living. The main function of phosphoglycerate kinase 1 is to participate in the glycolysis procedure. It can catalyze 1,3-diphosphoglyceric acid into 3-phosphoglyceric acid. Some studies have shown that phosphoglycerate kinase 1 can affect DNA replication and repair the cell nucleus. It can also serve as an mRNA binding protein[29]. Other studies showed that phosphoglycerate kinase 1 up-regulation can cause carcinoma and vascularization. The oxidation status of larvaceous intra-cellular protein is a biological marker in human colon carcinoma[30]. These studies indicated that overexpressed phosphoglycerate kinase 1 can promote cellular proliferation, suggesting that phosphoglycerate kinase 1 is involved in colon compensation by inducing cell proliferation. The bioinformatics analysis in this study showed that phosphoglycerate kinase 1 acts as a hub in the protein-protein interaction networks involved in colon adaptation, suggesting that phosphoglycerate kinase 1 played a key role in colon adaptation and compensation.

Mitochondrial ribosomal proteins were also richly expressed in this study. Some studies have shown that mitochondrial ribosomal proteins can be regulated in translation as chondriogene mRNA. Up-regulation of mitochondrial ribosomal proteins is related to dell differentiation in tunica mucosal coli and colon carcinoma[31]. It has also been implied that mitochondrial ribosomal proteins participate in colon compensation since the number of chondriosomes is markedly increased in USBS rats.

Bioinformatics analysis and previous reports showed that eight proteins were closely related to sugar, fat metabolism, protein synthesis, redox, and molecular chaperones. These proteins likely play an important role in colonic compensation in USBS rats. Further studies to understand the characteristics of these differential proteins will help elucidate the molecular mechanisms of colonic compensation in USBS, and develop drugs to promote adaptation and alimentation.

ACKNOWLEDGMENTS

We would thank the staff of the Institute of Life and Health Engineering and National Engineering and Research Center for Genetic Medicine, Jinan University for their technical and administrative assistance. We also are thankful to the staff of Medical College, Jinan University for providing the operation environment.

COMMENTS

Background

People with short small bowel may have difficulties in nutritious absorption and may struggle to maintain nutritious balance. The increased morbility in intestinal tumors, traumas and mesenteric vascular thrombosis resulted in the increased incidence of short small bowel. It has been evidenced that rats can live without small intestine for a long period as their colon can undergo compensation.

Research frontiers

More studies of intestinal compensation have focused on compensatory mechanisms, adsorptive function and cellular hyperplasia of remnant intestines. Mechanism and treatment of the disease will eventually be reflected in the protein expression levels and changes of post-translational modifications.

Innovations and breakthroughs

The protein expression changes in normal rats and short bowel rats have been studied based on mass spectrometric analysis and other advanced technologies. Eight proteins were found to be closely related with sugar, fat metabolism, protein synthesis, redox, and molecular chaperone. They might play important roles in nutritious absorption, and treatment of short bowel patients.

Applications

The authors offered the methodology for exploring the proteins in short bowel rats. Besides mass spectrometry, immunohistochemistry may be also a smart choice. Once the mechanism is clear and the therapeutic target is established, the malabsorption resulting from extensive small bowel loss will be hopefully solved.

Terminology

Short bowel syndrome is a chronic malabsorptive syndrome resulting from extensive small bowel resections. Mass spectrometry is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds.

Peer review

This study combines proteomics with short bowel syndrome systematically. And it may form the theoretic foundation for treating short bowel syndrome.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i20.2580

World J Gastroenterol 2011 May 28; 17(20): 2580-2584
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Non-cirrhotic portal hypertension with large regenerative nodules: A diagnostic challenge

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Received: November 27, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: May 28, 2011

Abstract

Non-cirrhotic portal hypertension is a poorly understood condition characterized by portal hypertension in the absence of conventional hepatic cirrhosis and described in association with blood coagulation disorders, myeloproliferative and immunological diseases and with exposure to toxic drugs. Very recently, precise classification criteria have been proposed in order to define four distinct subcategories. The present case highlights how the clinical presentation, the confounding results from imaging studies, and the difficulties in the histological evaluation often render cases of non-cirrhotic portal hypertension a real diagnostic challenge. It also underscores the classification problems which can be faced once this diagnosis is performed. Indeed, the different subcategories proposed result from the prevalent subtypes in a spectrum of hepatic regenerative responses to a variety of injuries determining microcirculatory disturbances. More flexibility in classification should derive from this etiopathogenic background.

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Key words: Non-cirrhotic portal hypertension; Large regenerative nodules; Nodular regenerative hyperplasia

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Vespasiani Gentilucci U, Gallo P, Perrone G, Del Vescovo R, Galati G, Spataro S, Mazzarelli C, Pellicelli A, Afeltra A, Picardi A. Non-cirrhotic portal hypertension with large regenerative nodules: A diagnostic challenge. *World J Gastroenterol* 2011; 17(20): 2580-2584 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i20/2580.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i20.2580

INTRODUCTION

Non-cirrhotic portal hypertension (NCPH) is a poorly understood condition characterized by the presence of portal hypertension in the absence of conventional hepatic cirrhosis (defined as diffuse nodules totally surrounded by fibrous septa). Its greatest prevalence has been observed in India, where it represents 20%-25% of the cases that bleed due to esophageal varices[1]. It has been described in association with blood coagulation disorders and myeloproliferative diseases[2,3], with exposure to toxic substances or to drugs[4,5], and with immunological diseases[6,7]. Most of the cases present with thrombosis or sclerosis of the intrahepatic portal venous system, with variable involvement of the prehepatic portal system[8]. Several clinical and pathological pictures are included within this syndrome.

The liver morphology of patients with NCPH shows greatly varying alterations which frequently include archi-

tectural distortion associated with irregular hyperplastic lesions[9,10]. Considering the features of architectural distortion, hyperplastic nodules and fibrosis, an attempt was made to classify each liver into one of 4 diagnostic categories, following the terminology for hepatic nodular lesions recommended by an International Working Party and the definitional criteria used by Nakanuma *et al*[11,12]: idiopathic portal hypertension, diffuse nodular regenerative hyperplasia (NRH), partial nodular transformation, and incomplete septal cirrhosis. Differential diagnosis between these categories is often difficult because single cases frequently do not match the estimated definitions, suggesting that each case of NCPH represents the peculiar pattern of expression of a spectrum of lesions with a common or similar etiopathogenetic background[10].

Here we report a case of NCPH with large regenerative nodules, which highlights the difficulties both in reaching the diagnosis and in classifying the single case into one of the proposed categories.

CASE REPORT

In July 2009, a 62-year-old Italian man was admitted to the Clinical Medicine and Hepatology Unit of our Hospital for recurrent melena.

He had been in good general condition until November 2008, when a first episode of melena occurred in the absence of hematemesis and abdominal pain. Therefore, he was admitted to another hospital, where a severe anemia was found (hemoglobin 5.5 g/dL). A pancolonscopy was negative. Upper intestinal endoscopy showed esophageal varices (not better classified), portal hypertensive gastropathy, and the presence of coagulated blood in the stomach. Upper abdominal ultrasonography revealed hepatomegaly, with caudate lobe hypertrophy, a diffusely dishomogenous echotexture due to the presence of multiple hypoechoic nodules, mainly in the left lobe, the biggest of which was 3 cm in diameter, and mild splenomegaly (transverse diameter: 13.2 cm). Since the patient had no history of chronic liver disease and presented normal liver enzymes and function tests, a transjugular catheterism was performed in order to measure the hepatic pressure venous gradient (HPVG), which confirmed severe portal hypertension (23 mmHg), and to obtain a transjugular liver biopsy, which was negative for cirrhosis-the report of which was the following: "Mild hepatocyte regenerative activity". The patient had been discharged without a definite diagnosis and with the indication to a strict clinical, biochemical and radiological follow-up.

At the admission to our hospital, laboratory tests showed anemia and leucopenia (hemoglobin 9.9 g/dL, hematocrit 32.9%, leucocyte count 2680/μL, platelets 162000/μL), a good renal function (urea 36 mg/dL, creatinine 0.78 mg/dL), and normal liver enzymes and hepatic function tests: AST 24 U/L [upper normal limit (u.n.l.) 35 U/L], ALT 21 U/L (u.n.l. 45 U/L), alkaline phosphatase 59 U/L (u.n.l. 120 U/L), GGT 18 U/L (u.n.l. 55 U/L), total bilirubin 0.7 mg/dL, total protein 6.6 g/dL, albumin 58%, gamma-globulins 17.1%, INR 1.1. The patient had no history of associated diseases and was not taking any medication. The blood tests performed in the last few years were also reassessed and showed only impaired glucose tolerance and intermittent mild elevation of the GGT (1.5-2 X u.n.l.).

Viral serology was negative for hepatitis B surface antigen and antibodies to hepatitis C virus. To rule out autoimmune hepatitis, primary biliary cirrhosis, α-1-antitrypsin deficiency, Wilson's disease, and hemochromatosis, antinuclear, anti-mitochondrial, anti-smooth muscle, and anti-liver and kidney antibodies, α-1-antityripsin, serum iron, transferrin and ferritin, ceruloplasmin, cupremia and cupruria were assessed and found to be negative or within the normal ranges. Also serum α-fetoprotein level was within the normal ranges (1.7 ng/mL, u.n.l. 8.1 ng/mL). Upper intestinal endoscopy confirmed esophageal varices (F2 blue, without cherry red spots, hematocystic spots or diffuse redness, but with red wale markings ++/+++), and severe portal hypertensive gastropathy.

Contrast-enhanced computed tomography (CT) and magnetic resonance (MR) of the upper abdomen were performed. On CT, several slightly hypodense liver lesions (almost 10, on both hepatic lobes) were visualized, showing an inhomogeneous contrast uptake due to the presence of a hypodense central core. On MR, liver morphology was not that of a cirrhotic liver. In basal conditions, the hepatic parenchyma was heterogeneous due to the presence of multiple nodules, the biggest of which was 3 cm in diameter, hypointense on T1-weighted sequences and slightly hyperintense on T2-weighted images (Figure 1). Nodules were better represented after gadolinium injection, showing mild signal hyperintensity. The very first radiological impression was that of multiple liver metastases from endocrine tumor, melanoma or renal cell carcinoma. The lesions were also compatible with large regenerative nodules in a radiologically noncirrhotic liver. The hypothesis of hepatocellular carcinoma was also considered, but not supported by the poor hypervascularization in the arterial phase and by the lack of hypointensity in portal venous and delayed phases.

In order to rule out malignancy, a US-guided liver biopsy was performed on the 2-cm-sized lesion in the second hepatic segment. Histologically, small foci of necrosis of isolated hepatocytes with focal accumulation of macrophages and other inflammatory cells, and infiltration of portal tracts by scattered mononuclear cells, were observed. The inflammatory infiltrates slightly involved some portal tracts, and were not accompanied by piecemeal necrosis or fibrosis. As recommended in cases of non-cirrhotic portal hypertension, the silver impregnation stain was performed and did not show any architectural alteration. The final histological diagnosis was nonspecific mild chronic hepatitis. In addition, sections from the transjugular liver biopsy performed in the first Hospital were obtained and reassessed by silver impregnation stain (Figure 2). A vaguely nodular arrangement of liver parenchyma was detected, in which areas with hyperplastic hepatocytes, arranged in plates more than one cell thick, were alternated with areas in which the

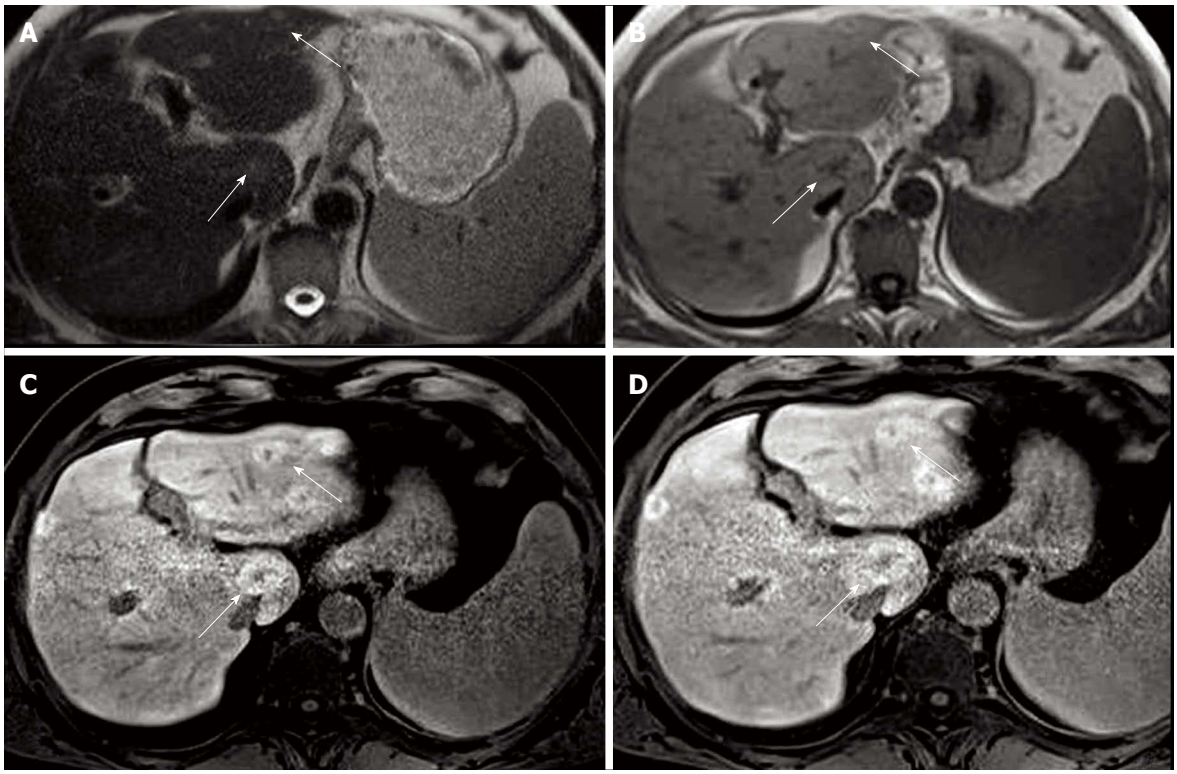

Figure 1 Magnetic resonance images before and after gadolinium injection. Half fourier acquisition single shot turbo spin echo T2-weighted sequences showed slightly hyperintense nodules (panel A), which were hypointense on T1-weighted sequences (panel B). After gadolinium injection, lesions were better represented, showing a mild signal hyperintensity (panels C and D). The white arrow in each panel indicates the biggest lesion, 3 cm in transverse diameter, on the caudate lobe.

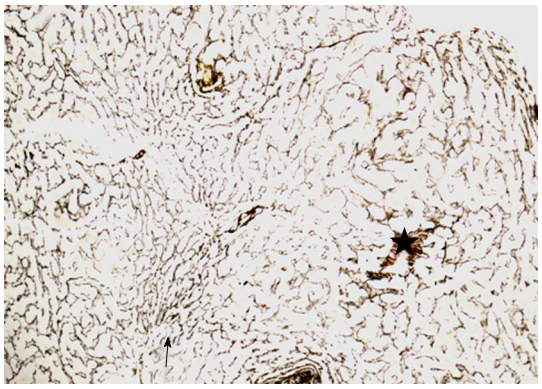

Figure 2 Liver biopsy, reticulin silver impregnation, original magnification, 100 ×. Hyperplastic parenchymal nodules with thickened liver-cell plates are seen (black star), whereas the parenchymal cells adjacent to the nodules are compressed and atrophic (black arrow). This growth pattern is not accompanied by fibrosis.

trabeculae were compressed and atrophic. The interface between nodules was not defined by fibrous septa. There was only minimal inflammation and no evidence of degenerative changes of hepatocytes. Histologically, the diagnosis was NRH. Clinically, the diagnosis was NCPH, in the form of NRH associated with large regenerative nodules.

As NCPH has been described in association with blood coagulation disorders, myeloproliferative diseases, immunological alterations, systemic or intra-abdominal infections and exposure to toxic substances or to drugs, all these conditions were evaluated and excluded by questioning the patient and performing the appropriate investigations. In particular, the complete panel for hereditary thrombotic risk factors was performed. Lupus anticoagulant, protein-C, protein-S, antithrombin, homocysteine, cardiolipin IgG and IgM antibodies, and anti-β2-glycoprotein-I antibodies were in the normal range. The molecular study was negative for factor V H1299R and G1691A, methylenetetrahydrofolate reductase C677T and A1298C, Plasminogen Activator Inhibitor-1 4G/5G, and for prothrombin G20210A gene polymorphisms. In order to rule out myeloproliferative diseases, the V617F Janus kinase-2 gene mutation was investigated and found to be negative. Bone marrow aspiration was not performed due to the lack of sufficient clinical suspicion justifying the procedure.

A first-degree atrioventricular block partially contraindicated beta-blocker therapy and the patient underwent band ligation of esophageal varices. An upper intestinal endoscopy was repeated after one month, revealing persistence of two F1 varices with red wale markings +/+++, which were band-ligated again. A third endoscopic control, performed after another month, was finally negative.

At present, after nearly 1 year of follow-up, the patient is in good clinical condition. The last image studies, MR in January 2010 and ultrasonography in June 2010, did not show significant modifications of the hepatic picture. The last upper intestinal endoscopy, performed in August 2010, showed only one F1 esophageal varix without red wale markings, hematocystic spots or diffuse redness. A

long-term clinical, radiological and endoscopic follow-up is warranted.

DISCUSSION

The present case highlights how the clinical presentation, the confounding results from imaging studies, and the difficulties in the histological evaluation often render cases of NCPH a real diagnostic challenge. It also underscores the classification problems which can be faced once the diagnosis of NCPH is performed.

Even if the exact prevalence and clinical load of NCPH are not known, there are a number of non-cirrhotic patients with long-standing portal hypertension of unknown etiology. Several clinical and pathological pictures are included within this syndrome. Among these, there are some that are morphologically very similar and, differing from country to country and author to author, they have been called idiopathic portal hypertension[13], hepatoportal sclerosis[14], and non-cirrhotic portal fibrosis[15]. In them, the hepatic architecture is mildly distorted due to the existence of irregular distances between the portal venous and hepatic venous structure[16], and nodular regenerative foci are frequently found[17]. In other types of NCPH, greater architectural distortion and prominent regenerative nodulation are found[12]. Only very recently, precise classification criteria have been proposed in order to define only 4 distinct subcategories: idiopathic portal hypertension, diffuse NRH, partial nodular transformation, and incomplete septal cirrhosis[11,12].

The clinical presentation of our case is typical of NCPH, bleeding from esophageal varices, and laboratory data correspond, since liver enzymes are usually normal or slightly elevated and hepatic function is preserved. As in the present case, in needle biopsies of the liver the changes of regeneration and atrophy may be very subtle on routine hematoxylin-eosin stains, and any case of suspected NCPH should be investigated further using reticulin stains[18]. Even the lack of coexisting diseases which are usually associated with NCPH is not infrequent[10]. However, the dominant finding in all the imaging studies performed in our case, i.e. multiple nodules ranging from a few millimetres to 3 cm in diameter, should not be considered typical of NCPH. Indeed, this picture is neither consistent with partial nodular transformation, where macronodules are concentrated around the hepatic ilus[12], nor with NRH, where nodules are usually too small to be radiologically detectable[19]. NRH is characterized by a diffuse nodular transformation of the hepatic parenchyma, without fibrosis separating the nodules. These histological features, consisting of diffuse hyperplastic nodules composed of cells resembling normal hepatocytes, and little or no inflammation or cholestasis, are concordant with the histological findings in our case. However, nodules characterizing NRH are usually 1-3 mm in size, and, although clusters of nodules up to 10 mm in diameter centered on a large portal tract are occasionally found[11], many nodules found in the liver of our patient are significantly bigger. Therefore, in the present case, on the histological background of NRH, nodules much larger than the typical NRH ones were detected by imaging studies.

It has been suggested that all the categories constituting NCPH could represent the same lesional spectrum, the different stages of the same disease or the final stage common to different diseases with varying etiologies. Probably, personal anatomy, patterns of vascularization and damage location influence the final picture. The nomenclature proposed, in many cases, provides an inadequate description of the hepatic morphology, and combinations of morphological patterns are often reported in literature. For example, as Nakanuma *et al*[12] evidenced in their study, nodular parenchymal hyperplasia without fibrous rim was prominent in all cases of NRH but was found also in one fourth of idiopathic portal hypertension cases, in all partial nodular transformation cases, and in one third of incomplete septal cirrhosis cases, in which it was present in a mild degree and with a focal distribution. Moreover, Ibarrola *et al*[10] were able to classify only 3 out of their 9 NCPH patients according to accepted terminology, using combined terms to describe the hepatic morphology of another 3 cases, and coined the descriptive terms of "irregular architectural transformation" for the last 3 cases, which showed very grossly deformed livers with irregularly distributed nodules of variable size.

In conclusion, the present case highlights the typical difficulties encountered in the diagnostic and classification processes of NCPH. The different categories of NCPH represent the prevalent subtypes of hepatic regenerative response to a variety of injuries determining microcirculatory disturbances. More flexibility in classification should derive from this etiopathogenic background.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 May 28; 17(20): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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World J Gastroenterol 2011 May 28; 17(20): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, 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 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 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

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World J Gastroenterol 2011 May 28; 17(20): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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World Journal of Gastroenterology

ISSN and EISSN
ISSN 1007-9327 (print)
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Organization as author

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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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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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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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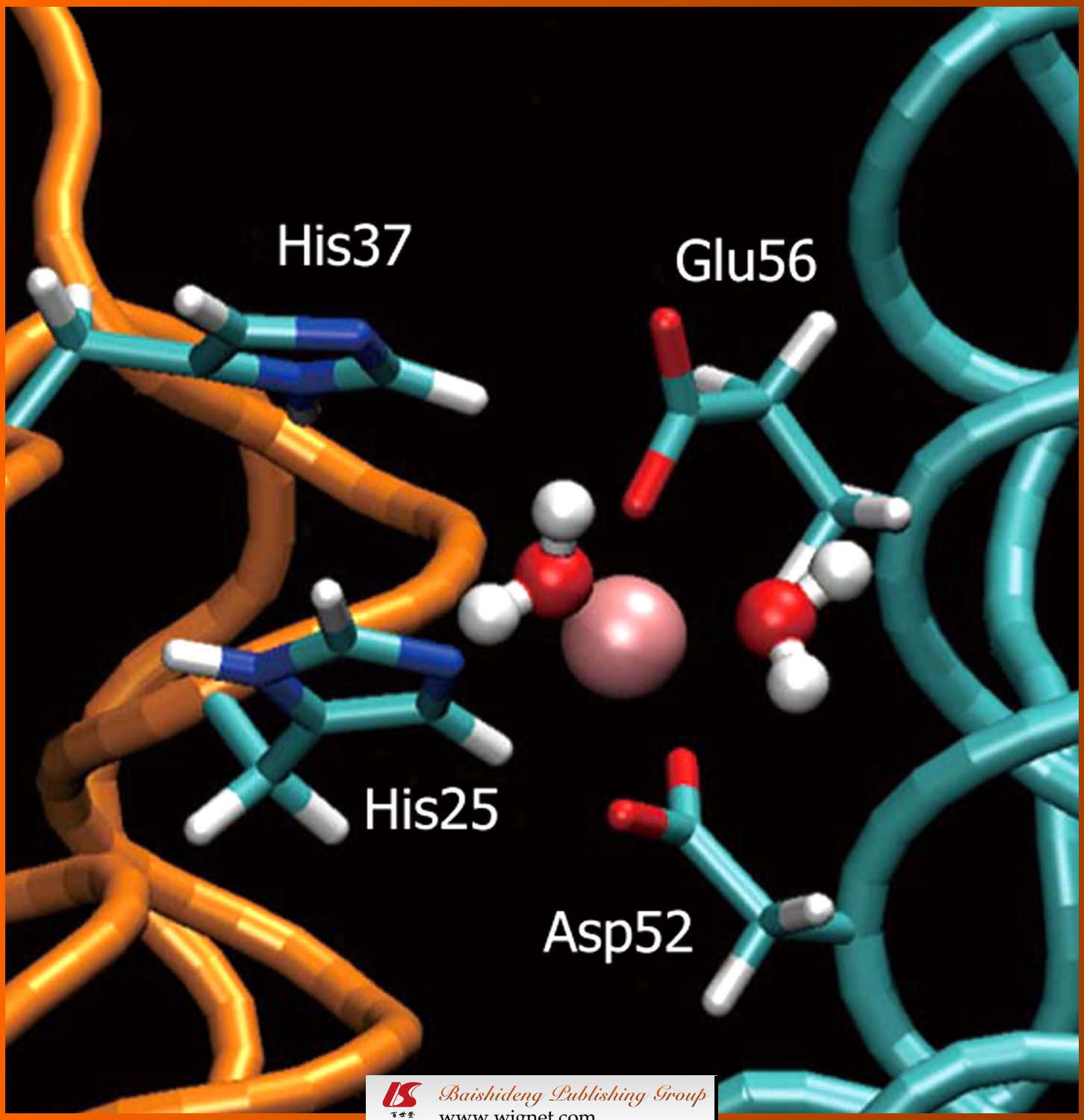
World Journal of *Gastroenterology*

World J Gastroenterol 2011 June 7; 17(21): 2585-2682



Baishideng Publishing Group
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World Journal of Gastroenterology

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

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Sang Geon Kim, *Seoul*
Seon Hahn Kim, *Seoul*
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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 21 June 7, 2011

EDITORIAL

2585 *Helicobacter pylori* neutrophil activating protein as target for new drugs against *H. pylori* inflammation
Choli-Papadopoulou T, Kottakis F, Papadopoulos G, Pendas S

TOPIC HIGHLIGHT

2592 Current status of endoscopic submucosal dissection for the management of early gastric cancer: A Korean perspective
Chun HJ, Keum B, Kim JH, Seol SY

2597 Do we have enough evidence for expanding the indications of ESD for EGC?
Lee HL, Choi CH, Cheung DY

2602 Diagnosis of gastric epithelial neoplasia: Dilemma for Korean pathologists
Kim JM, Cho MY, Sohn JH, Kang DY, Park CK, Kim WH, Jin SY, Kim KM, Chang HK, Yu E, Jung ES, Chang MS, Joo JE, Joo M, Kim YW, Park DY, Kang YK, Park SH, Han HS, Kim YB, Park HS, Chae YS, Kwon KW, Chang HJ, The Gastrointestinal Pathology Study Group of Korean Society of Pathologists

2611 Worldwide experiences of endoscopic submucosal dissection: Not just Eastern acrobatics
Cho KB, Jeon WJ, Kim JJ

2618 Chicken soup for teaching and learning ESD
Kim EY, Jeon SW, Kim GH

2623 Endoscopic submucosal dissection for early gastric cancer: Quo vadis?
Cho WY, Cho JY, Chung IK, Kim JI, Jang JS, Kim JH

ORIGINAL ARTICLE

2626 Hepatobiliary scintigraphy for detecting biliary strictures after living donor liver transplantation
Kim YJ, Lee KT, Jo YC, Lee KH, Lee JK, Joh JW, Kwon CHD

2632 Keratinocyte growth factor gene therapy ameliorates ulcerative colitis in rats
Liu CJ, Jin JD, Lv TD, Wu ZZ, Ha XQ

BRIEF ARTICLE

2641 Chronic constipation: Facilitator factor for development of varicocele
Kilciler G, Sancaktutar AA, Avcı A, Kilciler M, Kaya E, Dayanc M

2646 Secretion of melatonin and 6-sulfatoxymelatonin urinary excretion in functional dyspepsia
Chojnacki C, Poplawski T, Klupinska G, Blasiak J, Chojnacki J, Reiter RJ

2652 Endoscopic removal and trimming of distal self-expandable metallic biliary stents
Ishii K, Itoi T, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Tsuji S, Ikeuchi N, Umeda J, Moriyasu F, Tsuchida A

2658 Clinical significance of serum expression of GROβ in esophageal squamous cell carcinoma
Dong QM, Zhang JQ, Li Q, Bracher JC, Hendricks DT, Zhao XH

2663 Protective effects of 2,4-dihydroxybenzophenone against acetaminophen-induced hepatotoxicity in mice
He YY, Zhang BX, Jia FL

2667 PAd-shRNA-PTN reduces pleiotrophin of pancreatic cancer cells and inhibits neurite outgrowth of DRG
Yao J, Zhang M, Ma QY, Wang Z, Wang LC, Zhang D

2674 Enhanced proliferation, invasion, and epithelial-mesenchymal transition of nicotine-promoted gastric cancer by periostin
Liu Y, Liu BA

LETTERS TO THE EDITOR

2681 Lower body weight and female gender: Hyperphosphatemia risk factors after sodium phosphate preparations
Deepak P, Ehrenpreis ED

World Journal of Gastroenterology
Volume 17 Number 21 June 7, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Theodora CP, Filippos K, Georgios P, Stefanos P. Helicobacter pylori neutrophil activating protein as target for new drugs against Helicobacter pylori inflammation. *World J Gastroenterol* 2011; 17(21): 2585-2591 http://www.wjgnet.com/1007-9327/full/v17/i21/2585.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.
FLYLEAF	I-VII	Editorial Board

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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

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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.

PUBLICATION DATE
June 7, 2011

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

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SPECIAL STATEMENT
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2585

World J Gastroenterol 2011 June 7; 17(21): 2585-2591
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Helicobacter pylori neutrophil activating protein as target for new drugs against *H. pylori* inflammation

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Supported by A Grant from the General Secretariat of Research and Technology, Ministry of Development of Greece, by the Program HERAKLITOS I as well as by Chios Gum Mastic Growers Association
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Received: October 12, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: June 7, 2011

Abstract

Helicobacter pylori (*H. pylori*) infection is among the most common human infections and the major risk factor for peptic ulcer disease and gastric cancer. Within this work we present the implication of C-terminal region of *H. pylori* neutrophil activating protein in the stimulation of neutrophil activation as well as the evidence that the C-terminal region of *H. pylori* activating protein is indispensable for neutrophil adhesion to endothelial cells, a step necessary to *H. pylori* inflammation. In addition we show that arabino galactan proteins derived from chios mastic gum, the natural resin of the plant *Pistacia lentiscus var. Chia* inhibit neutrophil activation *in vitro*.

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Key words: *Helicobacter pylori* neutrophil activating protein; *Helicobacter pylori*; Peptic ulcer disease; Gastric cancer

Peer reviewer: Takashi Kawai, MD, PhD, Professor and Director of Endoscopy Center, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Choli-Papadopoulou T, Kottakis F, Papadopoulos G, Pendas S. *Helicobacter pylori* neutrophil activating protein as target for new drugs against *H. pylori* inflammation. *World J Gastroenterol* 2011; 17(21): 2585-2591 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2585.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2585

INTRODUCTION

The *Helicobacter pylori* (*H. pylori*) neutrophil activating protein (HPNAP) is one of a number of virulence factors produced by the bacterium *H. pylori*[1]. This protein originally purified from aqueous extracts of *H. pylori*, was shown to induce neutrophil adhesion to endothelial cells *in vitro*[1] as well as *in vivo*[2], to increase the adhesion of neutrophils to endothelial cells[3], to induce migration and activation of human neutrophils and monocytes[4,5] and to be a potent stimulant of mast cells[6]. Its binding to neutrophil-glycosphingolipids[7] and mucin, a component of the stomach mucus layer[8], has also been reported. HPNAP induced reactive oxygen intermediates (ROI) production involves a cascade of intracellular activation events, including increase of cytosolic calcium ion concentration and phosphorylation of cytosolic proteins, leading to the assembly of the superoxide-forming nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase on the neutrophil plasma membrane[5,9,10].

Free radicals produced by neutrophils are a key component of the innate immune system and an effective antimicrobial agent against *H. pylori* as well as a factor that perpetuates mucosal damage and gastritis. Activation of neutrophils results in NADPH oxidase-mediated superoxide anion production which is highly destructive for the gastric mucosa, induces oxidative DNA damage and leads to substantial mucocal disruption. NADPH oxidase targeting is disrupted such that active enzyme complexes are present in patches at the cell surface but not on *H. pylori* phagosomes. Consequently, superoxide accumulates in the extracellular space but not near ingested bacteria. By this unusual mechanism, *H. pylori* evades oxidative killing and promotes tissue damage and ulceration. A possible blocking of reactive species production may lead to improvement of *H. pylori* induced chronic gastritis and reduction of signs of inflammation.

THE TWO ROLES OF HPNAP

Bacterial protection

HPNAP is a dodecameric protein consisting of 17 kDa monomers with a central cavity where iron can bind[11,12]. The observation that its synthesis is not affected by the iron content of the growth medium, led to the proposal that the primary role of HPNAP *in vivo* may not be to scavenge iron[13]. The primary sequence and overall structure of HPNAP[14] is similar to those of Dps family of iron-binding and DNA-protecting proteins[15]. Dps family proteins protect DNA from oxidative damage through direct interaction. Dps and DNA form a highly ordered and stable nucleoprotein complex called a biocrystal so that DNA is "sheltered" from the attack of the free oxidative radicals[16] by preventing the production of hydroxyl radicals produced by the Fenton reaction[17]. These proteins are present in many prokaryotes[18-22]. They bind ferrous ions and some of them lack the ability to bind DNA *in vitro*[12,19,23].

The role of HPNAP in protecting *H. pylori* from oxidative damage was first suggested by the observation that loss of alkyl hydroperoxide reductase (AhpC) leads to a concomitant increase in HPNAP expression[24]. Like other Dps proteins, HPNAP production is maximal in stationary-phase cells, and an *H. pylori* napA mutant survives less than the wild type strain upon exposure to oxidative stress conditions[25].

Our previous studies[26] revealed that HPNAP does not bind to DNA and therefore protection of the bacterial DNA by means of ferroxidase activity ensues by a mechanism similar to that suggested for other non DNA binding Dps. Molecular dynamics simulations (MDS) revealed that the ferroxidase site amino acids are indispensable for dimer formation and that ferrous ions contribute extensively to the stability of the dimers in solution.

HPNAP's inflammatory role

The inflammatory role of HPNAP concerns the attraction and activation of neutrophils. In particular, the 150 kDa oligomeric protein isolated from *H. pylori* has been found to promote neutrophil adhesion to endothelial cells[1,11]. This protein was designated the HPNAP because of its ability to induce neutrophils to produce reactive oxygen radicals[5,11]. HPNAP is released in the medium, probably after cell lysis, and binds to the bacterial surface where it can act as an adhesin, mediating binding to mucin or to polymorphonuclear leukocyte sphingomyelin[7,8]. Purified recombinant HPNAP has been produced in *Bacillus subtilis* to avoid contamination by *Escherichia coli* lipopolysaccharide. This purified material was found to be chemotactic for human neutrophils and monocytes *in vitro*[5]. Moreover, using intravital microscopy, it has recently been demonstrated that in rats HPNAP is able to cross the endothelia efficiently and to promote rapid neutrophil adhesion *in vivo*[27]. HPNAP-induced adhesivity depends on the induction of expression and on the acquisition of a high-affinity state of β2-integrin on the plasma membrane of PMNs[5,27]. This conformational change requires a functional p38 mitogen-activated protein kinase (MAPK). Collectively, these observations suggest that HPNAP plays a central role in the accumulation of leukocytes at the site of infection[5,11,27]. HPNAP stimulates PMNs to synthesize and release several chemokines, including CXCL8 (interleukin-8), CCL3 (MIP-1α) and CCL4 (MIP-1β)[27]. Because neutrophils rapidly migrate in large numbers at infection sites, the fact that they also serve as a chemokine source may contribute to the generation of the conditions necessary for both the recruitment and activation not only of additional neutrophils, *via* CXCL8, but also of monocytes, dendritic cells, and lymphocytes through CCL3 and CCL4. After crossing epithelial monolayers, HPNAP is also able to activate the underlying mast cells to release tumor necrosis factor-α (TNF-α) and other pro-inflammatory molecules[6,13].

NEUTROPHIL ACTIVATION IN FOCUS

According to the literature HPNAP is the only protein of the DPs family capable of activating human leukocytes. Zanotti *et al*[14] investigated this unique property of HPNAP by analyzing not only its surface but also the surfaces of the structurally similar Flp, Dlp-1 and Dlp-2 which failed to activate human neutrophils. That was an attempt to identify regions located on the surface of these proteins whose different properties could account for such biological difference. They found that the surface of HPNAP was characterized by a large presence of positively charged residues, a property that was not shared by the other members of Dps family. The strong prevalence of positive charges of the electrostatic surface potential of HPNAP conferred a basic character on it. By taking into account the fact that positively charged residues of several proteins, including those of some chemokines which was believed to play a role in the activation of neutrophils[28,29] they suggested that the presence of the large number of basic residues on the HPNAP dodecamer surface was responsible for its neutrophil activating property.

However according to Kottakis *et al*[26] by replacing His25, His37, Asp52 and Lys134, that are located within the ferroxidase site, with Ala, a total loss of ferroxidase activity, dodecamer formation and DNA protection in

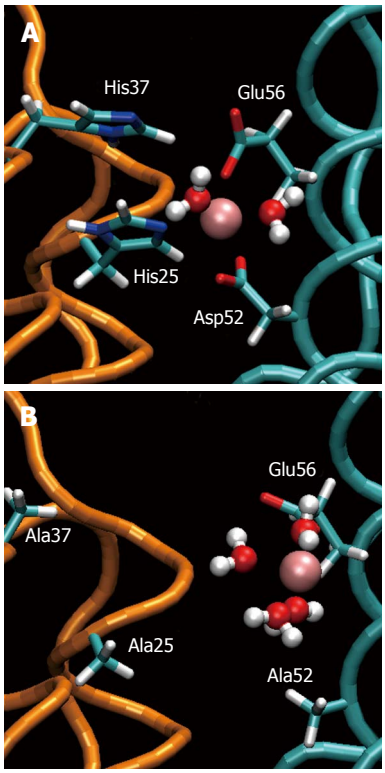

Figure 1 Ferroxidase site of *Helicobacter pylori* neutrophil activating protein. A: The "ferroxidase site" in the equilibrated wild type. The iron ion (pink) is kept in position by Asp52, Glu56, His25 and His37. Two water molecules are attracted by Fe(Ⅱ); B: The same site in the equilibrated mutant. The ferrous ion is attracted one-sidedly by Glu56 and Asp53 (not shown) loosing its ability to stabilize the dimer. Four water molecules are attracted by Fe(Ⅱ).

environments rich in free radicals was observed.

MDS revealed that dimer formation was highly unlikely following mutation of the above amino acids, since the ferrous ion is not attracted equally strongly by both subunits (Figure 1A and B). These findings indicate that iron plays an important role in the conformation of HPNAP by initiating the formation of stable dimers that are indispensable for the ensuing dodecamer structure. In addition, according to our experiments both HPNAP wild type as well as HPNAP mutant were able to activate neutrophils. In particular, by incubating neutrophils separately with the above proteins we observed a similar degree of activation for both cases[26]. Very surprisingly, neutrophil activation was stimulated by structural elements that are localized within the broad C-terminal region of both HPNAPmut and dodecamer HPNAP-wild type. In particular, it was found that the dodecamer conformation was not necessary for activation and that helices H3 (Leu69-Leu75), H4 (Lys89-Leu114) or the linking coils (His63-Thr68 and Thr76-Ser88) were critical in stimulating neutrophil activation (Figures 2 and 3).

It was recently reported that HPNAP promotes a Th1 immune response by inducing the expression of IL-12 and IL-23 in neutrophils and monocytes, and also elicits an antigen-specific Th1-polarized T cell response in gastric mucosa of *H. pylori*-infected patients *in vivo*[30]. It has been shown that HPNAP is able to shift antigen activated

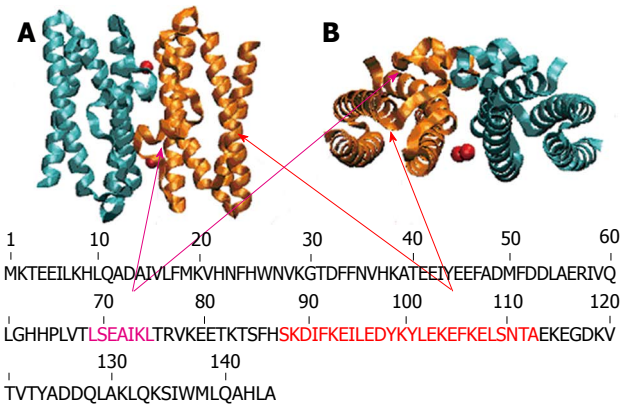

Figure 2 Schematic representation of exposed helices of *Helicobacter pylori* neutrophil activating protein. *Helicobacter pylori* neutrophil activating protein dimer in stand up (A) and top view (B) with the exposed helices H3 and H4 (therefore possible candidates for interacting with the neutrophils) colored in violet and orange respectively.

human T cells from a Th2 to a Th1 cytotoxic phenotype characterized by production of IFN-γ and TNF-α. Since HPNAP is a powerful stimulant for the production of ROS, mediating damage to DNA and enhancing cell turnover[19], it may be a risk factor for *H. pylori*-associated gastric cancer. Considering the upregulatory effects of HPNAP on the innate immune system, it could possibly be argued that the chronic inflammatory response mediated by HPNAP may be associated with an increased danger of occurrence of gastric cancer in view of the fact that *H. pylori* is classified as class 1 carcinogen. As a matter of fact, a recent impressive work[31] studied the serum positivity and mean absorbance value of HPNAP-specific antibodies in patients with gastric cancer in comparison to patients with chronic gastritis. Interestingly, HPNAP antibodies were significantly higher in the gastric cancer group indicating a possible pathogenetic role of HPNAP in gastric carcinogenesis.

ACTIVATED NEUTROPHILS ATTACH TO ENDOTHELIAL CELLS

Human neutrophils were separately incubated with HPNAP-6xHis, HPNAP$_{1-57}$-6xHis (N-terminal region) and HPNAP$_{58-144}$-6xHis (C-terminal region) on micro wells with pre attached endothelial cells and their attachment was quantified by using the myeloperoxidase (MPO) assay[32]. Besides the entire protein and its truncated forms neutrophils were also incubated by the same manner with the neutrophil stimulator formyl-Met-Leu-Pro peptide (fMLP) in order to control their "bioactivity". In addition, a synthetic hexa-histidine peptide (6xHis) was also used for neutrophil activation in order to exclude the possibility that the obtained activation was attributed to the existence of the tailed histidines. Figure 4A shows that HPNAP-6xHis and the C-terminal region display almost the same ability to promote neutrophil adhesion to endothelial cells while the N-terminal region lacks this ability.

Considering the existence of lipopolysaccharides (LPS)

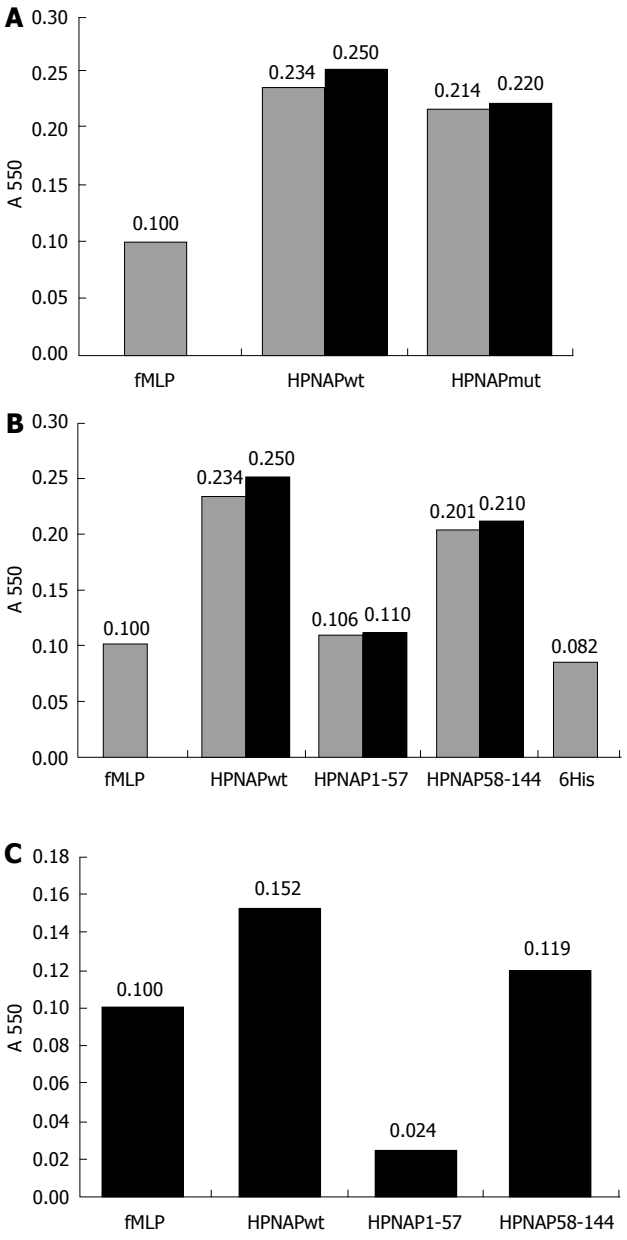

Figure 3 Neutrophil activation by *Helicobacter pylori* neutrophil activating protein. A: Neutrophil activation by *Helicobacter pylori* neutrophil activating protein (HPNAP)-wt and HPNAPmut. Bar 1, activation by formyl-met-leu-pro peptide (fMLP) (control), bar 2, activation by HPNAPwt, bar 3, activation by HPNAPmut; B: Neutrophil activation by HPNAPwt-hexa-histidine peptide (6xHis), HPNAP1-57-6His, HPNAP58-144-6His and 6His peptide. Bar 1, activation by fMLP (control), bar 2, activation by HPNAPwt-6His, bar 3, activation by HPNAP1-57-6His, bar 4, activation by HPNAP58-144-6His, bar 5, activation by 6His peptide; C: Values after subtraction of "6His" value from these of "HPNAPwt", "HPNAP1-57" and "HPNAP58-144".

and their involvement in the activation it is clearly shown that even after their removal the activation effects did not change significantly (Figure 4A). These results are consistent with previously published data[26], indicating that the 58-144 region of the HPNAP protein is the key component in neutrophil recruitment, activation and subsequent adhesion to endothelial cells, leading to oxidative burst and inflammation during *H. pylori* infection. Considering recently published data[33], on the safety and immunogenicity of an intramuscular vaccine comprising VacA, CagA and HPNAP, we suggest that the obtained neutrophil activation by the C-terminal region of HPNAP opens new ways for drug design dealing with *H. pylori* inflammation.

NEUTROPHIL ATTACHMENT INHIBITION BY ARABINO-GALACTAN-PROTEINS FROM CHIOS MASTIC GUM

Chios mastic gum (CMG) and its derivatives were largely used in traditional medicine to ease the discomfort in patients suffering from gastric pain. Its *in vitro* antibacterial properties against a great variety of bacteria are well established[34,35]. In this study we demonstrate that AGPs extracted from CMG as described in[34] inhibit the neutrophil attachment to endothelial cells caused by the HPNAP and its C-terminal region. In particular, human neutrophils were incubated with either HPNAP-6xHis or HPNAP$_{58-144}$-6xHis both in the presence and in the absence of AGPs and their attachment to endothelial cells was investigated as above. Figure 4B shows the inhibition of neutrophil attachment to endothelial cells after co-incubation of entire HPNAP and its truncated forms (N-terminal and C-terminal) with the AGPs. In particular, bar 3 shows the absence of any influence of AGPs on neutrophil attachment to endothelial cells. The designation "control" on the figure represents the found attachment of isolated neutrophils to endothelial cells after incubation, without any other addition of proteins or AGPs. The marked percentages of all other combinations are calculated by taking into account the control values. Thus, comparison of the bars 4 (HPNAP entire) to 5 (HPNAP entire plus AGPs) and 6 (HPNAP$_{58-144}$) to 7 (HPNAP$_{58-144}$ plus AGPs) reveals that neutrophil activation and their subsequent attachment to endothelial cells is inhibited by the AGPs from CMG.

A recent study[36] focused on HPNAP mediated neutrophil activation before and after 2 mo of per os administration of CMG. According to this work, CMG induces a significant reduction in neutrophil activation when incubated with AGP plus HPNAP in *H. pylori*-infected patients and controls. CMG also induces a significant reduction in neutrophil activation when incubated with HPNAP in *H. pylori*-infected patients. These results indicate a substantial down-regulation of the innate cellular immune effectors, which - according to unpublished clinical data in the context of this study - are accompanied by a significant clinical improvement of the patients' complaints (dyspepsia, epigastric discomfort, distention). However a demonstration of the histopathological improvement of the patients' chronic gastritis would provide even more valuable evidence, concerning the potential anti-inflammatory and gastritis-suppressive effects of CMG.

Summarizing our results presented within this work we evidenced that the broad C-terminal region of HPNAP stimulates neutrophil adhesion and that the AGPs from CMG disrupt the process of neutrophil-endothelial cell attachment caused by HPNAP, an effect that should

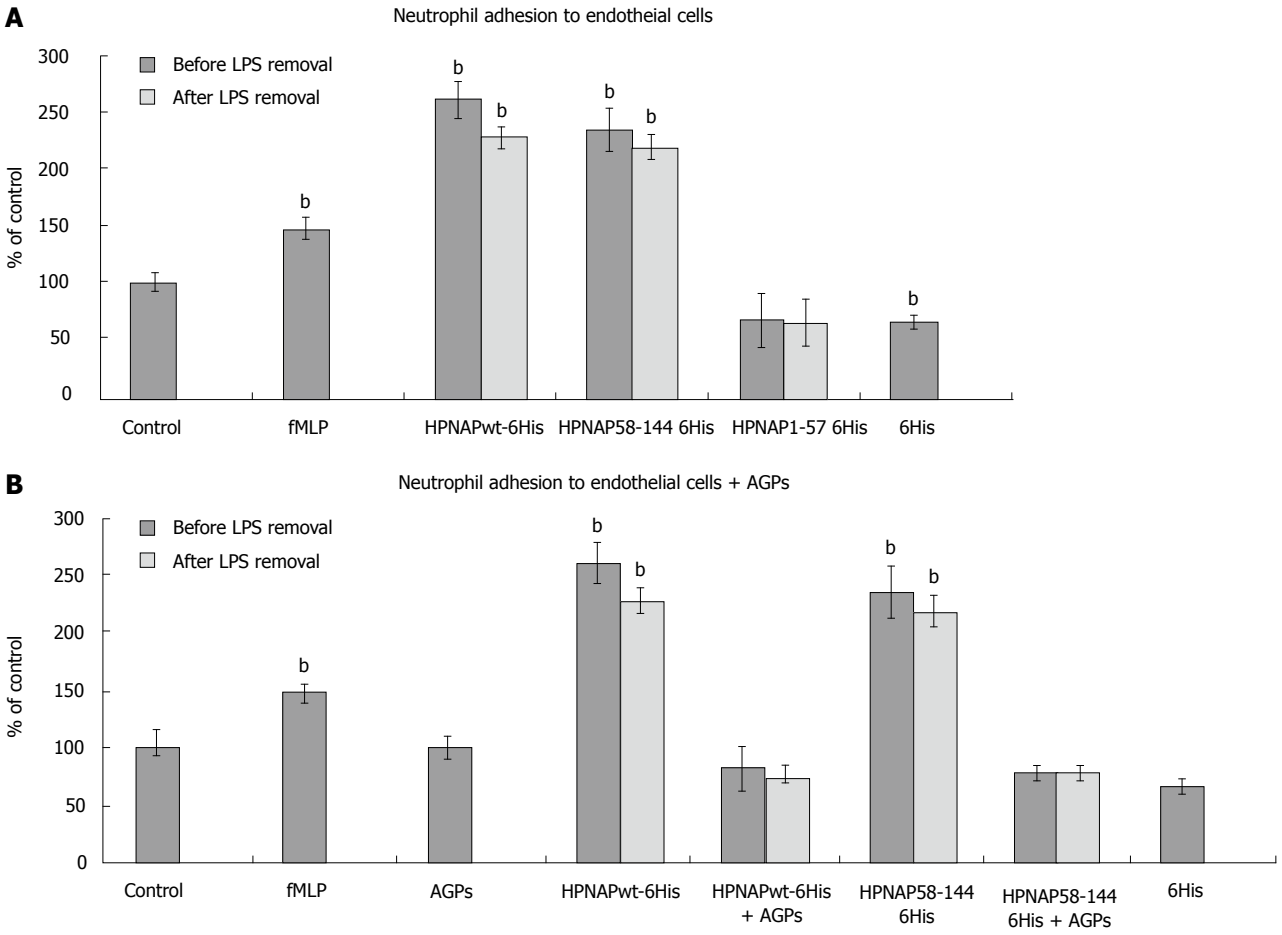

Figure 4 Neutrophil adhesion to endothelial cells. A: Neutrophil adhesion to endothelial cells. Black bars indicate neutrophil adhesion to endothelial cells prior to lipopolysaccharides (LPS) removal while grey bars indicate the adhesion after LPS removal. The data represent triplicates from at least three independent experiments. Error bars indicate standard deviation (SD). Statistical evaluation was performed by Mann-Whitney test. Significant differences with the control values are marked by ($^bP < 0.001$) bar 1, control, bar 2, formyl-met-leu-pro peptide (fMLP), positive control of the procedure, bar 3, *Helicobacter pylori* neutrophil activating protein (HPNAP)wt-hexa-histidine peptide (6xHis) effect on neutrophil adhesion to endothelial cells before and after LPS removal, bar 4, HPNAP58-144-6xHis effect before and after LPS removal, bar 5, HPNAP1-57-6xHis effect before and after LPS removal, bar 6, 6xHis effect; B: Effect of arabino galactan proteins (AGPs) on neutrophil adhesion to endothelial cells, bar 1, control, bar 2, fMLP, positive control of the procedure, bar 3, effect of AGPs, bar 4, HPNAPwt-6xHis effect before and after LPS removal, bar 5, HPNAPwt-6xHis and AGPs co-effect, before and after LPS removal, bar 6, HPNAP58-144-6xHis effect before and after LPS removal, bar 7, HPNAP58-144-6xHis and mastic gum extract co-effect, before and after LPS removal, bar 8, 6xHis effect.

be further investigated and maybe exploited in a future anti-inflammatory therapy for *H. pylori* patients.

FAILURE OF ANTIBIOTIC THERAPY AND RECURRENCE OF *H. PYLORI* INFECTION - IS *H. PYLORI* AN INVINCIBLE ENEMY?

Triple as well as quadruple treatment regimens are so far a well established therapy of *H. pylori* infection. Eradication rates of over 80% (triple therapy) and 96% for the quadruple regimen with bismuth or ecabet sodium are documented[37,38]. However, a significant number of mutants is emerging-especially in developing countries - which confers resistance to standard antibiotics (amoxicillin, metronidazole, clarithromycin)[39-41] as well as to fluoroquinolones. Resistance rates of 35% for clarithromycin and 26% for fluoroquinolones are documented, a very worrying fact, which limits considerably the future perspectives of successful antibiotic treatment of *H. pylori* infection. Moreover, a high prevalence of recurrence of *H. pylori* infection in adults as well as in children could possibly render our efforts to eradicate this pathogen futile[42-45]. Indeed, an incidence of *H. pylori* annual recurrence of 2.67% and 13.00% in developed and developing countries respectively is documented in a recent study[46]. In view of these data, alternative methods of treatment as adjunct or main therapy regimens should be considered. HPNAP, a major stimulant of neutrophil recruitment and activation could be effectively targeted by natural agents that reduce inflammation (due to their activity as antioxidants) or suppress the production of inflammatory cytokines that attract neutrophils[47-50]. On the other side, the advent of the structure oriented drug design era would eventually provide us with valuable weapons to fight the *H. pylori* infection by the direct inhibition of HPNAP *in vivo*. The identification of structural elements at the C-terminal region of HPNAP monomer as a stimulus for neutrophil migration through endothelial cells and subsequent release of ROIs[26,51] renders this region eligible for drug mediated

inhibition of its immunological effects upon neutrophils. Indeed, additional studies should be carried out in order to validate this assumption and open the way for new and alternative perspectives to fight this ubiquitous pathogen.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2592

World J Gastroenterol 2011 June 7; 17(21): 2592-2596
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Current status of endoscopic submucosal dissection for the management of early gastric cancer: A Korean perspective

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Received: November 11, 2010 Revised: January 11, 2011
Accepted: January 18, 2011
Published online: June 7, 2011

Abstract

The early diagnosis of gastric cancer allows patients and physicians to pursue the option of endoscopic resection, which is significantly less invasive than conventional surgical resection. In Korea, the use of endoscopic submucosal dissection (ESD) has been increasing, and many reports on ESD have been published. In addition, Korean gastroenterologists from several hospitals performing ESD have conducted formal meetings to discuss useful information regarding ESD. Here, we discuss the Korean experience with ESD, including outcomes and prospects of endoscopic treatments.

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Key words: Early gastric cancer; Endoscopic submucosal dissection; Endoscopic mucosal resection

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Chun HJ, Keum B, Kim JH, Seol SY. Current status of endoscopic submucosal dissection for the management of early gastric cancer: A Korean perspective. *World J Gastroenterol* 2011; 17(21): 2592-2596 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2592.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2592

INTRODUCTION

Early gastric cancer (EGC) is defined as gastric carcinoma confined to the mucosa or submucosa, regardless of the presence of regional lymph node metastases[1]. In the 1980s, surgery was typically used to treat early gastric cancer. However, the development of endoscopic devices and skills has resulted in a different approach to treatment. Endoscopic mucosal resection (EMR) is a procedure for the resection of gastrointestinal (GI) neoplasms that was first introduced in Japan. However, since it is difficult to achieve *en bloc* resection of specimens larger than 20 mm with EMR, this procedure has recently been replaced by endoscopic submucosal dissection (ESD)[2,3]. The improvement in ESD technique has led to extension of the indications for endoscopic treatment to that of early gastric cancer. In Korea, ESD has become an effective alternative treatment method to manage early gastric cancer. In this review, we provide an overview of ESD in Korea.

THE DEVELOPMENT OF ENDOSCOPIC RESECTION

Endoscopic therapy has been in continuous development since the injection of saline into submucosal tissue for cutting sessile rectal polyps by Dehyle in 1973[4] In 1984, strip-off biopsy (strip biopsy) was introduced for gastric cancer treatment in Japan[5]. Although successfully used to

Table 1 Endoscopic mucosal resection/endoscopic submucosal dissection therapeutic outcomes

Author	Yr	*n*	Method	Complete resection (%)	Local recurrence (%)	Bleeding (%)	Perforation (%)
Lee	1996	19	Strip biopsy	37.8	28.6		
Hyun	1996	20	Strip biopsy				
Cheon	2000	28	Strip biopsy	64.3	3.6		
Seong	2002	35	Strip biopsy	94.3	6.1		
Hyun	2003	45	Strip biopsy	55.6	0.0	24.4	0.0
Kim	2000	20	EMR-L	85.0	5.9	0.0	0.0
Kim	2005	109	Strip biopsy, EMR-C, EMR-P	67.9	1.4	8.3	2.8
Youn	2006	149	Strip biopsy, EMR-C, EMR-L, ESD	84.6	4.0	22.8	1.3
Kim	2007	514	Strip biopsy, EMR-C, EMR-L, EMR-P, ESD	77.6	6.0	13.8	0.6
Jung	2007	360	EMR-P	82.8		10.6	1.1
Min	2009	103	EMR-P	75.7	0.0	3.9	1.9
Jung	2007	264	ESD	87.9		9.8	3.8
Kang	2008	456	ESD	80.3	0.0		
Park	2008	434	ESD	77.4	1.8	8.1	2.3
Min	2009	243	ESD	88.9	0.0	5.3	4.5
Chung	2009	534	ESD	87.7		15.6	1.2

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EMR-C: EMR by cap-fitted panendoscope; EMR-L: EMR with ligation; EMR-P: Precutting followed by snare resection.

treat early gastric cancer, strip biopsies cannot be used to resect depressed ulcers or neoplasms. In the 1990s, EMR by cap-fitted panendoscope (EMR-C) and EMR with ligation (EMR-L) methods were developed in Japan for resection of early gastric cancer[6,7]. However, lesions larger than 2 cm cannot be removed *en bloc* with these techniques. Because piecemeal resection carries a high risk of recurrence and does not facilitate exact pathological staging, ESD was developed to remove early gastric neoplasm *en bloc*[8].

ESD was introduced in the late 1990s and allows direct dissection of the submucosa. ESD allows for certain histological diagnoses and reduces the rate of recurrence compared to EMR. The *en bloc* resection of large lesions is possible with insulation-tipped (IT) electrosurgical knives[9], as well as hook, flex, triangle and flush knives[8].

THE DEVELOPMENT AND OUTCOMES OF ENDOSCOPIC RESECTION IN KOREA

EMR for early gastric cancer was performed for the first time in Korea in 1996 by Hyun[10]. Several small-scale studies of ESD and EMR have been performed in Korea since then[11] (Table 1).

A large-scale, multicenter, retrospective study of EMR treatment was reported in 2007[12]. From January 2000 to December 2002, 514 EGCs in 506 patients were treated by EMR at 13 institutions. The most commonly used procedure was circumferential precutting followed by snare resection (EMR-P, n = 269, 52.3%). Complete resection and incomplete resection after EMR were confirmed in 399 lesions (77.6%) and 103 lesions (20.0%), respectively. In the completely resected mucosal cancer group (n = 399), local recurrence was detected in 24 cases (6.0%) with a median interval between EMR and recurrence of 17.9 mo (range, 3.5-51.7 mo). There were 3 cases of perforation and 71 cases of bleeding. There were no deaths related to the recurrence of gastric cancer during the overall median follow-up period of 39 mo[12].

The use of ESD has increased in many Korean hospitals over the last 5 years, accompanied by a subsequent increase in published reports about ESD treatment. Park *et al*[13] initially reported 27 cases of ESD treatment using the IT knife in Korea in 2004, after which use of the ESD procedure increased rapidly in Korea. The Korean Society of Gastrointestinal Endoscopy (KSGE) organized an ESD research group in 2003 to exchange professional opinions and develop ESD techniques. The KSGE held hands-on courses to introduce ESD procedures and devices using animal models. Live international ESD demonstrations have been held over a telemedicine network since 2006[14].

In 2009, a large multicenter study of ESD treatment was published[2]. From January 2006 to June 2007, 1000 early gastric cancers in 952 patients (502 men, 450 women; mean age 62.1 years, range 43-90 years) were treated by using ESD at six Korean ESD study group (KESG)–related university hospitals in Korea. The rates of *en bloc* resection, complete *en bloc* resection, vertical incomplete resection, and piecemeal resection were 95.3%, 87.7%, 1.8% and 4.1%, respectively. The rates of delayed bleeding, significant bleeding, perforation, and surgery related to complications were 15.6%, 0.6%, 1.2% and 0.2%, respectively. The rates of *en bloc* resection differed significantly based on the location of the lesions (upper portion *vs* middle portion *vs* lower portion of the stomach, 88.6% *vs* 95.2% *vs* 96.0%, respectively; P = 0.002), presence of a scar (no *vs* yes, 96.0% *vs* 89.5%, respectively; P = 0.002), and histologic type (low-grade adenoma *vs* high-grade adenoma *vs* differentiated early gastric cancer *vs* undifferentiated early gastric cancer, 95.8% *vs* 94.6% *vs* 96.2% *vs* 83.8%, respectively; P = 0.007)[2]. The results of this study suggested that the ESD techniques used in Korea achieve high rates of complete resection (87.7%) with an acceptable rate of complications. In addition, ESD outcomes from several hospitals show that *en bloc* and complete resection rates are 87%-100%, which are superior to those of conventional EMR treatment[15] (Table 2).

Table 2 Endoscopic submucosal dissection outcomes

Result of resection	*n* (%)
Complete resection	877 (87.7)
En bloc resection	953 (95.3)
Free margin of *en bloc* resection	901 (90.1)
Failure of *en bloc* resection	6 (0.6)
ESD time (min), mean ± SD	47.8 ± 38.3
Submucosal invasion of tumor	74 (7.4)
Lymphovascular invasion of tumor	30 (3.0)

ESD: Endoscopic submucosal dissection.

THE CHALLENGE OF ESD IN KOREA

Currently accepted classical indications for EMR according to the gastric cancer treatment guidelines published in 2001 by the Japanese Gastric Cancer Association are: (1) well-differentiated elevated cancers less than 2 cm in diameter; (2) small (< 1 cm) depressed lesions without ulceration; (3) moderately- or well-differentiated cancers confined to the mucosa; and (4) no lymphatic or vascular involvement[8,16]. However, the classical indications for EMR may be too strict and lead to unnecessary surgery[8].

Japanese studies have expanded the indications for endoscopic treatment of gastric cancer[8,17]. ESD has been developed to allow dissection directly along the submucosal layer using electronic knives. Gotoda *et al*[8,18] defined the risk of lymph node metastasis in patients with EGC using a large database of more than 5000 patients who underwent gastrectomy with meticulous R2 level lymph node dissection. This group of patients was characterized by having either no risk or a lower risk of lymph node metastasis compared to risk of mortality from surgery. Thus, expanded criteria for endoscopic resection were proposed: (1) mucosal cancer without ulcer findings, irrespective of tumor size; (2) mucosal cancer with an ulcer < 3 cm in diameter; and (3) min (< 500 μm from the muscularis mucosa) submucosal invasive cancer < 3 cm in size.

Ryu *et al*[19] investigated surgery as indicated for non-curative endoscopic resection in EGC in Korea. Neither residual cancer nor lymph node metastasis was found in patients with less than 500 μm submucosal invasion without margin involvement in ER specimens. An *et al*[20] studied predictive factors for lymph node metastasis in EGC with submucosal invasion. They concluded that lymphatic involvement and tumor size are independent risk factors for lymph node metastasis in EGC with submucosal invasion. Minimally invasive treatment such as EMR may be feasible for highly selective submucosal cancers with no lymphatic involvement, SM1 invasion, and tumor size < 1 cm. In addition, Park *et al*[21] reported that EGC with signet ring cell histology can be treated by endoscopic mucosal resection, if it is smaller than 25 mm, limited to the SM2 layer, and does not involve the lymphatic-vascular structure. Another Korean study showed that poorly differentiated EGC confined to the mucosa or with minimal submucosal infiltration (< or = 500 μm) could be considered for curative EMR due to the low risk of lymph node metastasis[22]. However, extending the indications for ESD remains controversial because the long-term outcomes of these procedures have not been fully documented.

Histopathologic diagnosis of EMR/ESD specimens is very important, but the diagnostic criteria, terminology and grading systems differ between the East and the West. Most Western pathologists focus on structural invasion to diagnose carcinoma, but Japanese pathologists emphasize severe dysplastic cytologic atypia irrespective of the presence of invasion[23,24]. In 2000, the Vienna classification was proposed to reduce diagnostic discrepancies between Japanese and Western pathologists[25]. However, there is still confusion about the pathological diagnosis of gastric epithelial lesions[26]. In Korea, gastroenterologists and pathologists did not have clear guidelines for diagnosis, and efforts were made to improve consensus. Korean ESD techniques have been influenced by Japan, but Korean doctors are also influenced by advances in Western medical science in general.

Pathologic diagnosis of gastric cancer tissue obtained by ESD was discussed among gastroenterologists and pathologists at a joint symposium of the Gastrointestinal Pathology Study Group of Korean Society of Pathologists (GIPS-KSP) and the ESD study group held in Korea in 2007. This collaboration continues, with the goal of establishing consensus for ESD indications in Korea, as well as internationally.

The following criteria have been suggested for the pathological diagnosis of gastric epithelial lesions: (1) standardization of the number of biopsy specimens (introduction of the number of biopsy specimens according to the size of the lesion); (2) introduction of criteria for determination of the adequacy of a biopsy specimen (marking of muscularis mucosa, submucosa); (3) establishing criteria for diagnosis of advanced adenomas (including nuclear findings and structural abnormalities); and (4) establishing criteria of lamina propria invasion and submucosal invasion[27].

THE PROSPECTS OF KOREAN ESD

Improvements in the ability to endoscopically identify and distinguish cancer and the development of diagnostic tools will enable the diagnosis of ultra-early lesions. The detection of these ultra-early lesions will eventually result in more active use of ESD for treatment.

The rapid evolution of ESD has allowed the procedure to be more widely indicated. In addition, new diagnostic and therapeutic techniques have become available. One example is natural orifice transluminal endoscopic surgery (NOTES), by which abdominal operations are performed with an endoscope passed through a natural orifice (e.g. mouth, urethra, anus) and then through an internal incision in the stomach, vagina or colon[28]. This procedure allows extension of a flexible endoscope to reach organs outside of the lumen of the bowel. NOTES is minimally invasive compared to open surgery and is associated with fewer risks. EMR and/or ESD can be combined with laparoscopic/thoracoscopic sentinel node

mapping to allow successful endoscopic treatment of gastrointestinal cancers with a potential risk of lymph node metastasis using NOTES[29]. The KSGE organized a NOTES research group to discuss, investigate and spread information about the use of NOTES.

Since the KSGE organized the Korean ESD study group, there have been several meetings of gastroenterologists and gastroenteropathologists from hospitals where ESD is performed to discuss standardization of the pathology. The short- and long-term outcomes of ESD for the treatment of early gastric cancer have also been discussed at these regular meetings regarding the progress and outcomes of ESD in Korea[2,12]. However, these evaluations are limited by their retrospective methods, and the absence of an international focus on long-term outcomes. There is agreement that multicenter clinical studies are necessary to obtain prospective results. Therefore, the National Evidence-Based Health Care Collaborating Agency (NECA) and KSGE suggested the prospective study of short-term and long-term clinical outcomes of EGC treated by ESD. Many tertiary university hospitals have been involved in this study, which is expected to provide indications for endoscopic treatment of EGC in Korea and worldwide.

CONCLUSION

ESD is a useful method for complete resection of EGCs, but has many limitations. To improve the safety and efficacy of ESD, many Korean research groups are currently concentrating on the development of new techniques and devices.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2597

World J Gastroenterol 2011 June 7; 17(21): 2597-2601
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Do we have enough evidence for expanding the indications of ESD for EGC?

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Received: June 25, 2010 Revised: September 20, 2010
Accepted: September 27, 2010
Published online: June 7, 2011

Abstract

Endoscopic submucosal dissection (ESD) is the most advanced and representative technique in the field of therapeutic endoscopy and has been used for the treatment of gastrointestinal neoplasms, including early gastric cancer. The major difference and advantage of ESD compared to existing endoscopic resection techniques, such as endoscopic mucosal resection (EMR) and polypectomy, are the width and depth of the resection. Newly developed cutting devices, distal attachable endoscopic accessories, and an advanced electrosurgical unit have helped to overcome the limitations of therapeutic endoscopy in terms of lesion size, location, presence of fibrotic scarring, and accompanying ulcers. As a result, the indications for ESD have been expanded from the classical indication for EMR and polypectomy, and there is now support for a further expansion of ESD indications. At present, the most critical factor to consider in the decision of whether to perform ESD is the probability of unexpected lymph node metastasis. The guidelines for ESD are continually being updated and debated. In this review, we discuss the strengths and weaknesses of the expanded guidelines, based on evidence found in the literature.

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Key words: Endoscopic submucosal dissection; Endoscopic mucosal resection; Early gastric cancer; Indications

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Lee HL, Choi CH, Cheung DY. Do we have enough evidence for expanding the indications of ESD for EGC? *World J Gastroenterol* 2011; 17(21): 2597-2601 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2597.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2597

INTRODUCTION

Early gastric cancer (EGC) is defined as a gastric cancer that is confined to the mucosa or submucosa, irrespective of the presence of regional lymph node metastasis[1,2]. Endoscopic submucosal dissection (ESD) is a novel endoscopic treatment that enables a clinician to resect a target lesion en bloc. For the last ten years, ESD has been performed in Korea for the management of early gastric cancer[3,4]. ESD for EGC is comparable to conventional surgery in many aspects, and it has the advantage of being less invasive and more economical. In this article, we

introduce the absolute and expanded indications of ESD and discuss their usefulness, safety, and limitations.

ABSOLUTE AND EXPANDED INDICATIONS OF ESD FOR EGC

The curability of EGC depends on the complete removal of the cancerous lesion and its metastatic lymph nodes. Fortunately, because a significant proportion of EGC has no lymph node metastasis, limited surgery, such as ESD, can be legitimately performed in many countries.

Traditionally accepted indications for endoscopic resection of EGC are small intramucosal EGCs of intestinal histology type. The rationale for this recommendation is based on the knowledge that larger lesions or diffuse histology lesions are more likely to extend into the submucosal layer and thus have a higher risk of lymph node metastasis. In addition, resection of a large lesion was not technically feasible until the ESD procedure was developed. Therefore, at present, the accepted indications for endoscopic mucosal resection (EMR) according to the gastric cancer treatment guidelines published in 2001 by the Japanese Gastric Cancer Association are: (1) well-differentiated elevated cancers less than 2 cm in diameter; and (2) small (< 1 cm) depressed lesions without ulceration. These lesions must also be moderately or well-differentiated cancers confined to the mucosa, and have no lymphatic or vascular involvement[5,6]. However, it has been clinically observed that currently accepted indications for EMR may be too strict, leading to unnecessary surgery[7].

Further studies by Gotoda *et al*[8] have defined new criteria to expand the indications for endoscopic treatment of gastric cancer. The ESD method has been developed to dissect directly along the submucosal layer using specialized devices. Preliminary studies have been published on the advantage of ESD over conventional EMR for the removal of larger or ulcerated EGC lesions en bloc. Thus, ESD allows the precise histological assessment of the resected specimen and may prevent residual disease and local recurrence. Gotoda *et al*[8] analyzed 5265 EGC patients who underwent gastrectomy with lymph node dissection. They provided important information on the risks of lymph node metastasis, wherein the differentiated gastric cancer with a nominal risk of lymph node metastasis was defined. They proposed expanded criteria for endoscopic resection: (1) mucosal cancer without ulcer findings, irrespective of tumor size; (2) mucosal cancer with an ulcer ≤ 3 cm in diameter; and (3) minimal (≤ 500 μm from the muscularis mucosa) submucosal invasive cancer ≤ 3 cm in size (Figure 1)[8,9]. However, extending the indications for ESD remains controversial because the long-term outcomes of these procedures have not been fully documented.

RECENT EVIDENCE FOR EXPANDED INDICATIONS OF ESD FOR EGC

Over the past 20 years, intraluminal endoscopic surgery, referred to as EMR, has been advanced in Japan and Korea. Recently, EMR and ESD have been widely used to treat EGC without lymph node metastasis. EMR is indicated when the risk of lymph node metastasis is minimal and when the tumor can be removed en bloc with a loop snare. Therefore, in the guidelines issued by the Japanese Gastric Cancer Association (JGCA), differentiated mucosal cancers measuring less than 2 cm in diameter best fit the above criteria. However, endoscopic resection has also been used for larger lesions without lymph node metastasis. In addition, improved techniques featuring ESD, which involve incision of the mucosa around the lesion followed by direct dissection of the submucosal layer, can provide en bloc resection, regardless of the tumor size. Therefore, provided that there is no metastatic lymph node, and the cancer is confined to mucosa and upper submucosal layer, endoscopic resection featuring ESD can be advocated as a proper management strategy for EGC.

To establish a safe and confident criteria and indication for ESD, we have to classify EGC with and without metastatic lymph nodes and to analyze long-term follow-up data after EMR and ESD. In a recent study of patients who had undergone radical gastrectomy for EGC, none of the 1230 well differentiated mucosa-confined cancers smaller than 3 cm in diameter had associated lymph node metastasis, regardless of the presence of ulceration[8]. Regarding the presence of ulceration in all EGC patients, the probability of lymph node involvement significantly

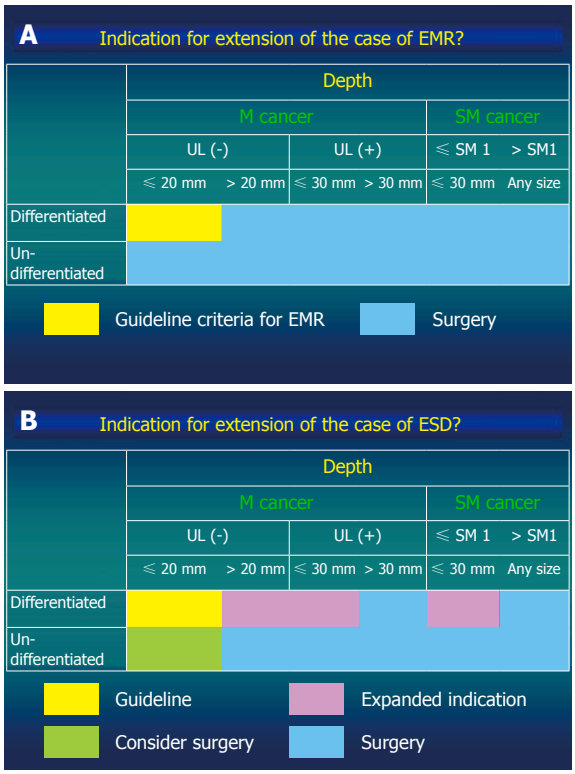

Figure 1 Guideline criteria for endoscopic mucosal resection (A) and expanded criteria for endoscopic submucosal dissection proposed by Gotoda *et al*[8] and Soetikno *et al*[9] (B). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

increases in EGC containing an ulcer (3.4%) compared to EGC without an ulcer (0.5%)[8]. In subgroup analysis, cancer that is confined to the mucosa without an ulcer has no lymph node involvement, regardless of size (95% CI, 0%-0.4%). Mucosal cancer with an ulcer showed a size limitation up to 30 mm to be free of lymph node involvement (95% CI, 0%-0.3%). Thus we can conclude that presence of an ulcer and size are factors for indication of ESD for EGC. However, such characterization of EGC based on morphological feature poses certain problems. First, we should consider the life cycle of a malignant ulcer[10]. About one-third of EGCs with depressed morphology can change over time[11]. An EGC with ulceration on initial EGD can change into non-ulcerative EGC with an antisecretory agent. In addition, EGCs with ulceration, which had not appeared ulcerous on previous examination, are sometimes encountered. Nevertheless, there is no evidence in the literature regarding the outcome of ESD for EGC with healing ulcers and fibrotic scarring. In practice, the decision of ESD for EGC with a shallow ulcer often depends on the timing of the diagnosis and on the willingness of the operator. Regarding the division of ulcerative and non-ulcerative as a criterion for ESD[8], there has not been sufficient research to allow a definite decision. Second, there can be inter-observer variation in defining an ulcer in EGC. By definition, ulcers measure 5 mm or larger in diameter and are on exposed submucosa. However, in real endoscopic examination, the differentiation between ulcer and erosion is not always clear. Third, the size of a lesion can be different according to the method of measurement. An endoscopic ruler or a standard size disc patch can be applicable but, in most cases, a lesion is measured by eye in comparing to opened grasp of forceps. Thus, a standard reliable measurement method is required.

Another factor for expanded indication of ESD concerns the invasion depth of EGC. Although the absolute indication for EMR is applicable to only mucosal cancer, there have been some studies of ESD in submucosal cancer. Of the 145 well-differentiated tumors that had invaded less than 500 μm into the submucosa, and were smaller than 30 mm in diameter, none showed evidence of lymph node metastasis, provided that there was no lymphatic or venous invasion. Based on these findings, it was suggested that the criteria for ESD for EGC could be expanded[12-16].

From the surgical literature, the risk of lymph node metastasis appears to depend on the presence of ulcer rather than on the depth of invasion of EGC. A retrospective analysis of patients who underwent surgery for EGC in Korea reported that, among 129 cases of mucosal cancer compatible with expanded indications for EMR or ESD, three patients (2.3%) had lymph node metastasis and, among 52 submucosal cancer cases that met the expanded indications for EMR or ESD, two patients (4%) had lymph node metastasis[17]. The authors suggest that if EMR or ESD had been performed in these patients, it would not have been curative. However, even in this report, differentiated mucosal cancers without ulcers did not have lymph node metastasis, irrespective of size. Thus, these data suggest that a well-differentiated mucosal cancer of any size without ulcer may be considered as an expanded indication for ESD.

Even in the expanded criteria, undifferentiated cancer an indication for surgery. However, studies on feasibility of ESD on undifferentiated EGC have been continuously performed and reported. Ye *et al*[18] reported that EGC with undifferentiated histology has no lymph node involvement, provided that the cancer is smaller than 25 mm, is confined to the mucosa or upper third of the submucosa, and has no lymphatic involvement. A similar study for signet ring cell carcinoma was reported by Park *et al*[19]. EGC with signet ring cell histology is a high risk for nodal and organ metastases, while smaller cancers of less than 25 mm that are confined to the SM2 layer and have no lymphatic-vascular involvement have no lymph node involvement. With regard to poorly differentiated EGC, a Korean study reported a somewhat lower risk of lymph node metastasis than expected[20]. On this retrospective analysis of 234 patients with poorly differentiated EGC who underwent radical gastrectomy with D2 lymph node dissection, half of the cases (n = 116) showed submucosal invasion in the resection specimen and 25.9% (30/116) of those were limited to the upper third (SM_1). Lymph node metastasis was found in 3.4% (4/118) of mucosa-confined cancer. For patients with minor submucosal infiltration (SM_1), the lymph node metastasis rate was non-existent (0/30). However, with $SM_{2/3}$ invasion, the lymph node metastasis rate increased sharply to around 30%. Another Korean study[21] focusing on endoscopic resection for undifferentiated-type cancer, such as poorly differentiated adenocarcinoma and signet ring cell carcinoma, showed interesting results. In this study, 58 lesions with undifferentiated EGC (17 poorly differentiated; 41 signet-ring cell) were treated by endoscopic resection. The en bloc and complete resection rates in poorly differentiated cases were 82.4% and 58.8%, respectively, whereas those in signet ring cell were 85.4% and 70.7%. The recurrence rate was 5.1% in complete resection during the follow-up period. Therefore, the authors suggested that endoscopic resection might be a feasible local treatment for undifferentiated EGC if complete resection can be achieved. Although the studies so far are still insufficient to form a conclusion, we could take poorly differentiated cancer with mucosa or minimal submucosal invasion into consideration as a possible candidate for ESD in high-risk surgical patients.

A prospective comparative study was reported in Japan[6] concerning the clinical outcomes of absolute and expanded indication of EMR and ESD. A total of 589 EGC lesions were divided into the guideline group and the expanded group. En bloc, complete, and curative resections were achieved in 98.6 and 93.0, 95.1 and 88.5, and 97.1 and 91.1% of the guideline and expanded criteria lesions, respectively, and the differences between the two groups were significant. The complication risks, such as procedure associated perforation and bleeding, were significantly higher and the completeness of resection was statistically superior in the expanded indication group. However, the overall survival was equally adequate in both groups, and the disease-specific survival rates were 100% in both groups.

LIMITATIONS OF ESD

Lymph node micrometastasis and delayed cancer dissemination after ESD is one of the concerns about endoscopic resection including ESD. Walter *et al*[22] reported a fulminant case in a 67-year-old male patient with EGC of 12 mm and moderate differentiation. The depth of submucosal invasion was 2.3 mm and the cancer free margin could not be established. The patient underwent gastrectomy and the postoperative stage was pT1 (sm3), pNO (0/58), cM0, L0, V0, G2 (UICC stage Ia). Three months later, an ultrasound revealed a new mass in the liver, and biopsy showed a rapidly growing metastasis of the gastric adenocarcinoma. This case highlights the risk of affected lymph nodes in early gastric cancer and the consequent risk of metastasis, which increases with greater depth of infiltration into the submucosa. Micrometastasis can be a reason for cancer recurrence even after curative surgery[23-27]. According to Cai *et al*[25], tumor size, macroscopic type, accompanying ulcers, and depth of invasion are strongly associated with micrometastasis in lymph nodes. Therefore, tumors with suspected submucosal invasion, large size, accompanying ulcers, and undifferentiated histology might have a risk of recurrence owing to micrometastasis, which would be contraindicated for EMR or ESD.

LONG-TERM FOLLOW UP DATA

Long-term follow up data are needed for the clinical application of the expanded criteria of ESD. One Japanese study[28] from the National Cancer Center Hospital involving 1955 EGC patients enrolled from January 1999 to December 2005 showed that there were no significant differences in the overall five-year survival rates between the curative resection group, as defined by the expanded indication, and the non-curative resection group, following additional surgery. This data suggest that ESD using the expanded criteria can show an excellent long-term outcome.

CONCLUSION

ESD makes it possible to perform complete resection for lesions larger than 20 mm, as well as those with ulceration, regardless of location. Many clinical data suggest that ESD might be adequate for lesions that fit both the current guidelines and the expanded criteria. In the near future, when long-term follow-up data accumulate and newer technology is available, endoscopic resection, including ESD, will be employed in the treatment of EGC with more expanded indications. However, we must keep in mind that accurate diagnosis, characterization of the lesion, and proper appreciation of technical aspects are most essential in therapeutic endoscopy.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2602

World J Gastroenterol 2011 June 7; 17(21): 2602-2610
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Diagnosis of gastric epithelial neoplasia: Dilemma for Korean pathologists

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Supported by Korean Society of Pathologists
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Received: June 29, 2010 Revised: September 9, 2010
Accepted: September 16, 2010
Published online: June 7, 2011

Abstract

The histopathological diagnosis of gastric mucosal biopsy and endoscopic mucosal resection/endoscopic submucosal dissection specimens is important, but the diagnostic criteria, terminology, and grading system are not the same in the East and West. A structurally invasive focus is necessary to diagnose carcinoma for most Western pathologists, but Japanese pathologists make a diagnosis of cancer based on severe dysplastic cytologic atypia irrespective of the presence of invasion. Although the Vienna classification was introduced to reduce diagnostic discrepancies, it has been difficult to adopt due to different concepts for gastric epithelial neoplastic lesions. Korean pathologists experience much difficulty making a diagnosis because we are influenced by Japanese pathologists as well as Western medicine. Japan is geographically close to Korea, and academic exchanges are active. Additionally, Korean doctors are familiar with Western style medical terminology. As a result, the terminology, definitions, and diagnostic criteria for gastric intraepithelial neoplasia are very heterogeneous in Korea. To solve this problem, the Gastrointestinal Pathology Study Group of the Korean Society of Pathologists has made an effort and has suggested guidelines for differential diagnosis: (1) a diagnosis of carcinoma is based on invasion; (2) the most important characteristic of low grade dysplasia is the architectural pattern such as regular distribution of crypts without severe branching, budding, or marked glandular crowding; (3) if nuclear pseudostratification occupies more than the basal half of the cryptal cells in three or more adjacent crypts, the lesion is considered high grade dysplasia; (4) if severe cytologic atypia is present, careful inspection for invasive foci is necessary, because the risk for invasion is very high; and (5) other structural or nuclear atypia should be evaluated to make a final decision such as cribriform pattern, papillae, ridges, vesicular nuclei, high nuclear/cytoplasmic ratio, loss of nuclear polarity, thick and irregular nuclear membrane, and nucleoli.

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Key words: Intraepithelial neoplasia; Stomach; Dysplasia; Adenoma; Carcinoma; Japanese; Western; Consensus; Vienna

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INTRODUCTION

Techniques for endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) have been developing rapidly, and pathologists more commonly encounter specimens derived from endoscopic resection. These procedures are sometimes performed for diagnostic purposes but mostly for therapeutic convenience compared with radical surgery. However, for both purposes, pathological diagnosis of gastric biopsies and EMR/ESD specimens is very important, because further treatment plans and a surveillance schedule must be established. The importance of a diagnosis is not only stressed from a clinical viewpoint, but also from an academic perspective. Many studies regarding early gastric neoplastic lesions based on a histopathological diagnosis have been performed, and they focus on various clinical and pathological aspects such as survival, recurrence, surveillance programs, and molecular pathology. However, if the pathological diagnosis is different among pathologists, the results cannot be compared. Additionally, socioeconomic problems are also important, because medical insurance is intimately associated with disease severity.

Although these issues are important, it is unfortunate that the definition, diagnostic criteria, and grading system for early stage gastric neoplastic lesions are not completely developed. In particular, it is well known that Eastern and Western pathologists use different criteria to make a gastric carcinoma diagnosis. A structurally invasive focus is necessary to diagnose carcinoma for most Western pathologists. The "Eastern" opinion is actually the "Japanese" concept, and diagnosis of gastric carcinoma is based on the cytological findings. We have realized that Korean pathologists have different diagnostic criteria for gastric epithelial neoplasia than Japanese or Western pathologists. In this article, we discuss the current problems for the pathological diagnosis of gastric neoplastic lesions, the Korean perspectives, and the path we should follow.

TERMINOLOGY: DYSPLASIA, ADENOMA, CARCINOMA *IN SITU*, AND INVASIVE CARCINOMA

Initially "dysplasia" was used for inflammatory bowel disease, but, now it is used throughout the gastrointestinal tract as well as for other organs. Dysplasia means an unequivocally neoplastic but non-invasive lesion distinguished from regenerative changes[1]. The term "gastric dysplasia" was used for the first time by Grundmann in 1975 to describe an exclusively precancerous gastric lesion[2]. Shortly thereafter, a WHO committee published a definition that characterizes dysplasia as cellular atypia, abnormal differentiation, and disorganized architecture[3,4].

Most Western pathologists use the term "dysplasia" to describe a neoplastic premalignant abnormality[1,5-11]. However, in Japan, the terminology for non-invasive neoplastic lesions is different. Since Nakamura *et al*[12] established

Table 1 Definitions and grading systems proposed for gastric epithelial neoplasia

References	Dysplasia and related lesions (from Rugge *et al*[40], modified)
Takagi *et al*[15]	Benign
	Borderline
	Carcinoma
Nagayo[14]	Atypical
	Borderline
	Probable cancer
	Definitive cancer
Grundmann[2]	Low-grade GED
	High-grade GED
	Invasive cancer
Oehlert *et al*[5]	Slight GED
	Moderate GED
	Severer GED
	Invasive cancer
Morson *et al*[4]	Regenerative
	Mild GED
	Moderate GED
	Severe GED
	Invasive cancer
Ming *et al*[7]	Grade 1 GED
	Grade 2 GED
	Grade 3 GED
	Grade 4 GED
	Invasive cancer
Japanese classification of gastric carcinoma[16]	Group Ⅰ lesions
	Group Ⅱ lesions
	Group Ⅲ-Ⅳ lesions
	Group Ⅴ lesions
Goldstein *et al*[9]	Reactive
	Indefinite for GED
	Low-grade GED
	High-grade GED
	Invasive cancer
Padova classification	Negative
	Indefinite for dysplasia
	Noninvasive neoplasia
	Suspect for invasive carcinoma
	Invasive carcinoma
Vienna classification	Negative
	Indefinite for dysplasia
	Low grade neoplasia
	High grade neoplasia
	Invasive neoplasia

GED: Gastric epithelial dysplasia.

specific histological atypical gastric epithelium criteria, which were classified as definitely benign, borderline, or carcinoma, the Japanese Society for Research on Gastric Cancer has recommended that gastric neoplastic lesions be subdivided into one of five categories: normal or benign without cellular atypia, benign with slight atypia, borderline, probable carcinoma, and obvious carcinoma[13,14]. "Atypia" has been used more frequently than "dysplasia" in Japan. Subsequently, Japanese authors suggested the definition of "group 3 or 4 lesions"[14-16] (Table 1).

In addition to the differences in terminology describing premalignant gastric lesions, some confusion exists for the terms adenoma and dysplasia. Originally, adenoma was considered a raised circumscribed lesion, either sessile or pedunculated, in contrast to dysplasia, which arose at flat or depressed mucosa. However, much confusing terminology has been introduced such as "flat adenoma", "depressed adenoma", "elevated dysplasia", and "polypoid dysplasia"[17-31]. Thus, WHO defined adenoma as "a circumscribed benign neoplasm composed of tubular and/or villous structures lined by dysplastic epithelium". In 1998, Lewin *et al*[10] suggested nomenclature using both adenoma and dysplasia; the former meant neoplastic circumscribed benign lesions unassociated with underlying inflammation whether pedunculated, sessile, flat or depressed, and the latter meant benign neoplastic lesions associated with underlying inflammation. Both were subdivided as low and high grade. Although there have been efforts to clarify the definition, a confusing situation still persists[9].

Another confusing concept is carcinoma *in situ*, which means carcinoma without invasion. However, a differential diagnosis of high grade dysplasia/atypia and carcinoma *in situ* is problematic. When cytological atypia and architectural complexity is marked, the term "carcinoma *in situ*" is used by some pathologists, but others do not make a distinction between "high grade dysplasia" and "carcinoma *in situ*" because the behavior and management are the same[10]. In the Japanese classification, there is no disease group describing adenocarcinoma *in situ*. The WHO International References Center for Histological/Classification of Precancerous Lesions of the Stomach met in 1978 and developed a consensus statement that stated that presumed precancerous lesions of the stomach should be termed "dysplasia" and that the term "intramucosal carcinoma" should replace "*in situ* carcinoma" for lesions that have invasive malignant cells confined to the lamina propria[3,4]. However, "adenocarcinoma *in situ*" is categorized in the AJCC and Vienna classifications as a non-invasive intraepithelial carcinoma, resulting in confusion.

The most important and surprising inconsistency between Western and Japanese criteria is in the diagnosis of adenocarcinoma. Japanese pathologists make a diagnosis of cancer based on severe dysplastic cytologic atypia with enlarged vesicular oval nuclei and prominent nucleoli irrespective of the presence of invasion. But, Western pathologists believe there must be evidence of invasion into the lamina propria to make a cancer diagnosis. This inconsistency causes serious problems understanding "early" cancer. Many investigators have pointed out this discrepancy and made some efforts to reduce the confusion. The Vienna classification was developed for common world terminology of gastrointestinal epithelial neoplasia[32]. Some Western pathologists agreed with the Japanese criteria and changed their view points[33].

GRADING SYSTEM

Dysplasia is regarded as a precancerous lesion with increased risk of carcinoma, and the risk increases in parallel with the histological grade of the atypia. Various grading systems have been introduced to predict a prognosis of dysplasia/adenoma with more accuracy (Tables 1 and 2).

Table 2 Differentiation of low and high grade dysplasia and gastric carcinomas

Histology	Feature	Low-grade dysplasia	High-grade dysplsia	Carcinoma
Structural atypia	Gland size	Uniform	Variable	Variable
	Gland arrangement	Regular	Slightly irregular	Irregular
	Glandular crowding	Slight	Moderate	Marked
	Glandular transition to surrounding mucosa	No	No	No
	Glandular branching/budding	Focal	Prominent	Prominent
	Glandular cribriform	No	Yes	Yes
	Surface maturation	No	No	No
Nuclear atypia	Shape	Elongated	Elongated and/or irregular	Oval/round
	Pseudostratification	Basal 1/2	Over basal 1/2	Irregular
	Membrane	Thin	Thick	Uneven
	Hyperchromasia	Hyperchromatic even	Hyperchromatic irregular	Vesicular
	Pleomorphism	No	Mild	Moderate to marked
	Prominent nucleoli	Absent	Present	Present
	Loss of polarity	No	No/yes	Yes
Stroma	Invasion	No	No	Yes

The most popular grading system is the three-tiered (mild, moderate, and severe) or two-tiered (low and high) system; the latter shows better inter-observer agreement[1,33-37], and most management protocols are based on the two-tiered system. There is no distinctive management protocol according to the three-tiered system that is practically significant[1,34-37].

The morphological features of low grade dysplasia/adenoma are characterized by simple tubules with little branching, nuclear stratification below half of the cytoplasm, tall columnar cells with dense spindle-shaped hyperchromatic nuclei, ample amphophilic cytoplasm, and sparse mitotic figures. High-grade dysplasia/adenoma is composed of tubules with elongation and complex budding, cribriform in the most extreme cases, greatly enlarged round to oval nuclei, markedly increased nuclear/cytoplasmic ratio, and loss of nuclear polarity (Table 2).

Although well-established low and high-grade dysplasia criteria are present, there are large scale interobserver or intraobserver discrepancies. Sometimes, regeneration causes serious confusion with carcinoma. The category of "indefinite for dysplasia" is maintained in the Vienna classification[32], and the histopathological finding of regeneration has been well described in many studies[9,10,38].

CURRENT STATUS OF PATHOLOGIC DIAGNOSIS OF GASTRIC EPITHELIAL NEOPLASTIC DISEASE IN KOREA

Korea is geographically close to Japan, and academic exchanges are active. Korean endoscopists introduced the EMR/ESD technique from Japan, and many discussions and cooperation continues. In the pathology field, there are many conferences and collaborations between Japanese and Korean pathologists. Furthermore, Korea's medical science is influenced by that in Western countries. Korean doctors are familiar with Western style medical terminology. As a result, the terminology, definition, and diagnostic criteria for gastric intraepithelial neoplasia are very heterogeneous in Korea.

To promote diagnostic consensus, The Gastrointestinal Pathology Study Group of the Korean Society of Pathologist (GIPS-KSP) established a grading system for gastric epithelial proliferative disease and produced a standardized pathological report for gastric cancer[38,39]. The standard guidelines for grading gastric epithelial proliferative disease are as follows: (1) proliferating gastric epithelium can be divided into hyperplastic and neoplastic; (2) the term "dysplasia" is reserved for the microscopic epithelial changes that are unequivocally neoplastic; (3) biopsy specimens are categorized as regenerative (negative for dysplasia), indefinite (questionable dysplasia), positive (positive for dysplasia) and overt carcinoma; and (4) the positive category is divided into two groups; high-grade dysplasia and low-grade dysplasia[40]. Another important criterion for the differential diagnosis of low and high-grade dysplasia is the extent of nuclear stratification; nuclear stratification below half of the cytoplasm is characteristic of low-grade dysplasia. If nuclear stratification above half of the cytoplasm is present at more than three contiguous glands, it is considered high-grade dysplasia[39]. This criterion is based on the definition of high-grade dysplasia associated with inflammatory bowel disease.

In Korea, most pathologists use the term "tubular/villous/villotubular adenoma with low/high grade dysplasia" to describe intraepithelial precancerous disease. "Dysplasia" is used to describe atypia due to neoplastic etiology, excluding regenerative changes. This concept of dysplasia unassociated with adenoma (foveolar type dysplasia) is not well established and needs further study. The term "carcinoma *in situ*" is used by some pathologists to describe a highly anaplastic lesion without lamina propria invasion, but this is not widely accepted.

At the 8th Japan-Korea Pathologist Symposium in 2008 in Yokohama, Japan, there was a consensus conference to discuss diagnostic differences in gastrointestinal neoplasia, and Japanese and Korean pathologists confirmed their different viewpoints.

In 2009, GIPS-KSP began to establish new Korean diagnostic criteria for gastric epithelial proliferative disease, and the effort is ongoing. We gathered 117 cases of gastric

Table 3 Inter-observer agreement rate before and after consensus conferences

Agreement rate (%)	Before (%)	After (%)
0-50	16 (38.1)	1 (2.4)
51-60	13 (31.0)	4 (9.5)
61-70	3 (7.1)	8 (19.0)
71-80	5 (11.9)	6 (14.3)
81-90	3 (7.1)	6 (14.3)
91-100	2 (4.8)	17 (40.5)
Total	42 (100)	42 (100)

biopsy specimens and ESD specimens from 14 institutes. Six pathologists screened the slides and selected 42 cases, which showed the difficulty of diagnosis. The selected cases were circulated and answers were gathered from 45 pathologists. The answer sheet was composed of five categories of diagnosis; regenerative atypia, low grade dysplasia, high grade dysplasia, carcinoma *in situ*, and carcinoma. In most cases, there was a wide range of interobserver discrepancy. We tried to simplify the diagnostic criteria to enhance diagnostic consistency but realized that it was impossible because determining low-grade dysplasia, high-grade dysplasia, and carcinoma was a complex process based on many kinds of diagnostic criteria. A consensus conference was held eight times and the pathological findings of each case were discussed and voted on anonymously. After the consensus conferences, the agreement rate increased (Table 3). Before the consensus conferences, only 10 cases among 42 showed a high agreement rate (more than 70%). After the conferences, the cases showing high agreement rate increased to 25 cases (Table 3). Although these data were not enough for a conclusion, it was suggested that there could be agreement for a pathologic diagnosis among Korean pathologists.

Many histological factors are helpful for the differential diagnosis of low and high-grade dysplasia, but these factors sometimes conflicted with each other. We attempted to identify a more simple and reproducible way to determine the dysplasia grade. We propose guidelines for differential diagnosis: (1) a diagnosis of carcinoma is based on invasion; (2) the most important characteristic of low-grade dysplasia is a regular distribution of crypts without severe branching, budding, or marked glandular crowding; (3) if nuclear pseudostratification occupies more than the basal half of the cryptal cells in three or more adjacent crypts, the lesion is considered high-grade dysplasia (this rule was based according to the previously mentioned Korean Standard of Pathology Report of Gastric Cancer[39]); (4) if severe cytologic atypia is present, careful inspection for invasive foci is necessary, because the risk of invasion is very high; and (5) other structural or nuclear atypia should be evaluated to make a final decision such as cribriform pattern, papillae, ridges, vesicular nuclei, high nuclear/cytoplasmic ratio, loss of nuclear polarity, thick and irregular nuclear membrane, and nucleoli. Based on these principles, the consensus rate was markedly increased, although not in every case.

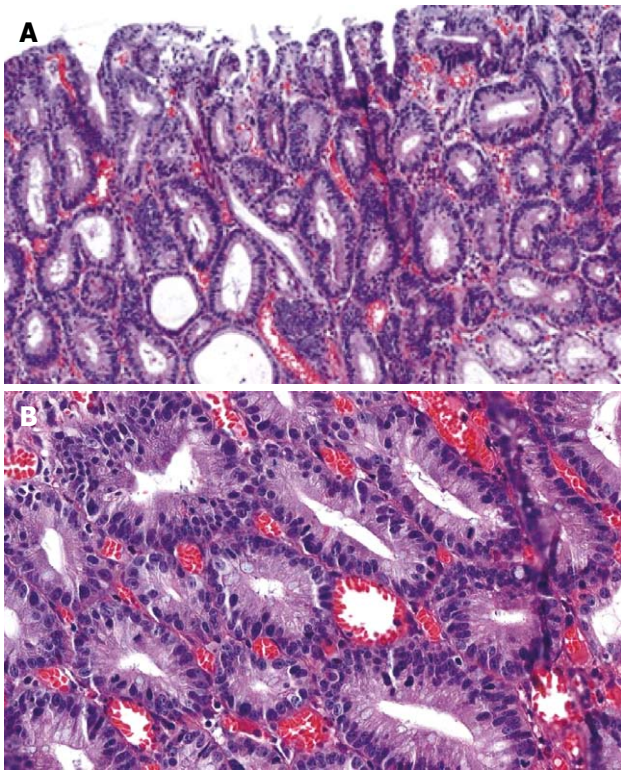

Figure 1 Consensus diagnosis of tubular adenoma with low grade dysplasia. A: Regular distribution of small proliferative glands without budding or branching (HE, × 100); B: Elongated nuclei with stratification below half of the cytoplasm (HE, × 200).

EXAMPLES OF CASES PRESENTED AT THE CONSENSUS CONFERENCE

Case 1

An ESD specimen revealed a regular distribution of small proliferative glands without budding or branching (Figure 1A). The nuclei were elongated and stratified below half of the cytoplasm (Figure 1B). Hyperchromasia and mitoses were present; 57.8% and 100% of the pathologists agreed with a diagnosis of tubular adenoma with low grade dysplasia before and after the consensus conference, respectively.

Case 2

An ESD specimen revealed regular distribution of small proliferative glands without budding or branching (Figure 2A). Glandular crowding was mild. The nuclei were ovoid and vesicular with conspicuous nucleoli but nuclear stratification did not exceed the basal half of the cell (Figure 2B); 73.3% and 60.9% of the pathologists agreed with a diagnosis of tubular adenoma with low-grade dysplasia before and after the consensus conference, respectively.

Case 3

An ESD specimen revealed compact small glandular proliferation with some variation in gland size (Figure 3A). Budding or branching was present. The nuclei were elongated and stratified with some ovoid nuclei. More than

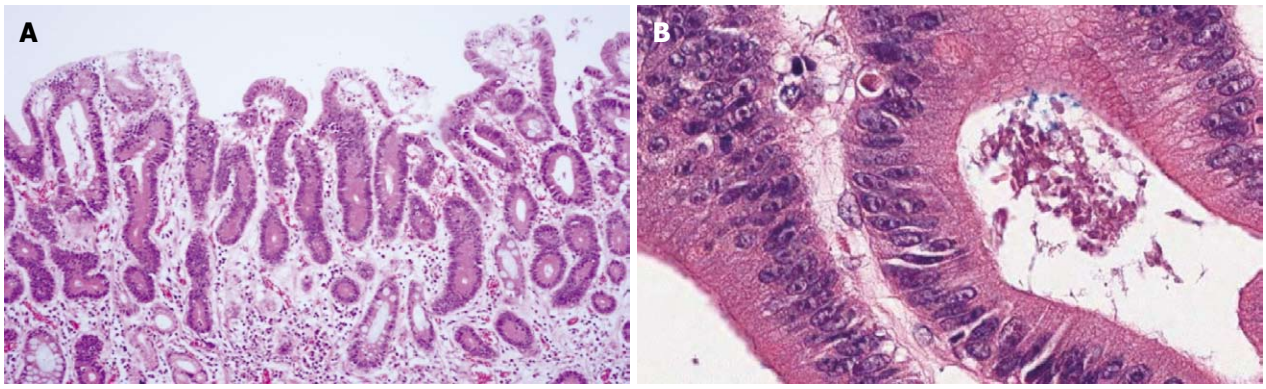

Figure 2 Major consensus diagnosis of tubular adenoma with low-grade dysplasia. A: Regular distribution of small proliferative glands without budding or branching (HE, × 100); B: Ovoid and vesicular nuclei with conspicuous nucleoli and nuclear stratification not exceeding basal half of the cell (HE, × 400).

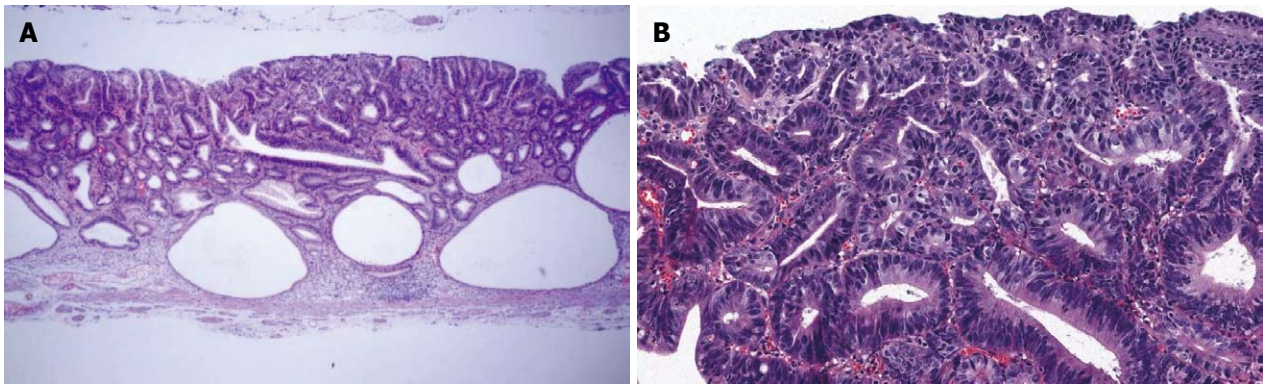

Figure 3 Consensus diagnosis of tubular adenoma with high-grade dysplasia. A: Compact small glandular proliferation with some variation of gland size, budding and branching (HE, × 40); B: Elongated or oval nuclei with stratification above half of the cytoplasm in more than three contiguous glands (HE, × 200).

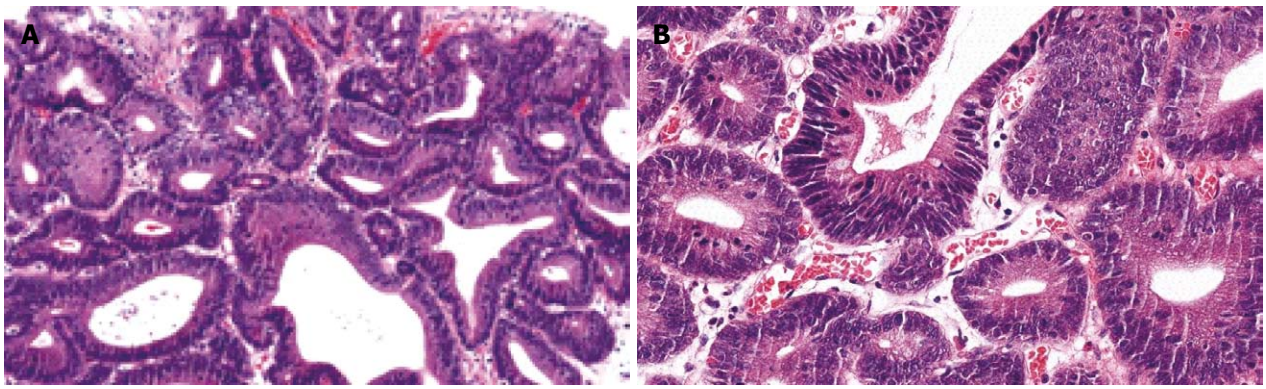

Figure 4 Major consensus diagnosis of tubular adenoma with high-grade dysplasia. A: Glandular crowding with some variation in gland size and budding (HE, × 100); B: Elongated or oval nuclei with stratification above basal half of the cytoplasm (HE, × 200).

three contiguous glands showed nuclear stratification above half of the cytoplasm (Figure 3B). Hyperchromasia and mitoses were present; 44.4% and 100% of the pathologists agreed with a diagnosis of tubular adenoma with high-grade dysplasia before and after the consensus conference, respectively.

Case 4

An ESD specimen revealed glandular crowding with some variation in gland size and budding (Figure 4A). The nuclei were elongated and stratified with some ovoid nuclei. Nuclear stratification above the basal half of the cytoplasm was present (Figure 4B). Hyperchromasia and mitoses were noted. Before the consensus conference, 44.4% of pathologists agreed with a diagnosis of tubular adenoma with high-grade dysplasia, which increased to 75% after the conference.

Case 5

An ESD specimen revealed compact small glandular

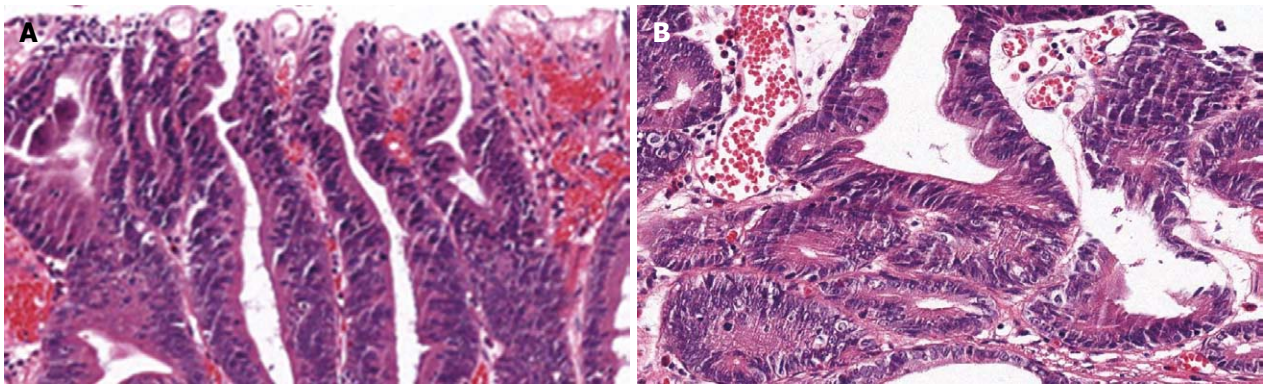

Figure 5 Major diagnosis of tubular adenoma with high-grade dysplasia. A: Compact small glandular proliferation with variation in gland size, budding and branching (HE, × 100); B: Elongated or oval nuclei with stratification above basal half of the cytoplasm in more than three contiguous glands. Glandular complexity without definite invasion (HE, × 200).

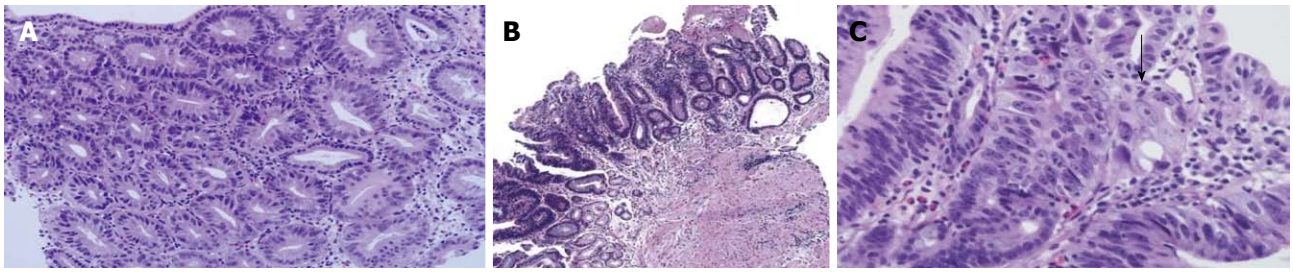

Figure 6 Consensus diagnosis of adenocarcinoma. A: Compact small glandular proliferation without budding or branching. Relatively regular glandular distribution but enlarged, oval to round, and pleomorphic nuclei (HE, × 100); B: Another section showing villous configuration (HE, × 40); C: Hyperchromasia and mitoses with invasion into the lamina propria (arrow) (HE, × 400).

proliferation with variation in gland size, budding and branching (Figure 5A). The nuclei were elongated and stratified with some ovoid nuclei (Figure 5B). More than three contiguous glands showed nuclear stratification above the basal half of the cytoplasm. Hyperchromasia and mitoses were present. Glandular complexity was present but definite invasion was not identified; 42.5% and 62.5% of the pathologists agreed with a diagnosis of tubular adenoma with high-grade dysplasia before and after the consensus conference, respectively.

Case 6

A mucosal biopsy specimen revealed compact small glandular proliferation without budding or branching (Figure 6A). Another section showed a villous configuration (Figure 6B). The glandular distribution was relatively regular but gland size was mildly variable. The nuclei were enlarged, oval to round, and pleomorphic. Nuclear stratification was not severe, but enlarged nuclei occupied more than the basal half of the cytoplasm. Hyperchromasia and mitoses were present. Invasion into the lamina propria was present (Figure 6C, arrow); 22.2% and 100% of the pathologists agreed with a diagnosis of adenocarcinoma before and after the consensus conference, respectively.

Case 7

A mucosal biopsy specimen revealed compact small glandular proliferation with budding or branching (Figure 7A). Glandular size and distribution were irregular. The nuclei were enlarged, oval to round, with vesicular chromatin. Severe nuclear stratification approaching the top of the cytoplasm in more than three contiguous glands was present. Marked hyperchromasia and mitoses were noted with invasion into the lamina propria (Figure 7B, arrow); 26.7% and 56.3% of the pathologists agreed with a diagnosis of adenocarcinoma before and after the consensus conference, respectively.

PROBLEMS TO BE SOLVED

The rate of agreement markedly increased after many consensus conferences (Table 3). However, this guideline has some limitations; (1) focal invasion into the lamina propria may not be detected on a biopsy specimen, which causes diagnostic discrepancy between a biopsy and resection specimen; and (2) a gray zone due to overlapping or mismatching of diagnostic criteria lowers the agreement rate. We must conduct a further study to verify the hypothesis in expanded cases and to determine that the guideline lowers inter and intraobserver discrepancies and correlates with clinical outcome. If more reliable pathological findings suggesting possible invasion into an adjacent area could be found, it would be very useful for small biopsy specimens. We will attempt to define the pathological criteria in a more simple and subjective way, and we would like to develop a diagnostic algorithm. Education is also important. Symposiums, workshops, and publishing of articles will be helpful.

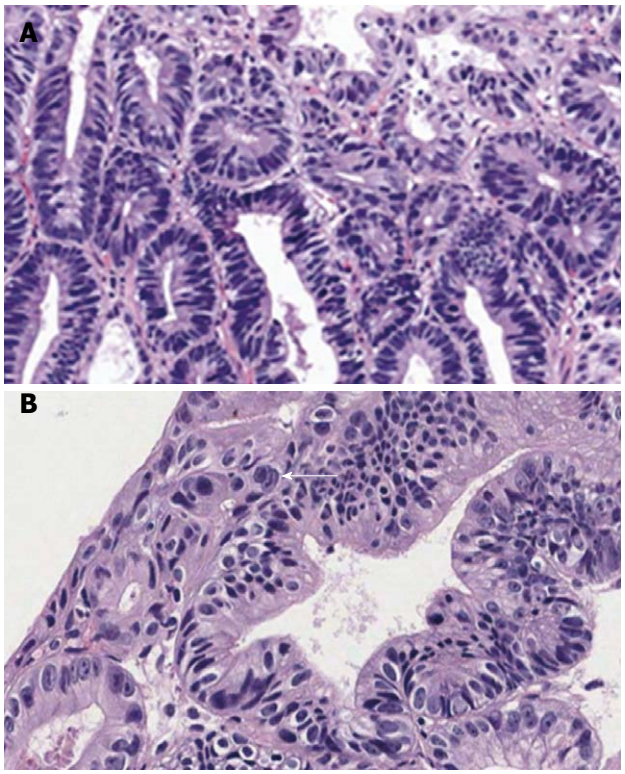

Figure 7 Major diagnosis after the consensus conference of adenocarcinoma. A: Compact small glandular proliferation with budding and branching. Regular glandular size and distribution (HE, × 100); B: Severe nuclear stratification approaching the top of the cytoplasm in more than three contiguous glands. Marked hyperchromasia and mitoses with invasion into the lamina propria (arrow) (HE, × 400).

We have additional problems to be solved, such as how to measure the invasion depth if submucosal invasion is present, the diagnostic policy for differentiation, judgment on lymphovascular invasion, and a fixation method for ESD specimens, which are all important decisions to develop a further treatment plan after EMR/ESD.

CONCLUSION

Eastern and Western pathologists have different terminology and diagnostic criteria for gastric intraepithelial neoplasia. In Korea, pathologists experience much difficulty when making a diagnosis, and have made efforts to increase the interobserver agreement rate. As a result, we have achieved improved diagnostic consensus, although it is not yet perfect. We tentatively suggest the guidelines for differential diagnosis: (1) a diagnosis of carcinoma is based on invasion; (2) the most important characteristic of low grade dysplasia is the architectural pattern such as regular distribution of crypts without severe branching, budding, or marked glandular crowding; (3) if nuclear pseudostratification occupies more than the basal half of the cryptal cells in three or more adjacent crypts, the lesion is considered high grade dysplasia; (4) if severe cytologic atypia is present, careful inspection for invasive foci is necessary, because the risk for invasion is very high; and (5) other structural or nuclear atypia should be evaluated to make a final decision such as cribriform pattern, papillae, ridges, vesicular nuclei, high nuclear/cytoplasmic ratio, loss of nuclear polarity, thick and irregular nuclear membrane, and nucleoli. Further study on the pathological findings and clinicopathological correlations as well as a follow-up study are necessary to increase diagnostic accuracy.

ACKNOWLEDGMENTS

We express great gratitude to Dr. Shimoda T who gave us very informative academic lectures, and to Dr. Ajioka Y who helped us create a Japanese-Korean pathologist consensus conference in 2008.

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S- Editor Shi ZF **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2611

World J Gastroenterol 2011 June 7; 17(21): 2611-2617
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Worldwide experiences of endoscopic submucosal dissection: Not just Eastern acrobatics

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Received: June 26, 2010 Revised: September 2, 2010
Accepted: September 9, 2010
Published online: June 7, 2011

Abstract

The high incidence of gastric cancer has led to the initiation of cancer screening programs. As a result, the number of early gastric cancer cases has increased and consequentially, the cancer mortality rate has decreased. Moreover, the development of minimally invasive endoscopic treatment has been introduced for these early lesions. Endoscopic submucosal dissection (ESD) is now recognized as one of the preferred treatment modalities for premalignant gastrointestinal epithelial lesions and early gastric cancer without lymph node metastasis. We review the results of ESD including experiences in Japan and Korea, as well as western countries.

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Key words: Experiences; Endoscopic submucosal dissection

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Cho KB, Jeon WJ, Kim JJ. Worldwide experiences of endoscopic submucosal dissection: Not just Eastern acrobatics. *World J Gastroenterol* 2011; 17(21): 2611-2617 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2611.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2611

INTRODUCTION

The high incidence of gastric cancer in Japan and Korea has led to the initiation of national cancer screening programs. As a result, the number of early gastric cancer (EGC) cases has increased dramatically and accounts for up to 50% of all gastric cancer diagnoses[1,2]. In addition, as a result of these programs, the mortality rate from gastric cancer has decreased[3]. Moreover, the development of minimally invasive endoscopic treatment has been introduced for these early lesions.

Initially, EGC was treated using endoscopic mucosal resection (EMR) techniques that included an injection that lifts the lesions, which are then cut. EMR can be used with a cap (EMR-C) and with ligation (EMR-L). These techniques are limited by the size of the lesions; only lesions of a relatively small size (< 2 cm) can be resected *en bloc*, due to the restricted size of the snare, cap and ligation devices. If EMR is attempted for lesions > 2 cm, the risk of piecemeal resection might increase, which makes it difficult to determine whether the lateral resection margins are free of disease. Previous studies of EMR have reported an approximately 75% *en bloc* resection rate. However, there is a high risk of local recurrence (2%-35%) with this procedure, especially when EMR cannot achieve *en bloc* resection[4-7].

To avoid the problems associated with EMR, endoscopic submucosal dissection (ESD) has been introduced

for safe *en bloc* resection. ESD can be used for larger lesions and those with ulceration, regardless of their location[7-9]. Although ESD techniques require advanced skill and might have a higher complication rate, including bleeding and perforation, they increase the rate of *en bloc* resection and complete histological analysis, and might ultimately reduce local disease recurrence rate[7,10].

ESD is now recognized as one of the preferred treatment modalities for premalignant gastrointestinal epithelial lesions and EGC without lymph node metastasis. The ESD procedure starts by making several marking dots around the lesion; a lifting solution is injected into the submucosal layer, followed by endoscopic circumferential incision of the lesion with various knives; dissection starts at the lateral edges, and proceeds through the lifted submucosal layer until the lesion is resected in one piece. According to a PubMed search, a total of 517 articles on ESD have been published up to January 2010. Most of the studies have been published from Asia, mainly Japan; however, recently there have been an increasing number of papers from Korea and China.

In this chapter, ESD for EGC is reviewed, including experiences from Japan and Korea, as well as western countries.

BACKGROUND OF ENDOSCOPIC RESECTION

EGC is defined by tumor invasion confined to the mucosa or submucosa, regardless of lymph node metastasis[11]. According to the outcomes after gastrectomy, 5- and 10-year survival rates for patients that have a diagnosis of mucosal EGC have been reported to be 96% and 92%, respectively[12]. The long-term outcomes after EMR for EGC < 2 cm has demonstrated excellent results with disease-specific 5- and 10-year survival rates of 99% and 99%, respectively; this was even with patients that had major organ complications, who were not good candidates for surgery[13]. Clinical experience suggests that complete resection of the cancer is possible, and cure can be achieved as long as the potential for metastatic spread is accurately excluded.

The accepted indications for EMR and ESD are lesions diagnosed as well-differentiated adenocarcinoma by histology, which are elevated and < 2 cm in diameter, and small (≤ 10 mm), depressed, well-differentiated tumors without ulcer formation[14]. However, these indications are rather strict, which leads to many patients being subjected to unnecessary surgery. Actually, endoscopic resection has also been used for larger lesions without lymph node metastasis by many Japanese investigators. Lymphovascular involvement, ulcer formation, and tumor size > 3 cm are independent risk factors for lymph node metastasis in EGC that is limited to the mucosa[15]. A large study on post-gastrectomy outcomes with lymph node dissection has shown that the overall risk for lymph node metastasis among patients with EGC that involved the mucosa was 2.7%. The risk increased to 18.6% when the cancer invaded the submucosa. The absence of submucosal lymphovascular involvement in moderately or well-differentiated adenocarcinoma was found to correlate with a nominal risk for lymph node metastasis. In lesions < 3 cm, the risk of lymph node metastasis is very low regardless of the presence of ulceration. In lesions without ulceration, the risk is unaffected by the size of the tumor[16]. Therefore, according to the *Treatment Guidelines for Gastric Cancer in Japan*, the expanded criteria for ESD are as follows: (1) a differentiated mucosal cancer without ulceration, no lymphatic-vascular invasion, regardless of size; (2) a differentiated mucosal cancer with ulceration, no lymphatic-vascular invasion, tumor < 3 cm; (3) an undifferentiated type of mucosal cancer without ulceration and tumor < 2 cm in diameter, and absence of lymphatic-vascular invasion; and (4) when a differentiated adenocarcinoma, which has not invaded deeper than submucosal level 1 (< 500 μm) and lymphovascular invasion is absent, additional lymph node dissection is not necessary[14].

However, expanding the indications for endoscopic resection remains controversial because the long-term outcomes of these indications have not been fully documented. Several publications have reported lymph node metastasis in EGC that meet the extended criteria[17-19]. Jee *et al*[19] have reported lymph node status in a total of 181 patients who met expanded indications for ESD and had undergone surgical resection. They reported lymph node metastasis in 2.3% of 129 patients with mucosal cancer. This included one ulcerated differentiated cancer < 3 cm in diameter, and two undifferentiated cancers < 2 cm in diameter without ulceration. Also, in a study of lymph node metastasis in 4% of 52 patients with submucosal cancer, those were two differentiated tumors.

Therefore, considering the indications for ESD, surgery is preferentially recommended for undifferentiated mucosal cancer, although several recent studies have shown that the rate of lymph node metastasis is negligible in small and undifferentiated mucosal cancer[20-24].

WORLDWIDE ESD RESULTS

The results of recent studies have suggested that the technique of ESD achieves a high rate of *en bloc* resection (92%-97%) and complete resection (73.6%-94.7%) with various rates of complications including bleeding (0.1%-15.6%) and perforation (1.2%-9.7%). Furthermore, they have revealed excellent long-term outcomes (5-year overall and disease-specific survival rates of 97.1% and 100%, respectively) (Table 1).

Japan

Oda *et al*[6] have performed a multicenter retrospective study to determine the nationwide results of endoscopic resection for EGC. Seven hundred and fourteen EGCs (EMR, 411; ESD, 303) that met the expanded criteria, except for the undifferentiated type of mucosal cancer, from 655 consecutive patients and 11 Japanese institutions were evaluated. Technically, 71.6% of the lesions were resected in one piece. The rate of *en bloc* resection by ESD (92.7%) was significantly higher than by EMR (56.0%). The rate of

Table 1 Recent outcomes of endoscopic resection for early gastric cancer

Author	Yr	No. (lesion/patient)	Method	*En bloc* rate (%)	Complete resection rate (%)	Follow-up (mo/range)	Complications (%)		Local recurrence rate (%)	3-yr residual/free recurrence rate (%)	3-yr overall survival rate (%)	5-yr overall survival rate (%)	5-yr disease-specific survival rate (%)
							Bleeding	Perforation					
Oda *et al*[6]	2006	714/655	EMR 411	56.0	61.1	38 (6-60)	0.1	1.2	7.5	92.5	99.2	NA	NA
	Retrospective		ESD 303	92.7	73.6			3.6	2.4	97.6			
Oka *et al*[7]	2006	1025/896	EMR 825/711	43.4	24.6	83.2	3.9	0.5	3.5 (31/825)	NA	NA	NA	NA
	Retrospective		ESD 195/185	92.8	92.8	19.4	6.2	9.7	0 (0/195)				
Imagawa *et al*[8]	2006	196/185	ESD	93.0	84.0	17.6 (1.3-45.7)		6.1	0 (0/164)	NA	NA	NA	NA
	Retrospective												
Jung *et al*[28]	2007	1327/NA	EMR-P 775	NA	91.0	NA	6.3	0.8	NA	NA	NA	NA	NA
	Retrospective		ESD 552		95.1		7.6	2.7					
Takenaka *et al*[54]	2008	306/275	ESD	NA	80.4	26 (26-64)	0.6	5.2	0 (0/177)	NA	NA	NA	NA
	Prospective												
Isomoto *et al*[10]	2009	589/551	ESD	94.9	94.7	30 (6-89)	1.8	4.5	0 (0/468)	NA	> 97.2	97.1	100
	Retrospective												
Goto *et al*[25]	2009	276/231	ESD	96.7	91.7	36 (2-93)	5.1	4.0	0.9 (2/212)	99.1	96.2	96.2	100
	Retrospective												
Nakamoto *et al*[26]	2009	202/177	EMR 80	53.8	37.5	54 (12-89)			17.5 (14/80)	82.5[1]	100	100	100
	Retrospective		ESD 122	94.3	92.6	34 (14-62)	1.6	2.5	0	0			
Chung *et al*[29]	2009	1000/952	ESD	95.3	87.7	NA	15.6	1.2	NA	NA	NA	NA	NA
	Retrospective												
Jang *et al*[30]	2009	402/402	ESD	89.7	87.9	30 (9-49)	7.4	2.9	5.1 (10/198)	94.9	NA	NA	NA
	Retrospective	(107 LGD/97 HGD/198 EGC)											
Min *et al*[31]	2009	346/243	EMR-P 103	77.7	75.7	29 (4-44)	3.9	1.9	0 (0/80)	NA	NA	NA	NA
	Retrospective		ESD 243	95.9	88.9	17 (4-37)	5.3	4.5	0 (0/191)				
Chang *et al*[32]	2009	70/70	ESD	91.4	92.8	NA	5.7	4.3	2.8	NA	NA	NA	NA
	Retrospective												

[1]Overall 5-year recurrence free rate. LGD: Low-grade dysplasia; HGD: High-grade dysplasia; NA: Not applicable; EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; EMR-P: precutting followed by snare resection.

curative resection by ESD (73.6%) was significantly higher than by EMR (61.1%). Bleeding was found in 0.1% of cases. The frequency of perforation with ESD and EMR was 3.6% and 1.2%, respectively. All complications were managed endoscopically, and there was no procedure-related mortality. The median follow-up period was 3.2 years. The 3-year cumulative residual-free/recurrence-free rate in the ESD group (97.6%) was significantly higher than that in the EMR group (92.5%).

Oka *et al*[7] have reported on a comparative study between EMR and ESD of 1020 EGCs that met the expanded criteria. Eight hundred and twenty-five EMRs and 195 ESDs were performed. In cases without ulceration, the *en bloc* and curative resection rates were significantly higher with ESD (both 92.8%) than with EMR (43.4% and 24.6%), regardless of tumor size. The average operation time was significantly longer for ESD than for EMR (84.4 min *vs* 12.6 min), regardless of tumor size. In addition, the frequency of intraoperative bleeding was significantly higher with ESD (22.6%) than with EMR (7.6%). The frequency of delayed bleeding did not differ. No patient experienced recurrence after ESD.

Imagawa *et al*[8] have analyzed 196 EGCs that met the expanded criteria and were treated by ESD, in relation to lesion size, location and the presence or absence of ulceration. The rate of *en bloc* resection was 93%, the curative resection rate was 84%, with a perforation rate of 6.1%, and a mean procedure time of 68 min. The rate of curative *en bloc* resection differed significantly depending on the location of the lesion (upper *vs* middle *vs* lower, 74% *vs* 77% *vs* 91%), as well as on the size of the lesion (> 20 mm *vs* ≤ 20 mm, 59% *vs* 89%). There were also significant differences in the mean procedure time in relation to the location of the lesion (upper *vs* middle *vs* lower, 105 min *vs* 81 min *vs* 45 min) and the size of the lesion (> 20 mm *vs* ≤ 20 mm, 124 min *vs* 55 min), as well as the presence of ulceration (positive *vs* negative, 97 min *vs* 65 min). They showed that the difficulty of ESD depends on the location and size of the lesion, as well as on the presence of ulceration.

Isomoto *et al*[10] have published the first long-term follow-up results of 589 EGCs treated by ESD, which met the expanded criteria. *En bloc* resection was achieved in 94.9%. Curative resection was achieved in 94.7%. *En bloc* resection was the only significant factor associated with curative ESD. Patients with a non-curative resection developed local recurrence more frequently. The 5-year overall and disease-specific survival rates were 97.1% and 100%, respectively.

Goto *et al*[25] have carried out a retrospective investigation of ESD to determine long-term outcomes. Two hundred and seventy-six node-negative EGCs that met the expanded criteria, except for the undifferentiated type of mucosal cancer, were enrolled. The *en bloc* and complete resection rates were 96.7% and 91.7%, respectively. During a median follow-up of 3 years, there were two local recurrences (0.9%). The 5-year overall and disease-specific survival rates were 96.2% and 100%, respectively.

Nakamoto *et al*[26] have performed a comparative study of EMR and ESD for 202 EGCs. The overall *en bloc* and complete resection rates were lower in patients undergoing EMR compared to ESD (*en bloc*: 53.8% *vs* 94.3%, complete: 37.5% *vs* 92.6%). The overall 5-year recurrence-free rate was lower in the EMR group than in the ESD group (82.5% *vs* 100%). However, with regard to tumor size, EMR was comparable to ESD for the small lesions (< 5 mm).

Korea

The number of publications on ESD in Korea has been increasing. The results of ESD including the *en bloc* resection rate, complete resection rate, and long-term follow-up survival rates are similar to those from Japan. The Health Insurance Review and Assessment Service of Korea have reported that 74 institutions, mainly tertiary hospitals, have performed ESD in 2008.

Kim *et al*[27] have published the first multicenter retrospective study of endoscopic resection in Korea. They collected 514 EGCs in 506 patients during January 2000 to December 2002 by use of the on-line database registry system. The most commonly used technique was circumferential precutting followed by snare resection (EMR-P, 52.3%). The second most common procedure was the injection and cut technique (24.3%). ESD was used only in 6.6% of cases at that time. Complete resection was confirmed in 77.6% of the lesions, and the mean tumor size was 1.76 cm. However, ESD is now in the mainstream for endoscopic resection of early gastric lesions in Korea.

Jung *et al*[28] have reported an 11-year experience of endoscopic resection performed by a single endoscopist. Seven hundred and seventy-five EMR procedures were performed after precutting (EMR-P) during the first 9 years and 552 ESDs over the following 2 years. The median specimen sizes were 33 mm for EMR-P and 45 mm for ESD. The complete resection rates were 91% and 95.1% and the respective complication rates were 7.1% and 10.7%.

The Korean ESD study group has published a retrospective six university hospital experience with ESD for 1000 gastric neoplasms[29]. The rate of *en bloc* resection and complete resection was 95.3% and 87.7%, respectively. The rate of delayed bleeding and perforation was 15.6% and 1.2%, respectively. The rate of *en bloc* resection differed significantly based on the location of the lesions and presence of a scar. Procedure times were increased in cases in the upper stomach that had a large lesion (> 40 mm), with the presence of an ulcer, and the presence of a scar.

Jang *et al*[30] have reported the results of ESD for 402 gastrointestinal neoplasms at a single hospital. *En bloc* resection and complete resection were achieved in 89.7% and 87.9%, respectively, and the local recurrence rate was 5.1%. The 3-year cancer-free survival rate was 94.9%.

Min *et al*[31] have published a comparative study of ESD and EMR after circumferential precutting (EMR-P) of 346 EGCs. *En bloc* resection and complete resection were achieved in 77.7% and 75.7% of the EMR-P group, respectively, and 95.9% and 88.9% of the ESD group. For EGCs > 20 mm, ESD demonstrated a significantly higher *en bloc* resection and complete resection rate compared to

EMR-P. In cases with completely resected differentiated cancer, neither group showed local recurrence during a median 29 and 17 mo follow-up, respectively.

Taiwan

Chang *et al*[32] have published a retrospective multicenter review of ESD of 70 EGCs in Taiwan. The *en bloc* resection rate was 91.4%. The bleeding and perforation rates were 5.7% and 4.3%, respectively. Emergency surgery was performed in the patients with perforations. The local recurrence rate was 2.8%. Another small study has been published in Taiwan[33]; however, the results have indicated that the procedure requires more experience.

Western countries

As a result of the low incidence of EGC in the west, relatively few institutions use ESD and there have been only a few clinical studies on the outcomes[34-42]. However, the reported outcomes of western studies have not been substantially different from those in eastern countries. Cardoso *et al*[35] have published an initial experience with ESD for 15 EGCs < 30 mm with no ulceration or scaring from Brazil. The mean procedure time was 140 min. The *en bloc* and complete resection rate was 80% and perforations occurred in 20% of cases. Catalano *et al*[36] have published their experience with EMR and ESD on 48 gastric lesions in Italy. After an initial experience with 36 EMRs, the procedure was changed to ESD. Out of 36 EMR procedures, *en bloc* and complete resection were achieved in 72% and 56%, respectively. However, among the 12 ESD cases, the rates both increased to 92%. Bleeding and perforation occurred in one case each. Dinis-Ribeiro *et al*[37] have published the results of ESD for 19 gastric lesions in Europe. ESD was performed under general anesthesia with a 79% *en bloc* resection rate. Probst *et al*[38] have published ESD results for 71 epithelial or submucosal tumors, and have demonstrated a learning curve that resulted in a decrease in the procedure duration and increased rate of complete *en bloc* resection over time (65.7% to 72.2%). Although there have been a limited number of studies outside Asia, the number of endoscopy centers that are performing ESD is slowly increasing worldwide[43].

WHAT IS UNIQUE ABOUT ESD IN EASTERN ASIA?

In Eastern Asia, endoscopic resection methods have been widely accepted as the standard treatment for gastric tumors, and many trained endoscopists are familiar with EMR techniques. Compared to other organs including the esophagus or colon, tumors of the stomach are relatively easy to remove by endoscopic resection. The basic techniques of EMR overlap with those of ESD. This allows for a stepwise approach to a variety of lesions. For example, one might start with the frequently encountered easier lesions in the distal portion of the stomach, move to lesions in the proximal stomach, and then lesions in the esophagus or colon as the final step[9,44]. However, in the west, because of the lower frequency of gastric cancer, endoscopists have relatively less experience with EMR techniques. In addition, the high incidence of Barrett's esophagus, the treatment for which is technically demanding, makes it difficult for beginners to gain extensive experience with ESD[45]. Training programs specifically aimed at advancing experience with ESD are useful[46,47]. Although data on the learning curve for ESD are limited, Choi *et al*[48] have shown that, for an experienced endoscopist, approximately 40 cases of gastric EMR with a circumferential mucosal incision in a low risk location are necessary for satisfactory training.

Kakushima *et al*[49] have reported a retrospective study on the learning curve for 383 ESD procedures by two principal operators and 11 (< 30 cases) endoscopists with less experience. For the two main operators, there was no significant difference between 25 consecutive patients with regard to the *en bloc* resection and complication rates. The size of the lesions increased as the number of patients increased, whereas the average procedure time decreased significantly. For the endoscopists with less experience, there was a similar treatment outcome and complication rate, mainly due to the easier location of the tumors in their cases. A constant rate of both treatment outcomes and complications was achieved over a 5-year period of experience with ESD. A decrease in the procedure time was found to be a marker for operator proficiency with this technique. Yamamoto *et al*[44] have reported on the learning curve for three resident endoscopists that had already learned the basic procedures. They performed ESD under supervision for 30 consecutive lesions each. They obtained a good overall complete resection rate of 93%, with an acceptable complication rate of 4.4% with appropriate supervision; however, there was difficulty in achieving a sufficient rate of finishing up alone for submucosal dissection.

ESD was first performed in Korea in 1999. The Korean Society of Gastrointestinal Endoscopy (KSGE) organized the ESD research group to investigate and expand the ESD procedure nationwide in 2003. Prior to 2006, only 22 hospitals had the facilities to perform ESD. The KSGE developed ESD hands-on courses and traveled nationwide to introduce the ESD procedure and the devices with animal models on eight occasions[50]. As a result, the number of registered ESD facilities increased to 77 according to data from National Health Insurance Review and Assessment Service in 2008. Furthermore, an international ESD live demonstration has been held every year since 2006. Such support from the KSGE, including the ESD research group, a joint symposium with the Korean Pathology Society, ESD live demonstrations, multicenter studies, and training models for teaching ESD have made it possible to standardize ESD guidelines and techniques in Korea.

ESD EAST TO WEST

Minimally invasive approaches provide a substantially better quality of life compared to conventional open surgery. There is currently not enough long-term follow-up outcome data on ESD compared to open surgery; however,

if careful patient selection is maintained, excellent oncological outcomes with ESD are attainable by experienced endoscopists. Although ESD procedures have not been performed in western countries with as much experience as in eastern countries, improved techniques can be achieved with additional experience. In spite of the low incidence of gastric lesions in the west, there is a relatively high incidence of colon lesions, including large sessile and flat polyps. The novice might start with a modified EMR technique including the adoption of some of the methods used for ESD, such as circumferential marginal incision, which has been used to teach ESD techniques during the past decade in Korea. Furthermore, training programs can be developed to teach ESD[47], and close collaboration between western and Asian centers and attending live demonstrations can help to gain such experience. On the other hand, modern medical science and technology continue to develop at a rapid pace. The recent development of accessories and traction devices might help in the acquisition of the skill and experience needed to make ESD easier to learn and apply[51-53].

CONCLUSION

ESD is an effective and safe therapeutic modality for management of early gastrointestinal tract neoplasms, although it has a relatively longer operation time and high risk of complications. In spite of the late start for ESD in the west, the results have been being similar to those from Japan. In the same manner, although there is a problem to overcome the flat learning curve in view of the low number of detected cases in western countries, close collaboration between western and Asian centers is required for improvement of the ESD technique and its clinical application.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2618

World J Gastroenterol 2011 June 7; 17(21): 2618-2622
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Chicken soup for teaching and learning ESD

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Received: June 26, 2010 Revised: September 20, 2010
Accepted: September 27, 2010
Published online: June 7, 2011

Abstract

Endoscopic submucosal dissection (ESD) is becoming a popular procedure for the diagnosis and treatment of superficial mucosal lesions, and has the advantage of *en bloc* resection which yields a higher complete resection and remission rate compared to endoscopic mucosal resection (EMR). However, the learning process of this advanced endoscopic procedure requires a lengthy training period and considerable experience to be proficient. A well framed training protocol which is safe, effective, easily reproducible and cost-effective is desirable to teach ESD. In addition, the training course may need to be tailored around settings such as ethnicity, culture, workload, and disease incidence. In Asian countries with a large volume of early gastric lesions which need endoscopic treatment, endoscopists would be able to learn ESD expanding their skills from EMR to ESD under the supervision of experts. Whereas, in Western countries due to the low incidence of superficial gastric tumors, trials have utilized simulator models to improve learning. In Korea, the Korean Society of Gastrointestinal Endoscopy (KSGE) is playing an important role in training many gastroenterologists who have shown an interest in performing ESD by providing an annual live demonstration and a nationwide tutoring program. The purpose of this article is to introduce our ESD tutoring experience, review the published papers related to this topic, and propose several suggestions for future directions in teaching and learning ESD.

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Key words: Endoscopic submucosal dissection; Learning; Teaching

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Kim EY, Jeon SW, Kim GH. Chicken soup for teaching and learning ESD. *World J Gastroenterol* 2011; 17(21): 2618-2622 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2618.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2618

INTRODUCTION

Endoscopic submucosal dissection (ESD) is an excellent procedure for the diagnosis and treatment of superficial mucosal lesions, but its clinical implementation has limitations due to technical difficulties and the risk of complications[1]. ESD has the advantage of *en bloc* resection, which yields a higher complete resection and remission rate compared to endoscopic mucosal resection (EMR). Because of this advantage, many gastroenterologists have shown an interest in performing ESD. Previous studies have indicated that ESD of gastric neoplasms is technically feasible when it is performed by competent endoscopists[2]. However, similar results from novice endoscopists are not

expected since the learning process of this advanced endoscopic procedure is long and requires considerable experience. This is one of the reasons why a well framed training protocol is necessary for this difficult procedure. It is our understanding that the training program should be tailored around needs based on ethnicity, culture, and/or country since the incidence of disease and working environment may be different. However, the training program should focus on safety, effectiveness, easy reproducibility and cost-effectiveness regardless of conditions. In this article, we describe our teaching experience and revisit published papers related to the teaching and learning of ESD.

ESD TRAINING EXPERIENCE IN KOREA

ESD was introduced in Korea in the late 1990s and is now widely accepted as one of the standard treatment modalities for early cancers and premalignant lesions of the stomach and colorectum. In 2008, according to the reference of the Korean Health Insurance Review and Assessment Service, ESD was performed in 70 tertiary hospitals, 3 community-based hospitals, and 1 primary physician center in Korea.

Live demonstrations have been held annually by the Korean Society of Gastrointestinal Endoscopy (KSGE) since 2004. It is a good opportunity for beginners to observe procedures performed by experts and to obtain information on ESD. However, we have noticed that simple observation of these procedures does not serve the needs of the endoscopists who are in the fellowship training course. For this reason, the ESD study group of KSGE held a nationwide tutoring program (7 provinces, 8 sessions) from January through October 2007. This program was divided into two sections and included a live demonstration of ESD in an *ex vivo* porcine stomach model and a hands-on training course using the same model. The well-irrigated porcine stomach was used in the experiment 24 h after the animal was slaughtered. The outlet portion of the stomach was closed with Kelly. A schematized frame was used for electrical patch attachment and water immersion was used for detection of air leakage. The inlet portion of the stomach was connected to the overtube and fixed within the frame (Figure 1). Before demonstrating the procedure, a mini lecture on the procedure was given to explain the procedure to the attendees. In the first section, tutors performed ESD with elaboration of the procedure. The endoscope was introduced through the overtube and the stomach was inflated with the endoscope. After selecting a target portion, the imaginary lesion was dissected after marking, injection and circumferential precutting. After completion of ESD, the integrity of the stomach wall was investigated by air insufflations with detection of leakage. In the second session, every participant performed this procedure once or twice under the supervision of the tutors. Although this program was not connected to the next step, such as *in-vivo* animal models or human studies, it permitted the trainee endoscopists to gain initial experience in performing this complex procedure and to accumulate knowledge on this therapeutic endoscopic technique. The previously mentioned annual live demonstrations by the KSGE provided valuable information for those who attended the training sessions and for those who led the courses.

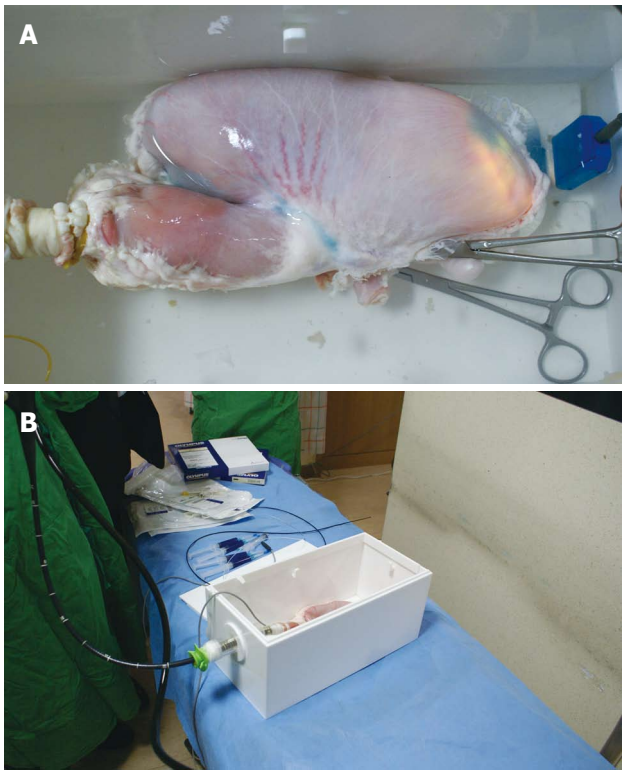

Figure 1 Porcine stomach model for endoscopic submucosal dissection. The stomach was immersed in water of the frame for detection of air leakage. The duodenum was sealed with Kelly. The electrical plate was attached to the bottom of the stomach. Bluish discoloration with light illumination was seen at the stomach antrum area (A); the endoscope was introduced through the overtube, which was fixed to the frame developed for endoscopic submucosal dissection tutoring (B).

JOURNAL REVIEW ON TEACHING AND LEARNING ESD

In Korea, a large volume of cases which need endoscopic therapy, especially in the stomach, enables endoscopists to extend their procedure from EMR to ESD step by step. Some early frontiers of ESD in Korea have accumulated their expertise as the EMR technique has evolved. EMR after circumferential precutting (EMR-P), which was first described as endoscopic resection with local injection of hypertonic saline-epinephrine (ERHSE) by Hirao *et al*[3] for superficial lesions less than 2 cm, was a common challenge towards the next step. With the EMR-P method, lesions are resected by a snare after circumferential precutting and *en bloc* resection of the lesion with low risk of complications is possible. For lesions less than 2 cm in diameter, the rates of *en bloc* resection and complications are comparable for EMR-P and ESD[4]. Choi *et al*[5] demonstrated a learning curve for the EMR-P technique, reporting an increase in the *en bloc* resection rate from 45% to 85% after 40 cases by summarizing their evolving self-taught experience on the EMR-P technique. There

were three perforations in the first 20 procedures (15%) and only one in the remaining 60 procedures (1.7%). They concluded that the trainee would need to perform 20-40 procedures to be able to use the technique safely and effectively. However, in the era of many ESD experts, the self-taught method of learning the EMR/ESD technique described in this study is unlikely to be acceptable as a good model for learning ESD[6].

In recognition of the complexity of ESD, the National Cancer Center Hospital in Japan, which is one of the highest-volume centers of ESD, has developed a rigorous training program. In this hospital, ESD is performed under the close supervision of an experienced endoscopist who offers advice and can complete the procedure when it is necessary for the benefit of the patient. In that setting, Gotoda *et al*[6] reported that experience of at least 30 cases is required for a beginner to gain early proficiency in this technique. In addition, they suggested that a major portion of the ESD training must be devoted to avoiding and managing its potential complications such as bleeding[7]. Recently, Yamamoto *et al*[8] reported a study on the assessment of the feasibility and learning curve in ESD performed by supervised residents. Before entry into this study, three supervised residents had experience of at least 1500 regular esophagogastroduodenal procedures and more than 10 EMRs. In addition, they had assisted ESD procedures performed by senior doctors for at least 1 year, and then attended a lecture on ESD techniques, using a manual and videos, by an experienced endoscopist. Each of them performed 30 consecutive ESD procedures for differentiated-type mucosal early gastric cancer without ulcers or scars, and smaller than 2 cm. Among the 90 procedures, there was a good overall complete resection rate of 93%, with an acceptable complication rate of 4.4%. The distribution of complete resection and complication rates were similar between operators. The self-completion rate and operation time were significantly worse for submucosal dissection than mucosal incision, which was mostly related to uncontrolled hemorrhage. Median operation time for mucosal incision did not change markedly and remained around 30 min for all operators. The median operation time for submucosal dissection became shorter than 30 min for one operator whose self-completion rate increased, but did not decrease for the other two operators. In this study, the authors stressed the importance of the assisting period. Trainees acquire the skills needed to troubleshoot various situations while assisting experienced endoscopists. Moreover, since most of the difficulties surrounding the procedure were related to uncontrollable hemorrhage, obtaining expertise in hemostasis before starting ESD is recommended. Kakushima *et al*[9] reported a learning curve for ESD for gastric epithelial neoplasms on the basis of the clinicopathological data from 383 ESD procedures by thirteen endoscopists. In study 1, the performance of the two principal operators (one performed 188 ESDs and the other performed 118 ESDs) was assessed every 25 cases. The endoscopist's experience did not affect either the treatment efficacy or the safety profile. However, the procedure time decreased with experience, regardless of the increase in lesion size and the increase in resected specimen size. In study 2, the performance of all thirteen operators (11 operators experienced fewer than 30 cases) was assessed according to their experience. There was no significant difference in treatment efficacy and complication rates between the operators throughout the study period. The lesions were mainly located in the lower part of the stomach in the procedures performed by the 11 less experienced endoscopists. The procedure times shortened as experience in the method increased. In this study, the authors were not able to demonstrate an optimal number of cases required to gain adequate experience. However, they suggested that a beginner could start to treat lesions in the lower part of the stomach independently after performing about 30 supervised ESD procedures.

One prospective study on ESD using a porcine model was reported in Hong Kong[10]. Before entry into the study, an ESD training workshop was held. It consisted of three components: (1) three days of an advanced intensive endoscopic course providing lectures on the basics of ESD; (2) a live demonstration of ESD performed for gastric, esophageal, and colonic lesions; and (3) a hands-on practical session of ESD using a porcine model under the supervision of local and overseas faculties. Twenty-four endoscopists were included and performed gastric and esophageal ESD using a porcine model. The mean procedural times were 52.1 ± 24.7 min for gastric ESD and 32.5 ± 8.5 min for esophageal ESD. Surprisingly, during gastric ESD, 15 participants (65.2%) encountered perforations, whereas bleeding occurred during 13 ESDs (56.5%). There were two procedure-related mortalities. The initial performances of ESD were associated with a high incidence of complications, the risk of which was not dependent on previous experience in endoscopy. The majority of the participants in this study agreed that the porcine model would be an appropriate simulation of human ESDs. Live porcine *ex vivo* or computer simulation models have gained much acceptance for training in upper endoscopy, and some evidence suggests that prior training with these simulation models enhances skill acquisition[11,12]. The use of simulation models may be helpful in attaining the skills required for safe performance of ESD, especially in the areas of a low volume of ESDs. From our experience and that of endoscopists in Palo Alto, California, USA[6] and Hong Kong[10], harvested porcine organs and live porcine models seem to provide a potential solution for learning ESD. Harvested porcine organs are a ready-to-use and inexpensive means of becoming proficient in a novel technique. Multiple large resections of the esophagus and the stomach may be practiced before the use of a live porcine model. The live model simulates a more realistic setting for endoscopic procedures and provides the opportunity to respond to and to treat potential complications such as bleeding and perforation. The use of models allows endoscopists to obtain knowledge in a relatively short time period, with a tutor on site. Further mentoring during the subsequent initial clinical experience would complement the animal model experience. Nevertheless, it

should be recognized that these simulation workshops are a means of augmenting training in skills in low-volume centers but will not replace patient-based training[11].

Vázquez-Sequeiros *et al*[13] from Spain reported their experience of learning and performing ESD in the absence of experts on ESD in their country. Four endoscopists with no experience in ESD underwent a four-step training program: (1) review of the literature and acquisition of theoretical concepts of ESD; (2) training in an *ex-vivo* animal model; (3) training in an *in-vivo* animal model; and (4) ESD of a gastric tumor in a patient. The four participants performed a total of 6 experiments using 6 porcine stomachs and esophagus for *ex-vivo* training. Six supervised ESDs were performed in a live porcine model under general anesthesia. After that, an ESD procedure in a patient was performed under general anesthesia in the operating room with a surgical team available. The procedure was successful but took quite a long time (210 min) and the resected specimen was 35 mm in size.

SUGGESTIONS ON TEACHING AND LEARNING ESD

It has been reported that closely supervised trainees can perform advanced surgery such as esophagogastrectomy, hepatectomy[14], or pancreatectomy[15] with similar outcomes to consultant surgeons. In those studies, surgeons with a large workload encouraged trainees to accept more opportunities to participate in such complex operations, with appropriate supervision, because this improved their learning of the surgical methods and did not jeopardize patient care. Similarly, proficiency in ESD cannot be achieved without the availability of a highly experienced supervisor[8], because a significant number of cases were not completed by the trainee alone and complications such as perforations were generally managed by the supervisor.

In Asian countries such as Korea and Japan where there is a large volume of early gastric lesions which need endoscopic treatment, endoscopists can learn ESD expanding their skill from EMR to ESD under the supervision of experts. Initial experience in ESD with a simulator model and accumulation of experience during the assisting period and supervised EMR-P and ESD procedures for easier sites such as the gastric antrum would be right step.

On the other hand, it is difficult to overcome a flat learning curve due to a low case volume in Western countries. According to previously published data, Western countries report a lower rate of complete resection and higher morbidity following ESD compared to most trials in Asian countries[14]. In these countries more time should be devoted to indirect learning with literature and videos. ESD videos are available at some web sites such as DAVE Project (http://daveproject.org/) and published papers with videos. When available, unedited full length videos of other operators may be more valuable for learning purposes. Attending live demonstrations or learning from a DVD which explains ESD technique (such as the one edited by the American Society of Gastrointestinal Endoscopy) is also helpful. In addition, the target lesion for ESD is different in Western countries and ESD could be more frequently used for resection of neoplasia in Barrett's esophagus or the colon due to their higher incidence compared with early gastric cancer[16,17]. Hence, *ex-vivo* and *in-vivo* training for esophageal and/or colonic ESD may be essential for Western trainees since ESD of these lesions is more difficult than gastric ESD[18].

CONCLUSION

ESD is a beneficial procedure which achieves higher rates of *en bloc* resection and complete resection for early cancer. However, training with a high enough volume to become proficient in ESD requires considerable time and patience especially in Western countries. A well structured training program is essential for the trainee, because the outcome of ESD is dependent on the experience of the endoscopist. Novice endoscopists can learn ESD by utilizing a simulation model, observing and/or assisting procedures performed by experts. The training course would be designed differently for Asian and Western countries according to the workload and incidence of disease. Close collaboration between Western and Asian countries will be helpful to improve ESD technique for various sites and to benefit patients who are suffering from early gastric, esophageal or colorectal cancer.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2623

World J Gastroenterol 2011 June 7; 17(21): 2623-2625
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Endoscopic submucosal dissection for early gastric cancer: Quo vadis?

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Received: June 29, 2010 Revised: September 2, 2010
Accepted: September 9, 2010
Published online: June 7, 2011

Abstract

The diagnosis of early gastric cancer (EGC) is of great interest because its endoscopic and surgical treatment presents the best chance for a cure. With technical development, endoscopic submucosal dissection (ESD) has been widely performed for the curative treatment of EGC in Korea. Multinational studies of ESD for EGC will be the next missions that overcome these limitations and global guidelines will be processed for ESD for EGC.

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Key words: Endoscopic submucosal dissection; Early gastric cancer

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Cho WY, Cho JY, Chung IK, Kim JI, Jang JS, Kim JH. Endoscopic submucosal dissection for early gastric cancer: Quo vadis? *World J Gastroenterol* 2011; 17(21): 2623-2625 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2623.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2623

INTRODUCTION

Gastrointestinal cancers represent the leading cause of cancer-related death worldwide. Prevalence and mortality rate of gastric cancer in malignant tumors is high in Asia, especially Korea and Japan. The diagnosis of early gastric cancer (EGC) is therefore of great interest because its endoscopic and surgical treatment presents the best chance for a cure. Advances in image-enhanced endoscopy allow improved visualization of lesions and can be used to gain insight into the pathology of the lesion, which, in turn, provides guidance to select the optimal treatment[1].

CURRENT STATUS OF ENDOSCOPIC SUBMUCOSAL DISSECTION IN KOREA

Prevalence of EGC is increased due to nationwide mass screening for gastric cancer in Korea, and patients have the opportunity to be treated with curative resection of the tumor by endoscopic therapy. This allows the patients to retain their organs and maintain their quality of life without surgical complications. Endoscopic mucosal resection (EMR) was performed in small-sized, differentiated mucosal EGC. But large scale surgical data reported the possibility of an increased indication of endoscopic resection, and technical and instrumental development enabled endoscopic submucosal dissection (ESD).

Since the early nineties, EMR has been performed as

a treatment modality of gastric neoplasia and ESD was first performed in Korea in 1999, with endoscopists performing ESD gradually. The Korean Society of Gastrointestinal Endoscopy (KSGE) organized an ESD research group in 2003 to discuss, investigate and spread ESD nationwide. Before 2006, only 22 hospitals had ESD facilities. The KSGE planned to hold a six session nationwide lecturing tour, with ESD hands-on courses to introduce the ESD procedure and devices with animal models. After that, the numbers strikingly increased in 2007, and the number of registered ESD facilities rose to 77 according to data from National Health Insurance Review & Assessment Service in 2008. Also, an annual international ESD live demonstration, *via* a telemedicine network, has been held since 2006, with more than a thousand endoscopists registered as audiences each year[2].

TROUBLESHOOTING THE LIMITATION OF ESD

Therapeutic and long-term outcomes of ESD for EGC were acceptable with absolute and expanded indications[3-6]. This revealed that, as described above, that ESD is a powerful technique with therapeutic efficacy for patients with EGC, which enables preservation of organs, increases the quality of life, and allows the complete removal of the primary tumor as an *en bloc* resection with a cancer cell-negative lateral and vertical margin regardless of the tumor location[7-9]. But, ESD has its limitations in that (1) it also cause additional gastrectomy if the depth of invasion is deeper than the SM2 layer, and (2) local resection can be less accurate at evaluating the exact status of lymphovascular invasion and lymph node metastasis than surgery. Current staging workup with endoscopic ultrasound, CT scan and PET-CT is also limited in its correct diagnosis of EGC[10-15]. Furthermore, gastric cancer generally shows greater histologic diversity than other types of cancer. Even tumors confined to the mucosa show histologic diversity, which tends to increase with deeper invasion and increased tumor diameter[16-19]. For these characteristics, additional gastrectomy was performed after pathologic mapping results of the ESD specimen revealed the possibility of lymph node metastasis. Endoscopists, surgeons and radiologists should discuss and overcome these situations to appropriate treatment for patients with EGC.

In Korea, the National Evidence-based Health Care Collaborating Agency and the KSGE have plans for prospective studies into the short term and long term clinical outcomes of EGC treated by ESD. More than eleven tertiary university-affiliated hospitals will be involved in this study. This will be the key to establishing when endoscopic treatment of EGC should be used.

EGC with potential node metastasis might also be treated by a laparoscopic lymph node dissection without a gastrectomy after ESD. Abe *et al*[20] previously demonstrated that this combination enabled the complete endoscopic resection of the primary tumor and histologic determination of lymph node status. However, remnant cancer cells in lymphatic and/or venous vessels in the gastric wall could potentially cause a cancer recurrence. Natural Orifice Transluminal Endoscopic Surgery (NOTES) has been applied to treat EGC with several case reports[21-25]. In Korea, Endoscopic Full-Thickness Gastric Resection (EFTGR) with laparoscopic lymph node dissection with hybrid NOTES has been performed and the data was reported to the NOSCAR conference in Boston, 2009. This consisted of five procedures; (1) marking around the lesion safety margin; (2) applying the ESD technique; a circumferential incision as deep as the submucosal layer was made around the lesion; (3) circumferential endoscopic full-thickness resection around the lesion through the submucosal incision line under laparoscopic guidance; (4) laparoscopic full-thickness resection around the remaining lesion through the EFTGR incision line inside the peritoneal cavity; and (5) laparoscopic closure of the resection margin. NOTES enables minimal tumor resection using the ESD technique, and a laparoscopic lymphadenectomy can be performed simultaneously during EGC, although there is a risk of lymph node metastasis. This procedure may be the bridge between ESD and gastric surgery[26].

CONCLUSION

In summary, ESD has become one of the mainstream methods for the treatment of EGC. Although long-term clinical outcomes of previous reports are promising, there still seem to be many obstacles to overcome in order to progress and stabilize the therapeutic range of endoscopic therapy. Multinational, prospective studies of therapeutic outcomes and survivals will be the next target that will overcome these limitations and global guidelines will be processed for ESD for EGC.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2626

World J Gastroenterol 2011 June 7; 17(21): 2626-2631
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Hepatobiliary scintigraphy for detecting biliary strictures after living donor liver transplantation

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Supported by The IN-Sung Foundation for Medical Research and Samsung Biomedical Research Institute, Grant No. SBRI C-B1-118-1
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Received: December 26, 2010 Revised: January 28, 2011
Accepted: February 4, 2011
Published online: June 7, 2011

Abstract

AIM: To investigate the diagnostic accuracy of hepatobiliary scintigraphy (HBS) in detecting biliary strictures in living donor liver transplantation (LDLT) patients.

METHODS: We retrospectively reviewed 104 adult LDLT recipients of the right hepatic lobe with duct-to-duct anastomosis, who underwent HBS and cholangiography. The HBS results were categorized as normal, parenchymal dysfunction, biliary obstruction, or bile leakage without re-interpretation. The presence of biliary strictures was determined by percutaneous cholangiography or endoscopic retrograde cholangiopancreatography (ERCP).

RESULTS: In 89 patients with biliary strictures, HBS showed biliary obstruction in 50 and no obstruction in 39, for a sensitivity of 56.2%. Of 15 patients with no biliary strictures, HBS showed no obstruction in 11, for a specificity of 73.3%. The positive predictive value (PPV) was 92.6% (50/54) and the negative predictive value (NPV) was 22% (11/50). We also analyzed the diagnostic accuracy of the change in bile duct size. The sensitivity, NPV, specificity, and PPV were 65.2%, 27.9%, 80% and 95%, respectively.

CONCLUSION: The absence of biliary obstruction on HBS is not reliable. Thus, when post-LDLT biliary strictures are suspected, early ERCP may be considered.

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Key words: Living donor liver transplantation; Tc99m mebrofenin; Radionucleotide imaging; Hepatobiliary scintigraphy; Biliary stricture

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Kim YJ, Lee KT, Jo YC, Lee KH, Lee JK, Joh JW, Kwon CHD. Hepatobiliary scintigraphy for detecting biliary strictures after living donor liver transplantation. *World J Gastroenterol* 2011; 17(21): 2626-2631 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2626.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2626

INTRODUCTION

Following the first adult-to-adult right lobe living donor liver transplantation (LDLT) in 1994[1], > 90% of organs for liver transplantation in Asian countries have come from live donations because of the shortage of cadaveric organ donation[2]. Even with the development in various refinements in surgical techniques, multiple biliary reconstruc-

tions and smaller bile ducts make right lobe LDLT difficult in comparison with cadaveric whole-size liver transplantation[2]. Although there has been overall improvement in the graft survival rate with advances in the organ preservation method and immunosuppressive management, the postoperative biliary complications have become the focus of concern.

A biliary stricture is one of the most common biliary complications following liver transplantation. The reported incidence of biliary strictures after right lobe LDLT is 28%-43%, which varies according to the type of biliary reconstruction, and with a higher incidence in patients undergoing duct-to-duct anastomosis[3-8]. The short-term consequences of biliary strictures are associated with cholangitis or sepsis, and long-term consequences are related to graft loss, re-transplantation, or even death[9].

Hepatobiliary scintigraphy (HBS) using technetium-99 attached to iminodiacetic acid is a physiological imaging method that is useful for detecting biliary obstruction[10]. There have been some studies to support the diagnostic role of hepatic iminodiacetic acid (HIDA) scans in liver-transplanted patients, mainly emphasizing that the abnormal HIDA scan results can predict the biliary complications[11-15]. The subjects in these studies were a relatively heterogeneous group of patients transplanted with different operative techniques (duct-to-duct or end-to-side anastomoses, cadaveric liver transplantation, or LDLT) and different biliary and non-biliary postoperative complications (biliary strictures, bilomas or bile leakage, and vascular compromise). Moreover, these studies were based on retrospective re-interpretations of the HIDA scans, and did not regard the usefulness of the HIDA scan in real clinical decision making. The purpose of the current study was to determine the usefulness of HIDA scans for detecting biliary strictures; specifically in patients with right lobe LDLT by duct-to-duct anastomosis.

MATERIALS AND METHODS

Patients and evaluation of biliary strictures

This study included 104 LDLT recipients who had HIDA scans and cholangiograms by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous cholangiography (PTC) for suspected biliary strictures between January 2001 and December 2009. The main clinical presentations that led to the evaluation were abnormal blood chemistry at the time of routine visits, or the appearance of symptomatic jaundice or cholangitis. Mebrofenin (bromo-2,4,6-trimethylacetanilidoiminodiacetic acid) was used as a radiopharmaceutical agent for HBS. All 104 LDLTs were performed using the right lobe of the living related donor, and the bile ducts were reconstructed by duct-to-duct anastomosis. The presence of significant biliary strictures on the cholangiogram was regarded as the diagnostic gold standard. We also made a diagnosis of biliary stricture only when a patient had showed improvement after endoscopic or percutaneous intervention, or biliary reconstructive surgery. We reviewed the medical records, and collected the clinical, radiological and laboratory data. In analysis of the ultrasonography (US) or computed tomography (CT) data, intrahepatic duct (IHD) dilatation of new onset or of progression in comparison with previous imaging studies was regarded as a significant change in bile duct size. The study protocol was approved by the Ethics Committee of Samsung Medical Center and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Classifications of HIDA scan results

Based on the nuclear physician's original interpretation, the results of HIDA scans were classified without retrospective re-interpretation. They were categorized as normal functioning grafts, parenchymal dysfunction, biliary obstruction, or bile leaks. A normal functioning graft was diagnosed when there was an immediate demonstration of hepatic parenchyma, followed sequentially by activity in the intra- and extrahepatic biliary duct system, gallbladder, and upper small bowel within 1 h[16]. Parenchymal dysfunction was considered to be present when abnormal hepatic uptake and/or abnormal excretion was noted. Scintigraphic diagnosis of biliary obstruction was made when bowel activity was not detected within 4 h, regardless of hepatic uptake. Bile leakage was diagnosed when accumulation of tracer was noted at an abnormal physiological site[12].

Statistical analysis

To describe the baseline patient characteristics, the mean ± SD was used for continuous variables with a normal distribution, and the median value was used for continuous variables without a normal distribution. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were used to investigate the accuracy as a diagnostic tool.

RESULTS

Baseline characteristics of the study subjects

Detailed baseline clinical characteristics of the study subjects are presented in Table 1. The mean age was 51.5 years, and there was a male predominance. Hepatic failure and hepatic malignancy constituted one-half of the indications for liver transplantation. Hepatitis B virus (HBV)-related hepatocellular carcinoma and HBV-related hepatic failure accounted for 39.4% and 31.7% of the cases, respectively. Other causes of hepatic failure not shown in Table 1 included drug-induced liver injury, chronic alcoholism, autoimmune hepatitis, and primary biliary cirrhosis. Other hepatic malignancies that led to LDLT were hepatocellular carcinoma associated with chronic alcoholism and hepatic angiosarcoma. The interval between LDLT and HIDA scan ranged between 25 and 1490 d (median: 294.5 d). Table 2 describes the baseline laboratory characteristics of the patients. All 104 patients had imaging studies (US or CT) for evaluation of biliary strictures, and 61 patients (58.7%) had significant changes in bile duct size. Cholangiograms were taken by

Table 1 Baseline clinical characteristics of the patients

Variable	Result
Age (yr), mean ± SD	51.5 ± 8.25
Male/female, *n* (%)	82/22 (79/21)
Indication for liver transplantation, *n* (%)	
Hepatic failure	52 (50)
HBV infection	33 (31.7)
HCV infection	5 (4.8)
Others	14 (13.5)
Malignancy	52 (50)
HCC, HBV-related	41 (39.4)
HCC, HCV-related	7 (6.7)
Others	4 (3.9)
Interval between LDLT and HIDA scan, d, median (range)	294.5 (25-1490)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; LDLT: Living donor liver transplantation; HIDA: Hepatic iminodiacetic acid.

Table 2 Baseline laboratory characteristics of the subjects

Laboratory test	Result
Bilirubin, mg/dL, median (range)	3.7 (0.6-44)
AST, U/L, median (range)	85 (15-336)
ALT, U/L, median (range)	132 (6-798)
ALP, U/L, median (range)	271 (65-988)
GGT, U/L, median (range)	297 (28-1491)
Change of bile duct size, *n* (%)	
Present	61 (58.7)
None	43 (41.3)
Stricture on the cholangiogram, *n* (%)	
Present	89 (85.6)
None	15 (14.4)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase.

ERCP in 56 patients (53.8%) and by PTC in 48 patients (46.2%), and a total of 89 patients (85.6%) had significant biliary strictures. Management of biliary stricture was successful using an endoscopic or percutaneous approach in 80 patients. However, interventions failed in nine patients and they required Roux-en-Y hepaticojejunostomy, eventually. The median value of the interval between the day of LDLT and the diagnosis of biliary stricture in 89 patients was 309 d (range: 37-1490 d).

Results of HIDA scans and cholangiograms

The results of HIDA scans and the presence of significant biliary strictures on the cholangiograms were matched, as in Table 3. Of 89 patients with biliary strictures, HIDA scans suggested a normal functioning liver graft (Figure 1) in six patients (6.7%), parenchymal dysfunction (Figure 2) in 32 (35.9%), biliary obstruction (Figure 3) in 50 (56.2%), and the presence of bile leakage in one (1.2%). The HIDA scans suggested the presence of biliary obstruction in four of 15 patients with no significant biliary strictures on cholangiograms as presented in Figure 4. All four patients with false-positive results on HIDA scans were confirmed to have allograft rejection after liver biopsy. Three of them received intravenous steroid pulse therapy

Table 3 Results of hepatic iminodiacetic acid scans and cholangiograms

	Normal	Parenchymal dysfunction	Biliary obstruction	Bilelea-kage	Total
	8 (7.7)	41 (39.4)	54 (51.9)	1 (1)	104 (100)
Stricture on cholangiogram	6	32	50	1	89
No stricture on cholangiogram	2	9	4	0	15

Data expressed as the number (%).

Table 4 Diagnostic accuracy of the change in bile duct size in detecting post-living donor liver transplantation biliary strictures

	Stricture on cholangiogram (+)	Stricture on cholangiogram (-)	Total
Change in bile duct size (+)	58	3	61
Change in bile duct size (-)	31	12	43
Total	89	15	104

and maintained graft function. However, one patient developed hepatic failure in spite of medical therapy, and he underwent re-transplantation.

Diagnostic accuracy of HIDA scans and changes in bile duct size to detect biliary strictures

The sensitivity, specificity, PPV, and NPV of HIDA scans to detect post-LDLT biliary strictures were 56.2% (50/89), 73.3% (11/15), 92.6% (50/54), and 22% (11/50), respectively. All 104 patients had imaging studies with US or CT in the evaluation process. When using the change in bile duct size on US or CT as a diagnostic tool (Table 4), the sensitivity, specificity, PPV, and NPV were 65.2% (58/89), 80% (12/15), 95% (58/61), and 27.9% (12/43).

DISCUSSION

Although there are various etiologies for liver function test abnormalities in liver transplant patients, rejection and biliary strictures are the most common and important causes of morbidity and mortality. These two conditions require different treatment strategies, so accurate differentiation is essential for proper management.

HBS using a radionuclide agent is a diagnostic imaging study that evaluates hepatocellular function and patency of the biliary system by tracing the production and flow of bile from the liver through the biliary system into the small intestine[16]. As a result of its non-invasiveness and convenience, HBS is commonly used to evaluate the presence of biliary strictures[3]. Even though older studies have concluded that HBS can differentiate intrahepatic cholestasis from pure hepatocyte damage in liver transplant patients[17,18], HBS cannot distinguish between cholestasis and rejection[18]. In the current study, the specificity, PPV, sensitivity, and NPV of HIDA scans in detecting post-LDLT biliary strictures were 73.3%, 92.6%, 56.2%, and 22%, respectively. Thus, HIDA

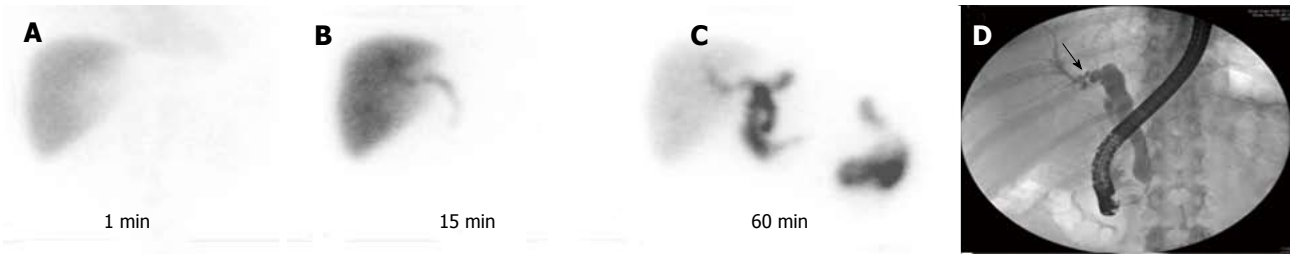

Figure 1 Hepatic iminodiacetic acid scan suggested normal graft function based on the normal hepatic uptake and normal excretion into the bile duct and bowel (A-C). However, the cholangiogram (D) obtained by endoscopic retrograde cholangiopancreatography showed a tight stricture (black arrow) at the anastomosis site.

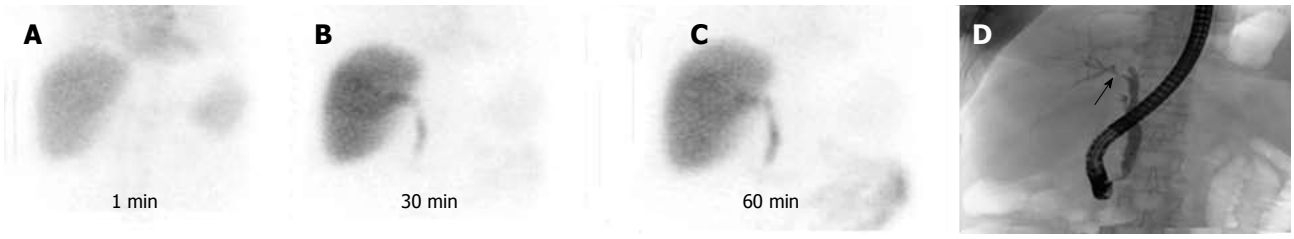

Figure 2 Hepatobiliary scintigraphy showed decreased hepatic uptake and normal excretion within 60 min, which suggested parenchymal dysfunction of the graft (A-C). However, the cholangiogram (D) revealed a significant anastomotic stricture (black arrow).

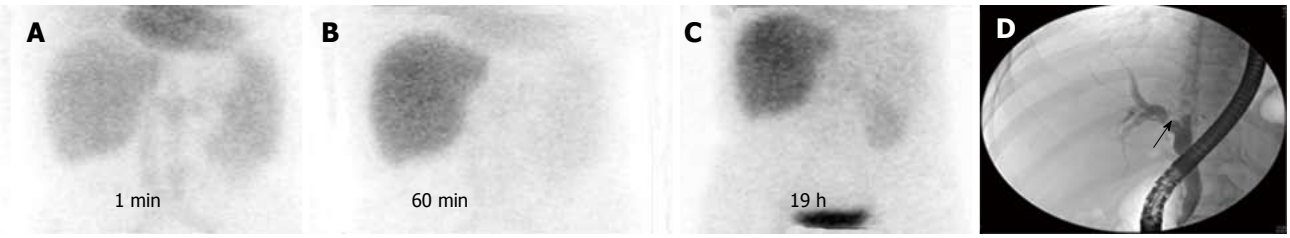

Figure 3 By Hepatic iminodiacetic acid scan, no bowel activity was noted after 19 h (A-C). Based on this finding, endoscopic retrograde cholangiopancreatography was performed (D), and a localized anastomotic stricture (black arrow) was demonstrated.

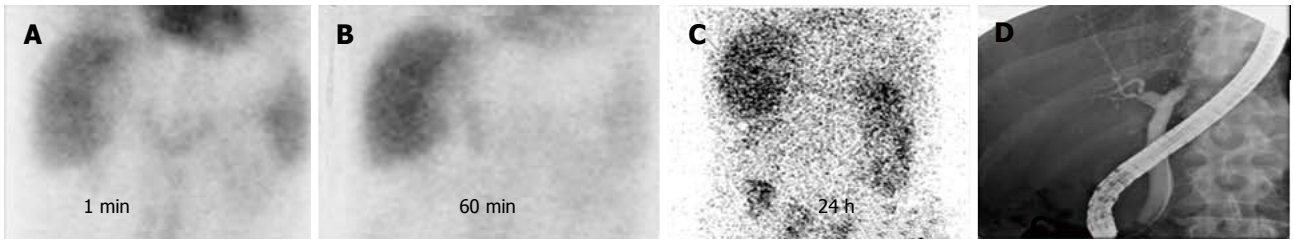

Figure 4 Hepatobiliary scintigraphy showed no clearance of the radiotracer into the bowel on the 24-h image (A-C), therefore, biliary obstruction was strongly suggested. However, no significant stricture was found on the cholangiogram (D).

scans cannot predict the presence of biliary obstruction in about 50% of the patients with significant biliary strictures. Surprisingly, six of eight patients with normal HIDA scan results were shown to have biliary strictures on cholangiograms. The normal HIDA scan results in patients with biliary strictures might be a reflection of post-LDLT biliary strictures that represent low-grade partial biliary obstruction. In the case of high-grade obstruction, there is usually prompt hepatic uptake, but no secretion of the radiotracer into biliary ducts after 4 h[12,13], and sometimes even after 24 h[10,16]. However, the typical scintigraphic imaging findings with low-grade or partial biliary obstruction include good hepatic uptake, normal secretion into the biliary ducts, and slightly delayed secretion into the bowel[10,16]. Another remarkable finding in the current study was that 32 of 41 patients who had parenchymal dysfunction on HIDA scans had biliary strictures on the cholangiograms. With prolonged biliary obstruction, concomitant hepatic dysfunction may occur, so decreased hepatic uptake of radionuclide trace can be seen prominently[16]. As shown in Table 3, four patients with no biliary strictures on cholangiograms had allograft rejection. Similar findings have been reported in previous

studies, and some patients with rejection and severely elevated serum bilirubin levels have scintigraphic findings of total biliary obstruction[12,17]. A possible explanation for this phenomenon is that the histological finding of rejection is characterized by the diagnostic triad of portal inflammatory mixed infiltrates, venous endotheliitis, and bile duct injury, and lack of hepatolysis as a dominant feature[19,20]. This result again confirms that HIDA scans do not have a role in the differential diagnosis of rejection and biliary strictures; the most important clinical situations.

Considering the relatively good PPV, the presence of biliary obstruction on HIDA scans can be quite reliable, and might offer a good rationale for clinicians proceeding to the next step (ERCP or PTC to confirm the presence of biliary strictures) and to initiate definitive treatment. The real problem in clinical practice occurs when the HIDA scan shows negative results, parenchymal dysfunction, or a normal functioning graft. This false negativity may guide the clinician to proceed to another diagnostic modality, most commonly a liver biopsy, and can create a delay in the proper diagnosis and management.

Cholangiography, either by ERCP or PTC, is considered to be the gold standard for post-liver transplantation biliary strictures, not only in establishing the diagnosis, but also in allowing therapeutic intervention in the same setting. ERCP has an advantage over PTC in that it is not only more physiological, but also less invasive. Moreover, recent studies have demonstrated an approximately 70% overall success rate for endoscopic treatment of post-LDLT biliary strictures with duct-to-duct anastomosis[5-9,21,22]. Biliary drainage with the percutaneous approach can be considered as a second option for cases not suitable for endoscopic management[22].

The suspicion for post-LDLT biliary strictures often begins with abnormal liver function tests or clinical evidence of obstructive cholangitis[9,23]. When a biliary stricture is suspected, imaging studies are commonly performed first because of non-invasiveness and convenience. The first step in establishing the diagnosis is usually US of the abdomen[9], which has the advantage that additional information about hepatic vascular patency is provided. However, US evaluation of the biliary tree is known to be of limited value because bile duct dilatation in liver transplant patients can be a non-specific postoperative finding, and the absence of dilatation has been noted to be an unreliable indicator of biliary obstruction[24]. The low sensitivity of US in evaluation of the biliary tree of 38%-66% has been the major drawback to US serving as a meaningful modality in the decision-making process[3]. However, these studies primarily reflect the outcomes during the period of cadaveric liver transplantation. There have been no recent data about the usefulness of change in bile duct size on US or CT in detecting post-transplantation biliary strictures in patients with LDLT. In the present study, to identify specifically clinically relevant changes in bile duct size, we only considered IHD dilatation of new onset or of marked progression. The sensitivity of this criterion was 65.2% and the NPV was 27.9%, which is still lower than that for a good diagnostic modality, but a higher rate than for HIDA scans. The specificity and the PPV were 80% and 95%, respectively, and again suggested that a significant change in bile duct caliber is mandatory to proceed in ERCP or PTC.

Recently, some studies have indicated that magnetic resonance cholangiography is a non-invasive and promising diagnostic modality with a sensitivity and specificity rate > 90%[25-27]. However, it has a limitation in that the therapeutic intervention is impossible, so cost-effectiveness is of concern. Also, it needs further validation in LDLT recipients by duct-to-duct anastomosis.

In conclusion, HIDA scans had relatively reliable specificity and PPV for detecting post-LDLT biliary strictures, but the sensitivity and NPV were poor. The absence of significant biliary obstruction on HBS cannot be a reliable marker for clinicians. Therefore, early performance of ERCP or PTC may be necessary to diagnose and treat biliary strictures, even though the HIDA scan results are negative, considering the successful outcome of ERCP or PTC in the treatment of biliary strictures.

COMMENTS

Background

Living donor liver transplantation (LDLT) has become the mainstream for liver transplantation to overcome the shortage of cadaveric donors. However, the higher risks or biliary complications remain the primary concern. Stricture is one of the most common biliary complications after LDLT.

Research frontiers

Hepatobiliary scan (HBS) is a diagnostic modality that is useful for detecting biliary obstruction and evaluating hepatocellular function. Several studies have evaluated the role of HBS for detecting biliary stricture in transplanted liver. However, the information is limited and insufficient because most of the studies were performed in the period of cadaveric LT.

Innovations and breakthroughs

To evaluate the role of HBS in real clinical settings, the authors included 104 patients who underwent LDLT and HBS. The HBS results were used without re-interpretation. Although HBS showed relatively reliable specificity and positive predictive value for detecting post-LDLT biliary strictures, the sensitivity and negative predictive value were poor. In particular, it did not distinguish between biliary stricture and rejection.

Applications

Even though the HBS results do not suggest the possibility of biliary obstruction, early performance of cholangiography may be necessary to diagnose and treat biliary strictures.

Peer review

The paper shows that scintigraphy is not a good indicator of modality in post-transplant biliary strictures.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2632

World J Gastroenterol 2011 June 7; 17(21): 2632-2640
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Keratinocyte growth factor gene therapy ameliorates ulcerative colitis in rats

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Supported by Postdoctoral Science Foundation of China, No. 20060390192, 200801243; and research grant from Science and Technology Department of Gansu Province, China, No. 0708NKCA128
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Received: October 5, 2010 Revised: March 23, 2011
Accepted: March 30, 2011
Published online: June 7, 2011

Abstract

AIM: To investigate the effect of keratinocyte growth factor (KGF) gene therapy in acetic acid-induced ulcerative colitis in rat model.

METHODS: The colitis of Sprague-Dawley rats was induced by intrarectal infusion of 1 mL 5% (v/v) acetic acid. Twenty-four hours after exposed to acetic acid, rats were divided into three experimental groups: control group, attenuated *Salmonella typhimurium* Ty21a strain (SP) group and SP strain carrying human *KGF* gene (SPK) group, and they were separately administered orally with 10% $NaHCO_3$, SP or SPK. Animals were sacrificed and colonic tissues were harvested respectively on day 3, 5, 7 and 10 after administration. Weights of rats, colonic weight/length ratio and stool score were evaluated. Histological changes of colonic tissues were examined by hematoxylin and eosin (HE) staining method. The expression of KGF, KGF receptor (KGFR) and TNF-α were measured either by enzyme-linked immunosorbent assay or Western blotting. Immunohistochemistry was used to detect the cellular localization of KGFR and Ki67. In addition, superoxide dismutase (SOD) activity and malondialdehyde (MDA) contents in the homogenate were measured.

RESULTS: Body weight and colonic weight/length ratio were declined in SPK group compared with SP and control groups (body weight: 272.78 ± 17.92 g *vs* 243.72 ± 14.02 g and 240.68 ± 12.63 g, $P < 0.01$; colonic weight/length ratio: 115.76 ± 7.47 *vs* 150.32 ± 5.99 and 153.67 ± 5.50 mg/cm, $P < 0.01$). Moreover, pathological changes of damaged colon were improved in SPK group as well. After administration of SPK strain, KGF expression increased markedly from the 3rd d, and remained at a high level till the 10th d. Furthermore, KGFR expression and Ki67 expression elevated, whereas TNF-α expression was inhibited in SPK group. In the group administered with SPK, SOD activity increased significantly (d 5: 26.18 ± 5.84 *vs* 18.12 ± 3.30 and 18.79 ± 4.74 U/mg, $P < 0.01$; d 7: 35.48 ± 3.35 *vs* 22.57 ± 3.44 and 21.69 ± 3.94 U/mg, $P < 0.01$; d 10: 46.10 ± 6.23 *vs* 25.35 ± 4.76 and 27.82 ± 6.42 U/mg, $P < 0.01$) and MDA contents decreased accordingly (d 7: 7.40 ± 0.88 *vs* 9.81 ± 1.21 and 10.45 ± 1.40 nmol/mg, $P < 0.01$; d 10: 4.36 ± 0.62 *vs* 8.41 ± 0.92 and 8.71 ± 1.27 nmol/mg, $P < 0.01$), compared with SP and control groups.

CONCLUSION: KGF gene therapy mediated by attenuated *Salmonella* ameliorates ulcerative colitis induced by acetic acids, and it may be a safe and effective treatment for ulcerative colitis.

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Key words: Keratinocyte growth factor; Ulcerative colitis; Gene therapy; Attenuated *Salmonella typhimurium*

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Liu CJ, Jin JD, Lv TD, Wu ZZ, Ha XQ. Keratinocyte growth factor gene therapy ameliorates ulcerative colitis in rats. *World J Gastroenterol* 2011; 17(21): 2632-2640 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2632.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2632

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder in gastrointestinal tract mainly involving ulcerative colitis (UC) and Crohn's disease (CD). It is characterized by tissue edema, inflammatory response and increased gut epithelial permeability[1,2]. Although many studies of IBD *in vivo* and *vitro* have been reported, the pathogenesis of IBD is still not completely understood. As it is reported, IBD may be related to a complex interaction of immune system, genetic and environmental factors[3,4]. Conventional drugs, such as corticosteroids and 5-aminosalicylate preparations, are effective in the treatment of IBD, however, long-term medication would induce severe side effects that have harmful impact on life quality of patients[5-7]. Hence, it is necessary to develop new approaches with fewer side effects for treatment of IBD.

Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (FGFs), could specifically stimulate proliferation and differentiation of various epithelial cells of different organs by a paracrine fashion including skin, lung, intestine and bladder[8]. In patients with IBD, which was mainly located in the lamina propria of gastrointestinal tract, endogenous KGF expression was increased markedly[9,10]. Previous studies indicated that KGF could promote proliferation and differentiation of intestinal epithelial cells both *in vivo* and *in vitro*, and protect the intestine[11,12]. Therefore, KGF may be a potential cytokine to ameliorate IBD. However, the recombinant protein of cytokines is limited in clinical application because of their instability, high dosage and repeated administration which increased the incidence of side effects[13]. We attempted to develop an oral gene therapy of KGF mediated by attenuated *Salmonella typhimurium* to improve the rat acetic acid-induced colitis, which has similar characteristics to human ulcerative colitis[14].

MATERIALS AND METHODS

Establishment of SPK and SP strains

Attenuated *Salmonella typhimurium* Ty21a strain (SP) and SP strain carrying human *KGF* gene (SPK) were constructed by electrotransformation with empty pCDNA3 plasmid (Invitrogen, Carlsbad, CA, USA) or recombinant pCDNA3 plasmid carrying human KGF gene.

Animals

Female Sprague-Dawley rats (aged 8-12 wk, n = 80, weighing 250-300 g), were purchased from the Animal Experimental Center of Lanzhou University (Lanzhou, China). They were housed in wire-bottom cages in a temperature-controlled room with a 12 h light-dark cycle. Rats were acclimatized to the environment for 7 d before experiment. All animal experiments were conducted following the institutional guidelines and approved by the Ethical Committee for Animal Care and Use, the General Hospital of Lanzhou Military Command.

Experimental protocol

Six rats were randomly assigned into normal group and 74 rats into experimental groups. Animals of experimental groups were anesthetized by intraperitoneal injection with pentobarbital sodium (30 mg/kg). A plastic catheter was inserted into the colon at 8 cm proximal to the anus, and 1 mL of 5% (v/v) acetic acid was infused into the rat colon. After 30 s, 2 mL saline was infused to remove the residual acid. Twenty-four hours after exposure to acetic acid, the rats were randomly divided into three groups: control group (n = 24), SP group (n = 25) and SPK group (n = 25). They were treated respectively with 1 mL solvent of 10% $NaHCO_3$, 1.0×10^8 colony forming unit (CFU) of SP strains, or SPK strains by gavage once every other day. Six rats of each group were sacrificed separately on day 3, 5, 7 and 10 after the first administration. The colons were quickly removed, freeze-clamped and dropped in liquid N_2 for various assays

Stool examination

The stool examination of each group was scored daily from day 1 to day 10 after drug administration. The score of stool was defined as 1 for normal, 2 for soft stool and 3 for watery diarrhea.

Histological examination

Histological analysis of rat tissues was performed on day 10. The colon tissues were fixed in 10% formalin, embedded in paraffin and sectioned at a thickness of 6 μm. Sections were stained with hematoxylin and eosin (HE) and evaluated under light microscope.

Detection of KGF and TNF-α by Enzyme-linked immunosorbent assay

The colon tissues were homogenized with nine-fold volume of ice-cold phosphate-buffered saline (PBS) using a glass homogenizer at 4℃. The homogenate was centrifuged at 855 × g for 10 min at 4℃ to remove the cell debris and the supernatant was obtained to determine the KGF and TNF-α content with Enzyme-linked immunosorbent assay (ELISA) kits (R and D, Minneapolis, MN, USA) according to the manufacturer's instructions. The optical density was detected at 450 nm with a microplate reader (Thermo, Pittsburgh, PA, USA).

Immunohistochemistry

Immunohistochemistry for KGFR and Ki67 was per-

formed as follows. The slides were incubated with a rabbit anti-rat primary antibody to KGFR (1:500, R and D, Minneapolis, MN, USA) or Ki67 (1:500, R and D, Minneapolis, MN, USA) in PBS at 4℃ overnight. After 3 washes in PBS, the sections were incubated with a horse radish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Beijing Zhongshan Biotechnology, Beijing, China) at a 1:1000 dilution in PBS at 37℃ for 2 h. The sections were then incubated with an avidin-biotin-peroxidase complex (Beijing Zhongshan Biotechnology, Beijing, China), followed by DAB (Beijing Zhongshan Biotechnology, Beijing, China) to induce a color reaction. The expression and localization of the KGFR and Ki67 were examined under light microscope (Olympus, Japan), and a brown color was indicated as positive.

Western blotting

To determine the effect of SPK strain on the KGFR in colon tissues, we detected the expression of KGFR using Western blotting techniques. The colonic homogenates were obtained as previously described, and the total protein in homogenates was quantified with the bicinchoninic acid (BCA) protein assay kit (Beijing Biosynthesis Biotechnology Beijing, China). Twenty μg of the total protein was resolved in SDS-PAGE, and protein was transferred onto PVDF membrane (Milipore, Temecula, CA, USA) electrophoretically at 80 mA for 2 h at 4℃. The membranes were blocked in 5% skim milk (w/v, in Tris-Buffered Saline with Tween-20 (TBST) for 2 h at room temperature. After 3 washes with TBST, the membranes were incubated with a rabbit anti-rat KGFR (1:500, R and D, Minneapolis, MN USA) and a rabbit anti-rat β-actin antibody (1:1000, R and D, Minneapolis, MN USA) overnight at 4℃, respectively. After 3 washes with TBST, the membranes were incubated with a HRP-conjugated goat anti-rabbit antibody (Beijing Zhongshan Biotechnology, Beijing, China) at a 1:2000 dilution for 3 h at 37℃. Bands on the membranes were imaged by X-ray with chemoluminescence reagents (Beijing Solarbio Science and Technology, Beijing, China). Finally, the bands were scanned and quantified using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Measurement of oxidative stress

The colonic homogenates were performed as mentioned above, and superoxide dismutase (SOD) activity and malondialdehyde (MDA) contents were determined by spectrophotometry according to the manufacturer's protocol. SOD and MDA detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis

The data from different groups at various time points were analyzed using one-way ANOVA with SPSS 11.5 software (IBM, Chicago, IL, USA). $P < 0.05$ was considered statistically significant, and data were presented as mean ± SD.

Table 1 Changes of body weight and colonic weight/length ratio

Group	Body weight (g)	Colonic weight/length ratio (mg/cm)
SPK	272.78 ± 17.92[a,b]	115.76 ± 7.47[a,b]
SP	243.72 ± 14.02	150.32 ± 5.99
Control	240.68 ± 12.63	153.67 ± 5.50
Normal	286.64 ± 18.95[a,b]	82.35 ± 4.63[a,b]

Data are presented as mean ± SD, $n = 6$. [a]$P < 0.05$, *vs* control group, [b]$P < 0.01$, *vs* SP group. SP: Attenuated *Salmonella typhimurium* Ty21a strain; SPK: Attenuated *Salmonella typhimurium* Ty21a strain carrying human *KGF* gene.

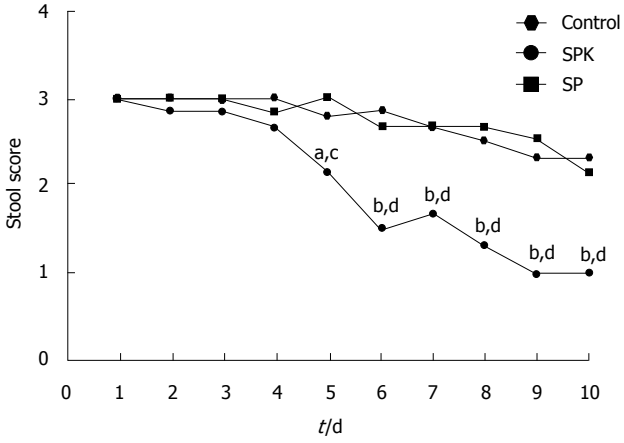

Figure 1 Stool score in each group after administration. Stool score decreased significantly on day 5 in SPK group, and recovered to normal on day 9. Data are presented as mean ± SD, $n = 6$. [a]$P < 0.05$, [b]$P < 0.01$ *vs* SP group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* control group at the indicated time point. SP: Attenuated *Salmonella typhimurium* Ty21a strain; SPK: Attenuated *Salmonella typhimurium* Ty21a strain carrying human *KGF* gene.

RESULTS

Changes of symptoms in rat ulcerative colitis

The rat body weight on day 10, as shown in Table 1, was significantly reduced ($P < 0.05$) in the SP and control groups, compared with the normal group. However, the body weight loss almost recovered in SPK group. The increase of the colonic weight/length ratio, an index of colonic edema associated with acetic acid-induced inflammatory reaction, was inhibited in SPK group compared with the SP and control groups ($P < 0.01$, Table 1). However, it was still higher than that in normal group ($P < 0.05$, Table 1).

Stool score

Diarrhea is one of the clinical signs of intestinal inflammation, so the effect of administration of SPK on the stool score was investigated. After treatment with acetic acid, diarrhea occurred in all groups on day 1. However, administration of SPK strain resulted in an obvious improvement of stool score on day 5 ($P < 0.05$, Figure 1), and a normal level was achieved on day 9 in SPK group.

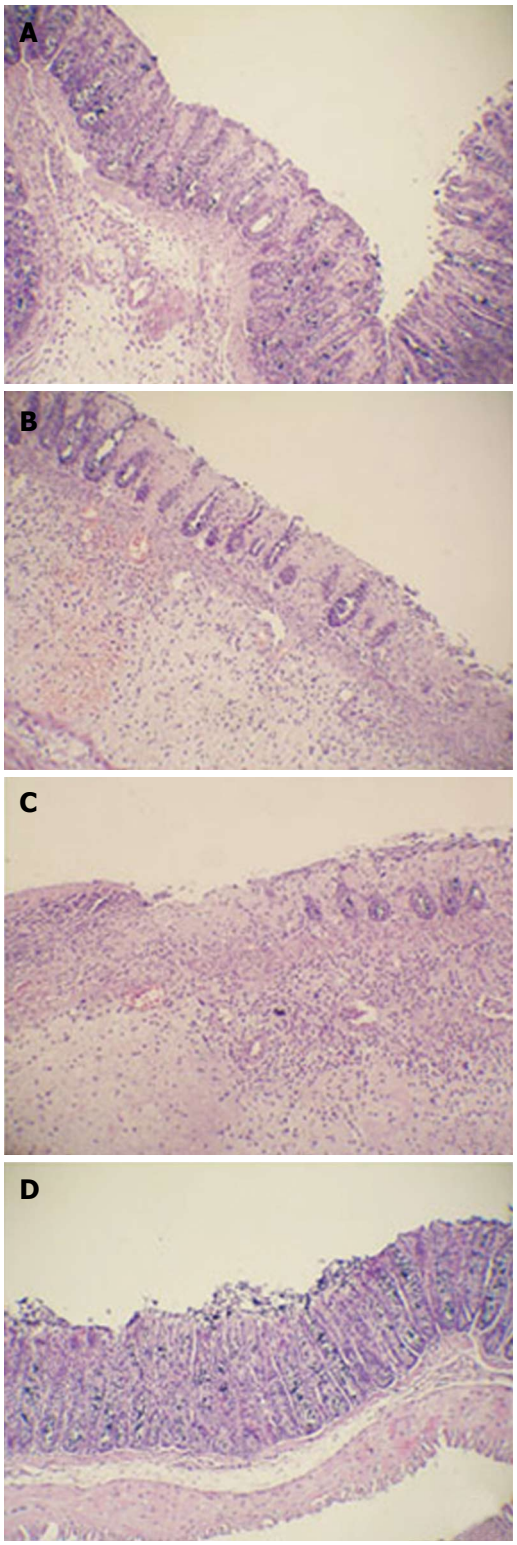

Figure 2 Histological sections of the colon on day 10 after treatment. Inflammatory cell infiltration and edema decreased evidently in attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene (SPK) group compared with attenuated *Salmonella typhimurium* Ty21a strain (SP) and control groups. Glands arranged more regularly and there was not obvious hyperemia in the lamina propria in SPK group. A: A representative colon from SPK group; B: A representative colon from SP group; C: A representative colon from control group. D: A representative colon from normal group (HE stain, × 200).

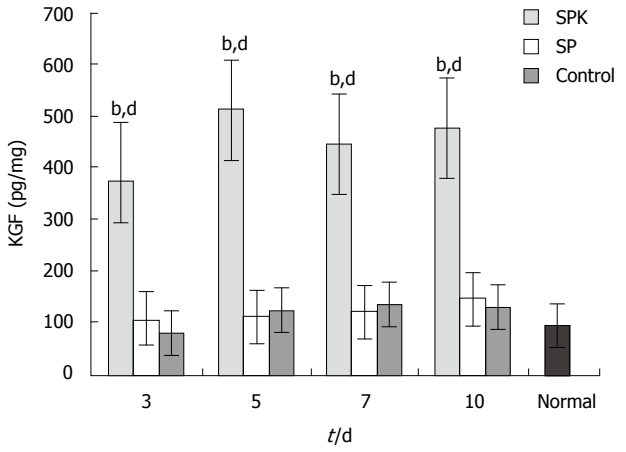

Figure 3 Keratinocyte growth factor concentrations in the homogenate of colon tissues. The concentration of keratinocyte growth factor (KGF) in the homogenate was measured by Enzyme-linked immunosorbent assay after administration, and the expression of KGF increased obviously in attenuated *Salmonella typhimurium* Ty21a strain carrying human *KGF* gene (SPK) group. Data are presented as mean ± SD, $n = 6$. $^{b}P < 0.01$ *vs* SP group at the same time point; $^{d}P < 0.01$ *vs* control group at the same time point. SP: Attenuated *Salmonella typhimurium* Ty21a strain.

Histological assessment

Histological sections were examined separately by two experienced observers. In SP and control groups, remarkable inflammatory cell infiltration and edema of the lamina propria were observed. The number of goblet cells dramatically decreased. Moreover, severely damaged mucous glands and thinner mucous layer were found in these two groups, and local hyperemia with thicker lamina propria was presented as well (Figure 2B and C). In contrast, the histological damage induced by acetic acid was improved by administration of SPK strain. Hyperemia was not found in the lamina propria, and inflammatory cell infiltration and edema were decreased in SPK group. The intestinal architecture recovered and glands arranged more regularly, as compared with the SP and control groups (Figure 2A).

Expression of KGF and KGFR in colon tissues

To investigate whether KGF could be expressed effectively in this gene therapy, the KGF concentration in the homogenates was measured by ELISA. In SPK group, the KGF concentration increased significantly at all time points, and peaked on day 5 (514.73 ± 103.30 pg/mg, Figure 3). The high level of KGF expression maintained between days 3 and 10. The result of high expression of KGF in SPK group demonstrated that *Salmonella typhimurium* Ty21a was an effective vector in the gene therapy on ulcerative colitis.

The effect of SPK on the expression of KGFR was confirmed by both Western blotting and immunohistochemistry. After exposure to acetic acid, the expression of KGFR in the colon tissues increased by more than 2-folds of the normal level (Figure 4). In SPK group, the expres-

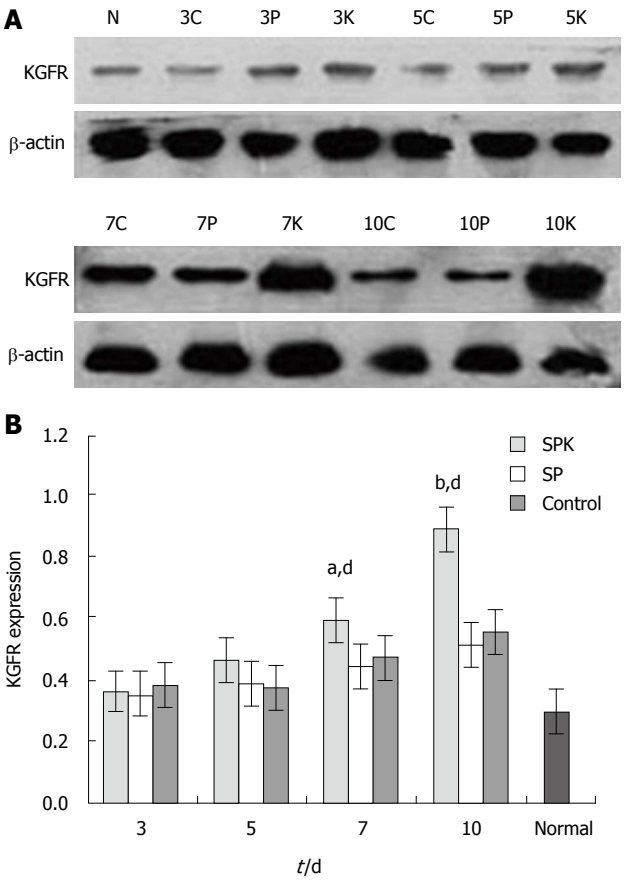

Figure 4 Western blotting of keratinocyte growth factor receptor in colon tissues. The expression of keratinocyte growth factor receptor (KGFR) in the colon tissues was measured by Western blotting after drug administration. The expression of KGFR on days 7 and 10 increased obviously in attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene group (SPK) compared with attenuated *Salmonella typhimurium* Ty21a strain (SP) and control groups. A: Western blotting analysis of KGFR. N: normal group; 3C, 3P and 3K represents control, SP and SPK groups, respectively, on day 3 of drug administration; 5C, 5P and 5K, on day 5; 7C, 7P and 7K, on day 7; 10 C, 10P and 10K, on day 10; B: Analysis of Western blotting of KGFR. The density of the bands was quantified using image-pro plus 6.0 software, and the data are presented as mean ± SD, $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$, *vs* SP group at the same time point.

sion of KGFR increased significantly on day 7 compared with the SP and control groups ($P < 0.05$, Figure 4B), and it increased by approximately 5-folds of the normal level on day 10 ($P < 0.01$, Figure 4B). As shown by immunohistochemistry, the expression of KGFR was located mainly in the epithelial lamina, and it was kept high in all groups, which was consistent with that detected by Western blotting (Figure 5).

Ki67 expression

To detect whether administration of SPK strain could promote intestinal epithelial cells proliferation, Ki67 expression, one of the markers of cell proliferation, was investigated by immunohistochemistry. Ten days after administration of SPK, the Ki67 expression in the epithelial lamina increased significantly compared with the SP and control groups (Figure 6).

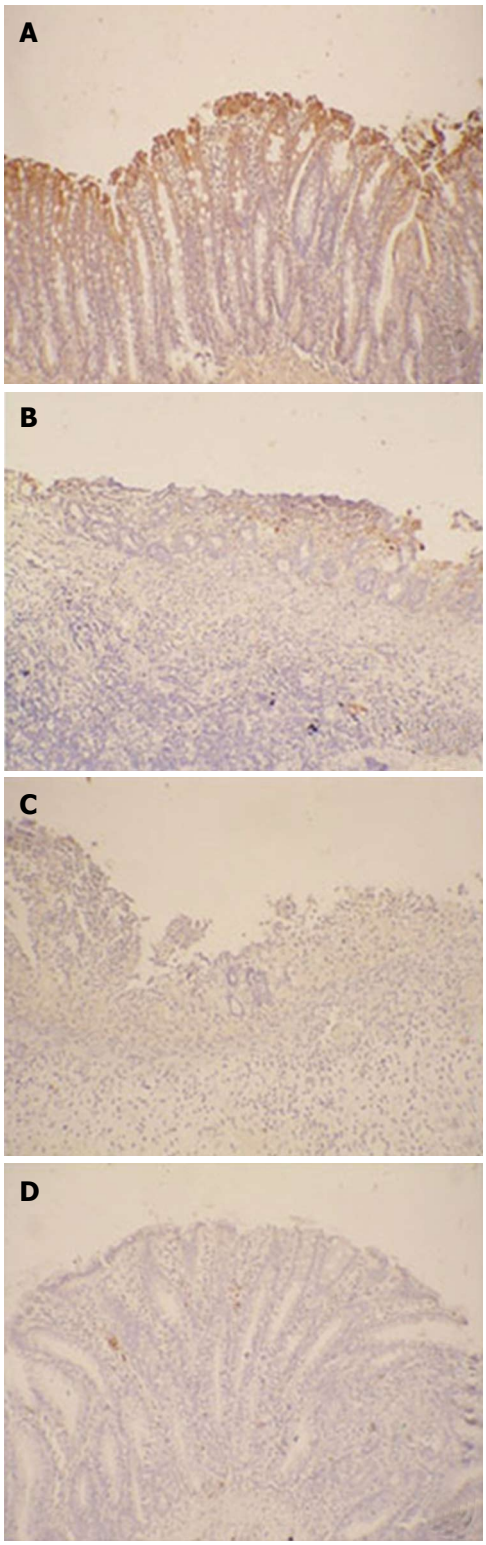

Figure 5 Immunohistochemistry of keratinocyte growth factor receptor in colon tissues. The expression of the keratinocyte growth factor receptor (KGFR) protein was confirmed by immunohistochemistry with a rabbit anti-rat KGFR antibody as the primary antibody and a horse radish peroxidase-conjugated goat anti-rabbit antibody as the secondary antibody, and the brown color was considered to be positive staining. The expression of KGFR elevated significantly in attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene (SPK) group which was consistent with the results of Western blotting, and KGFR is located mainly in epithelial lamina. A: Representative wound tissue from the SPK group; B: Representative wound tissue from attenuated *Salmonella typhimurium* Ty21a strain group; C: Representative wound tissue from control group; D: Representative wound tissue from normal group (× 200).

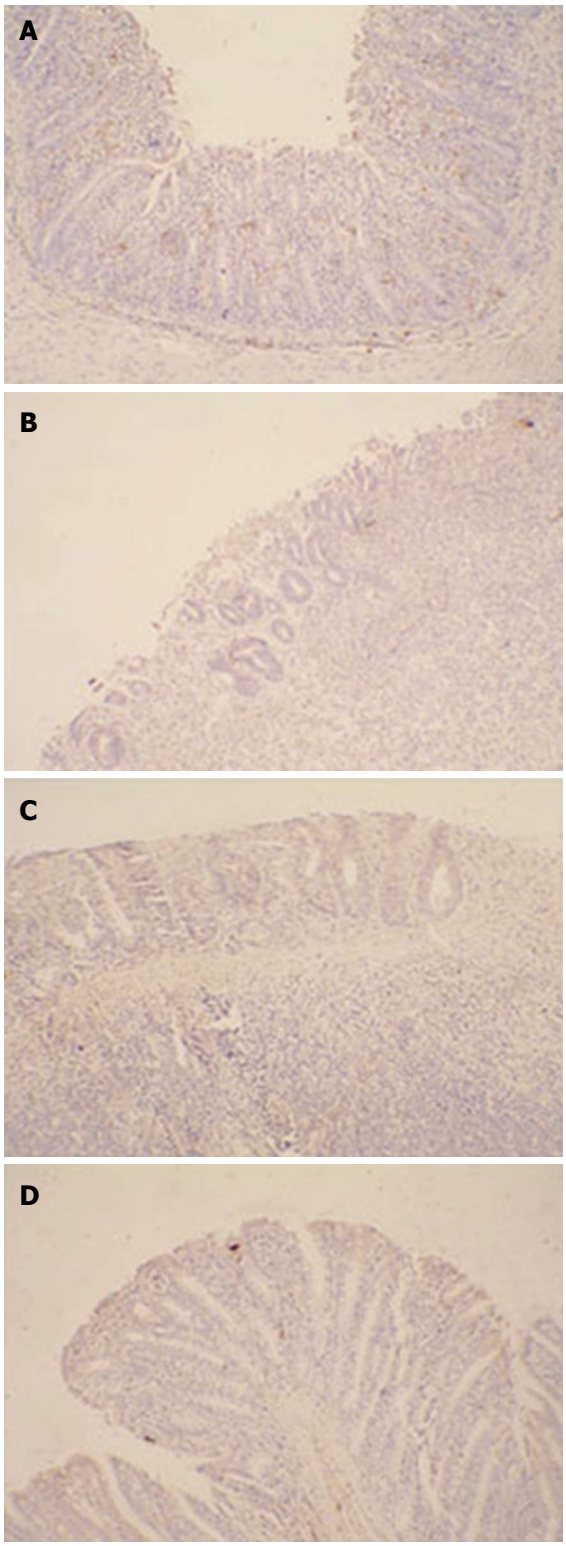

Figure 6 Immunohistochemistry of Ki67 in colon tissues. The expression of the Ki67 protein was detected by immunohistochemistry with a rabbit anti-rat Ki67 antibody as the primary antibody and a horse radish peroxidase-conjugated goat anti-rabbit antibody as the secondary antibody, and the brown color was considered to be positive staining. The expression of Ki67 in damaged colonic tissues increased evidently in the group administered with attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene (SPK) strain, indicating the proliferation of colonic epithelial cells. A: Representative wound tissue from SPK group; B: Representative wound tissue from attenuated *Salmonella typhimurium* Ty21a strain group; C: Representative wound tissue from control group; D: Representative wound tissue from normal group (× 200).

Table 2 Changes in superoxide dismutase activity (U/mg) in colon tissues

Group	D3	D5	D7	D10
SPK	19.92 ± 4.22	26.18 ± 5.84[b,d]	35.48 ± 3.35[b,d]	46.10 ± 6.23[b,d]
SP	18.29 ± 2.70	18.12 ± 3.30	22.57 ± 3.44	25.35 ± 4.76
Control	19.08 ± 3.57	18.79 ± 4.74	21.69 ± 3.94	27.82 ± 6.42
Normal	57.22 ± 4.03			

Data are presented as mean ± SD, $n = 6$. [b]$P < 0.01$, *vs* SP groups, [d]$P < 0.01$, *vs* control group at the indicated time points. SP: Attenuated *Salmonella typhimurium* Ty21a strain; SPK: Attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene.

Table 3 Changes in malondialdehyde contents (nmol/mg) in colon tissues

Group	D3	D5	D7	D10
SPK	11.40 ± 0.92	10.33 ± 1.09	7.40 ± 0.88[b,d]	4.36 ± 0.62[b,d]
SP	10.99 ± 0.73	10.96 ± 1.02	9.81 ± 1.21	8.41 ± 0.92
Control	11.37 ± 1.23	11.03 ± 0.87	10.45 ± 1.40	8.71 ± 1.27
Normal	2.54 ± 0.67			

Data are presented as mean ± SD, $n = 6$. [b]$P < 0.01$, *vs* SP and [d]$P < 0.01$, *vs* control group at the indicated time point. SP: Attenuated *Salmonella typhimurium* Ty21a strain; SPK: Attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene.

Changes in SOD activity and MDA contents in colon tissues

As revealed in Table 2, the decreased SOD activity induced by acetic acid was inhibited significantly by administration of SPK strain on day 5 ($P < 0.01$), and the SOD activity of SPK group became similar to the normal group on the 10th d. MDA contents increased significantly due to the inhibition of SOD activity induced by acetic acid, and reached to 4-folds approximately of the normal level (Table 3). However, administration of SPK strain resulted in a remarkable decrease of MDA contents ($P < 0.01$, Table 3) on day 7, compared with the SP group and control group. The decrease of MDA contents maintained until day 10, although they were still higher than normal levels ($P < 0.01$, Table 3).

Expression of TNF-α in colon tissues

To examine the effect of SPK strain on the expression of proinflammatory cytokine, the TNF-α level in colon was measured by ELISA. The TNF-α level of colon tissues elevated dramatically after exposure to acetic acid (Figure 7). However, administration of SPK strain significantly inhibited the overexpression of TNF-α between days 5 and 10, as compared with the SP and control groups ($P < 0.01$, Figure 7).

DISCUSSION

Ulcerative colitis is one of the refractory diseases, and there are still no specific and ideal drugs and therapies for this disease. In this study, we focused on the gene therapy of KGF mediated by attenuated *Salmonella typhimurium*

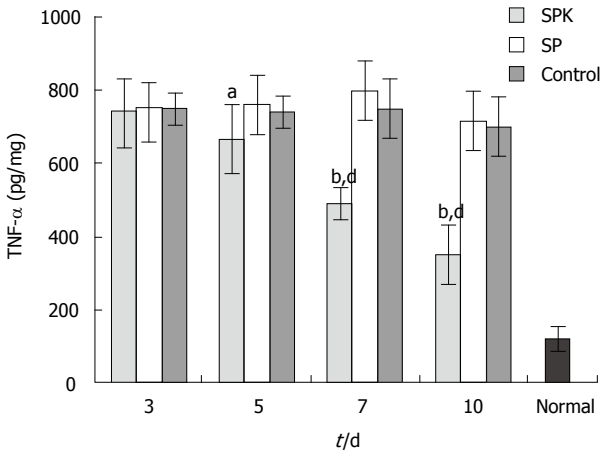

Figure 7 Tumor necrosis factor-α concentration in homogenate of colon tissues. The concentration of tumor necrosis factor (TNF)-α in the homogenate was measured by enzyme-linked immunosorbent assay after administration. The expression of TNF-α decreased significantly in attenuated *Salmonella typhimurium* Ty21a strain carrying human keratinocyte growth factor gene (SPK) group from day 5 to day 10. Data are presented as mean ± SD, $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* attenuated *Salmonella typhimurium* Ty21a strain (SP) group; $^{d}P < 0.01$ *vs* control group at the same time point.

Ty21a. Our results showed that oral administration of SPK strain ameliorated the colitis induced by acetic acid in rat model. Furthermore, no side effects induced by administration *Salmonella typhimurium* Ty21a, were detected. This gene therapy is potential for the treatment of ulcerative colitis.

Keratinocyte growth factor receptor (KGFR/FGFR2-ⅢⅠb), a splice variant of FGFR2, is a transmembrane tyrosine kinase receptor encoded by the *bek* gene[15]. KGFR, with a high affinity to KGF, expresses mainly in epithelial cell lineages, so KGF specifically stimulates epithelial cells *via* binding with KGFR. KGF promotes the intestinal epithelial cell proliferation and differentiation in a paracrine fashion[11]. KGF may help maintain and restore the integrity of the intestinal mucosa after injury[16]. And local administration of KGF could alleviate the inflammatory and promote the re-epithelialization and adaptative process after proctocolectomy[10]. In addition, the expression of KGF and KGFR in the intestinal tissues of IBD patients was enhanced significantly [16], suggesting that the interaction of KGF and KGFR may play a crucial role in the progress of IBD. In this study, we found that the expression of KGF increased remarkably compared with the control group at all time points after administration of SPK strain, and the expression of KGFR in SPK group also elevated obviously on day 7. The expression of Ki67, one of the markers of cell proliferation[17], in the epithelial lamina increased in SPK group, which indicated that KGF might directly promote the restoration of intestinal epithelial cells *via* binding with KGFR in the ulcerative colitis.

Many factors are involved in the process of the pathogenesis of IBD, although the accurate mechanisms are still unclear. The damage induced by reactive oxygen species (ROS) is one of the important factors. Accumulation of ROS in ulcerative colon tissues stimulates inflammation responses and secretion of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6[18,19]. ROS also impairs the integrity of the intestinal epithelial cells and increases the intestinal mucosal permeability, which subsequently attenuates the barrier function and host defense to exogenous bacteria and microorganisms[20,21]. In addition, ROS could induce DNA damage and stimulate activation of NF-κB that plays an important role in inflammation responses[19,22]. As it was reported previously, ROS levels increased significantly in IBD patients[23]. There are two main reasons for the ROS increase. The first one is that the release of ROS from lymphocytes, macrophages and neutrophils increases in the process of IBD; and the second one is the endogenous antioxidant enzymes, such as SOD and GSH, reduce and lead to the accumulation of ROS[22]. SOD is able to counteract ROS by catalyzing ROS to oxygen (O_2) and hydrogen peroxide (H_2O_2) which is subsequently decomposed to water (H_2O) and oxygen (O_2)[24]. At present, many therapies based on SOD have been applied in treatment of IBD. Suzuki *et al*[25] reported that PC-SOD (40 mg or 80 mg daily) is able to improve UC rapidly. In addition, SOD gene therapy mediated by *Lactobacillus gasser* also ameliorates the IBD in IL10-deficient mice *via* reducing the infiltration[26].

The present study showed that the SOD activity increased significantly on the 5th d after administration of SPK strain compared with the SP and control groups ($P < 0.01$, Table 2). The increase of SOD activity may be associated with KGF-induced promotion of the proliferation and repairing of intestinal epithelial cells[16], which secreted SOD in regenerative intestinal tissues[26]. MDA, one of the direct products of ROS, induces lipid peroxidation and its contents are usually used as a marker for free radicals-induced lipid peroxidation[19]. In this study, MDA contents in SPK group decreased significantly on day 7, accompanied with the increase of SOD activity. These results demonstrated that administration of SPK strain could protect intestinal tissues from damages induced by ROS.

TNF-α, mainly from activated macrophages in the gastrointestinal tract, plays an important role in the pathogenesis of IBD. It directly induces apoptosis of epithelial cells, promotes production of oxygen free radicals (ROS), interferes with the intestinal epithelial barrier, activates neutrophils and macrophages, and causes inflammation as well[27,28]. Moreover, by activating transcription factor nuclear factor κB (NF-κB) signal pathway, TNF-α indirectly induces production and secretion of many inflammatory cytokines and chemokines involving IL-6, IL-12, IL-18, IFN-γ and MMPs[27,29,30]. The expression of TNF-α in IBD patients increases dramatically, and Infliximab, an anti-TNF-α monoclonal antibody, has been applied clinically to treat Crohn's disease and ulcerative colitis[31]. The TNF-α levels in colon tissues are often used as a biomarker of the severity of colitis[29]. Similarly, the expression of TNF-α increased dramatically in the damaged

colonic tissues induced by acetic acids in this study. However, in SPK group, TNF-α level decreased significantly on day 7, which demonstrated that administration of SPK strain could ameliorate rat ulcerative colitis resulting from inhibition of TNF-α secretion. It has been reported that TNF-α could induce excessive expression of KGF in the intestinal tissues[32]. KGF, in return, could down-regulate the TNF-α expression in the lung or intestinal tract injury induced by bone marrow transplantation[33,34]. This is consistent with the results in this study. All these suggest that there may be a negative feedback mechanism between TNF-α and KGF.

Attenuated *Salmonella typhimurium* is one of the prevalent bacterial vectors in gene therapy and oral DNA vaccines, which could transfer multiple exogenous gene into host cells effectively[35,36]. *In vivo*, live attenuated *Salmonella* strains penetrate into the intestinal epithelial barrier *via* M cells and macrophages, and reach the Peyer's patches, where the targeting gene would be expressed. The exogenous gene would subsequently be expressed in the host cells[37]. Attenuated *Salmonella* has been proved to be a simple, effective and safe vector in gene therapy[36,38]. In addition, production of bacteria is relatively simple and low in cost compared with other vectors, such as virus and liposome. We chose the attenuated *Salmonella typhimurium* Ty21a as the vector of KGF gene therapy, an aroA mutant strain that is safe and effective for human and has been approved for human use by the U.S. FDA[39,40]. In the present study, no adverse effects were observed in the animals administrated orally with SPK and SP strain. Therefore, this attenuated *Salmonella* strain is safe for treatment of ulcerative colitis.

In conclusion, the oral gene therapy of KGF could improve the colitis physiologically and pathologically in rat model and this therapy may be a potential, safe and efficient for ulcerative colitis.

ACKNOWLEDGMENTS

We thank Professor Zhong-Xiong Tang for his critical reading and revising the manuscript.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder in gastrointestinal tract mainly involving ulcerative colitis (UC) and Crohn's disease (CD). It is characterized by tissue edema, inflammation and diarrhea. Although conventional drugs, such as corticosteroids and 5-aminosalicylate preparations, are effective in the treatment of IBD, long-term medication would induce severe side effects, affecting the life quality of patients. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (FGFs), could specifically stimulate proliferation and differentiation of various epithelial cells of different organs including skin, lung, intestine and bladder. So, the authors hypothesized that KGF could repair the damaged intestinal mucosa and relieve the inflammation.

Research frontiers

Previous studies indicated that KGF could promote proliferation and differentiation of intestinal epithelial cells both *in vivo* and *in vitro*, and protect the intestine. However, the recombinant proteins of cytokines are limited in clinical usage because of their instability.

Innovations and breakthroughs

To avoid the disadvantages of protein drugs, the authors attempted to develop an oral gene therapy of KGF mediated by attenuated *Salmonella typhimurium* to improve the ulcerative colitis in rats with acetic acid-induced colitis.

Applications

The oral gene therapy of KGF could improve the colitis physiologically and pathologically in rat model, and this therapy may be potential, safe and efficient for ulcerative colitis.

Peer review

Oral administration of the attenuated *Salmonella typhimurium* Ty21a strain carrying *KGF* gene could effectively relieve the ulcerative colitis induced by acetic acid, through promoting the proliferation of intestinal mucosal cells, inhibiting the expression of tumor necrosis factor-α, a inflammatory factor, and protecting the intestinal tissues from the damage of reactive oxygen species.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2641

World J Gastroenterol 2011 June 7; 17(21): 2641-2645
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Chronic constipation: Facilitator factor for development of varicocele

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Received: July 29, 2010 Revised: September 25, 2010
Accepted: October 2, 2010
Published online: June 7, 2011

Abstract

AIM: To evaluate the possible relationship between varicocele and chronic constipation.

METHODS: Between April 2009 and May 2010, a total of 135 patients with varicocele or constipation and 120 healthy controls were evaluated. Patients were divided into two groups. In both groups detailed medical history was taken and all patients were examined physically by the same urologist and gastroenterologist. All of them were evaluated by color Doppler ultrasonography. All patients with constipation, except for the healthy controls of the second group, underwent a colonoscopy to identify the etiology of the constipation. In the first group, we determined the rate of chronic constipation in patients with varicocele and in the second group, the rate of varicocele in patients with chronic constipation. In both groups, the rate of the disease was compared with age-matched healthy controls. In the second group, the results of colonoscopies in the patients with chronic constipations were also evaluated.

RESULTS: In the first group, mean age of the study and control groups were 22.9 ± 4.47 and 21.8 ± 7.21 years, respectively ($P < 0.05$). In the second group, mean age of the study and control groups were 52.8 ± 33.3 and 51.7 ± 54.3 years, respectively ($P < 0.05$). In the first group, chronic constipation was observed in 8 of the 69 patients with varicocele (11.6%) and 3 out of 60 in healthy controls (5%), respectively. In this regard, there was no statistical significance between varicocele patients and the healthy control ($P = 0.37$). In the second group, varicocele was observed in 16 of the 66 patients with chronic constipation (24.24%) and 12 out of 60 in healthy controls (20%) respectively. Similarly, there was no statistical significance between chronic constipation and healthy controls ($P = 0.72$). Internal/external hemorrhoids were detected in 4 of the 16 patients with chronic constipation and varicocele, in the second group. In the remaining 50 patients with chronic constipation 9 had internal/external hemorrhoids. In this regard, there was no statistical significance between chronic constipation and healthy controls ($P = 0.80$).

CONCLUSION: Chronic constipation may not be a major predictive factor for the development of varicocele, but it may be a facilitator factor for varicocele.

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Key words: Varicocele; Chronic constipation; Hemorrhoid; Intra-abdominal pressure; Relationship

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Kilciler G, Sancaktutar AA, Avcı A, Kilciler M, Kaya E, Dayanc M. Chronic constipation: Facilitator factor for development of varicocele. *World J Gastroenterol* 2011; 17(21): 2641-2645 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2641.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2641

INTRODUCTION

Varicocele, the most common surgically correctible cause of male infertility, is characterized as abnormal tortuosity and dilation of the pampiniform plexus within the spermatic cord[1]. Although the exact etiology of varicocele is not known[2], the pathogenesis may be associated with various factors resulting in increased retrograde blood flow or increased pressure in the pampiniform plexus and internal spermatic vein[3].

Constipation is a common, often chronic, gastrointestinal motility disorder described by such symptoms as straining, difficult stool, and infrequent defecation[4]. The prevalence in children ranges from 0.7% to 30%. In adults, constipation affects between 2% and 28% of the general population[5].

In this study we aimed to evaluate the possible relationship between varicocele and chronic constipation.

MATERIALS AND METHODS

Between April 2009 and May 2010, a total of 135 patients with varicocele or constipation and 120 healthy controls were evaluated. Patients were divided into two groups.

The first group was consisted of 69 patients with left varicocele and 60 age-matched healthy volunteers, and the second group was consisted of 66 patients with chronic constipation and 60 age-matched healthy volunteers.

Patients who were admitted to the outpatient clinic with complaints of scrotal pain and/or infertility were requited in the first group. In this group, the age-matched healthy controls were the people who were admitted to the outpatient clinic with no varicocele.

In the second group, patients diagnosed with chronic constipation by a gastroenterologist were consulted to the urology outpatient clinic and examined for varicocele. The age-matched healthy controls were the people who were admitted to the outpatient gastroenterology clinic with no constipation.

In both groups a detailed medical history was taken and all patients were examined physically by the same urologist and gastroenterologist in attention to those associated with varicocele and constipation. Afterwards, all of them underwent a standard gray scale and color Doppler ultrasonographic examination of the scrotum, which was performed by the same radiologist. For the ultrasonographic examination, a color Doppler scanner (GE Logic 9, Milwaukee, Wisconsin, USA) equipped with a 5- to 10-MHz linear transducer was used. The transverse diameter of the biggest vein in the pampiniform plexus was measured 3 times by a transducer probe with a frequency of 7.5-10 MHz during normal breathing, and the Valsalva maneuver. The arithmetic mean value of the diameters measured was used for the assessment. Patients with a varicose spermatic vein larger than 2.0 mm or having reversal flow during the Valsalva maneuver were included in the study group as a varicocele patient.

In the second group, patients were included according to the scope of the Rome Ⅲ criteria. The Rome Ⅲ criteria system was developed to classify functional gastrointestinal disorders based on clinical symptoms.

In this regard, patients who had straining and incomplete evacuation and hard stools more than 25% of the time, as well fewer than 3 bowel actions in a week were defined as "chronically constipated". The patients were questioned in terms of systemic diseases and other medications. Anal tonometry was performed to assess the colonic transit time. Colonoscopy and anoscopy was performed in the same session. The presence of hemorrhoid was confirmed. The patients in whom no reason for constipation was detected by colonic transit, anorectal manometry, anoscopy or colonoscopy studies, were enrolled in the study. The hemorrhoid was diagnosed by anoscopy. The other studies were performed for the other etiologies except hemorrhoids. The control group of Group-2 consisted of patients who underwent colonoscopy because of gastrointestinal symptoms other than constipation. All colonoscopies were performed by the same gastroenterologist with a Olympus Q160 AL 160 cm video colonoscope. During the colonoscopy 4 mg midazolam were given to all the patients for anesthesia.

Patients with drug use such as diuretics, hypotensive drugs, anticonvulsants anal fistulas and abscesses, and neurogenic and endocrine disorders associated with constipation such as paraplegia and hypothyroidism, were excluded. Due to the possible effects to spermatic veins, patients with a past medical history of inguinal or scrotal surgery were excluded from the study.

In the first group, we determined the rate of chronic constipation in patients with varicocele and in the second group, the rate of varicocele in patients with chronic constipation. In both groups, the rates of these diseases were compared with age-matched healthy controls. In the second group, the results of colonoscopies in the patients with chronic constipations were also evaluated.

Informed consent was obtained from all participants, and the study was approved by the local Ethical Committee.

SPSS for Windows Version 15 was used for statistical analysis. Statistical analysis was based on χ^2 test. Results are reported as mean ± SD where $P < 0.05$ was considered to be statistically significant.

RESULTS

In the first group, mean age of the study and control groups were 22.9 ± 4.47 and 21.8 ± 7.21 years, respectively. There was no statistical significance between the mean ages of the study and control groups ($P = 0.83$).

In the second group, mean age of the study and control groups were 52.8 ± 33.3 and 51.7 ± 54.3 years, respectively. There was no statistical significance between

Table 1 Constipation rates in the first group

Group	Varicocele group (n = 69)	Control group (n = 60)	P value
Constipation			
(+)	8	3	
(-)	61	57	0.37

Table 2 Varicocele rates in the second group

Group	Constipation group (n = 66)	Control group (n = 60)	P value
Varicocele			
(+)	16	12	
(-)	50	48	0.72

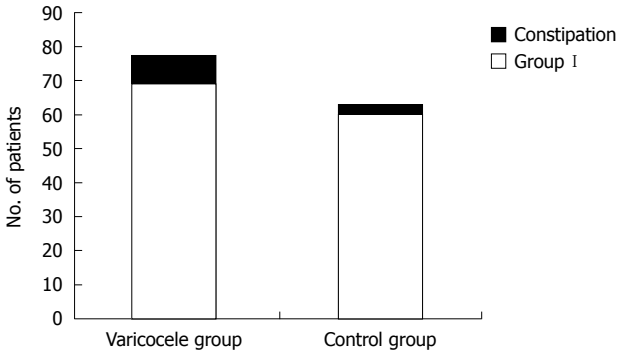

Figure 1 Constipation rates in the first group in graph.

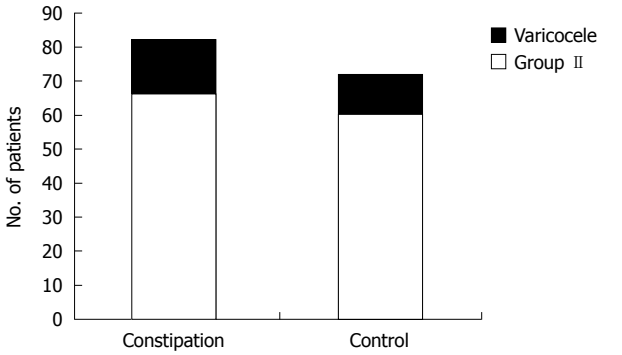

Figure 2 Varicocele rates in the second group in graph.

the mean ages of the study and control groups (P = 0.78).

In the first group, chronic constipation were observed in 8 of the 69 patients with varicocele (11.6%) and 3 of the 60 in healthy controls (5%), respectively. In this regard, there was no statistical significance between varicocele patients and healthy control (P = 0.37) (Table 1 and Figure 1).

In the second group, varicocele was observed in 16 of the 66 patients with chronic constipation (24.24%) and 12 of the 60 in healthy controls (20%) respectively. Again, there was no statistical significance between chronic constipation and healthy controls (P = 0.72) (Table 2 and Figure 2).

Internal/external hemorrhoids were detected in 4 of the 16 patients with chronic constipation and varicocele, in the second group. In the remaining 50 patients with chronic constipation 9 had internal/external hemorrhoids. In this regard, there was no statistical significance between chronic constipation and healthy controls (P = 0.80). Total colonoscopy findings in the second group is summarized in Table 3 and Figure 3.

Table 3 Colonoscopy results at the second group

Patients with chronic constipation (n = 66)	Varicocele (+) (n = 16)	Varicocele (-) (n = 50)	P value
Colonoscopy findings			
Hemorrhoid	4	9	
Other intestinal pathologies	-	24	
Normal	12	17	0.8

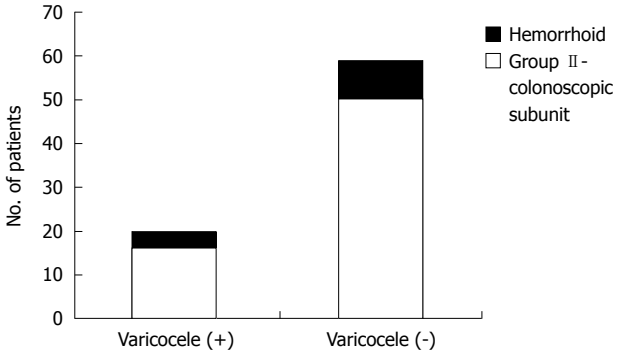

Figure 3 Colonoscopy results at the second group in graph.

No complication was observed during the colonoscopy procedure.

DISCUSSION

Varicocele, which is defined as the dilatation of veins of pampiniform venous plexus, is found in approximately 15% of the general population, 35% of men with primary infertility and 80% of men with secondary infertility[6].

Although the exact etiology has not been identified yet, several mechanisms have been proposed in the pathophysiology of varicocele involving insufficiency or absence of venous valves[6], anatomic inclination due to raised pressure in the left renal vein[7], "nut-cracker" phenomenon due to compression of left renal vein between aorta and superior mesenteric artery and oxidative stress[8].

Constipation may be clarified as having two of the following symptoms of straining, difficult stools, sensation of incomplete discharging, sensation of anorectal obstruction/blockage, requiring manual maneuvers to evacuate the stools, and having less than 3 evacuations per week using the Rome Ⅲ criteria[9]. For the constipation to be described as chronic, the Rome criteria need to have been met for the previous 3 mo, with the attack of symptoms 6 mo prior to diagnosis[10]. In our study the group was formed by patients with symptoms of constipation for at least 8 wk. A longer follow-up period is needed to evaluate the relationship between the duration

of constipation and varicocele. Therefore; in this study, the relation of the duration of constipation and varicocele was not investigated. This issue may to be another subject of study.

Several mechanisms have been proposed for the physiopathologic mechanism of chronic constipation, but the following 2 major disturbances are commonly accepted as related to the development of constipation: "colonic inertia"[4], causing a delay in the passage of the fecal material through the colon; and "outlet inertia" or "anorectal dysfunction"[11], by which the defecatory mechanism is disturbed, causing difficulty in ejected stool from the distal colon to the anus[12].

In the literature on the urinary tract abnormalities and constipation are extensive. The first study reported[13] that 31% of children with functional constipation had megaureter, and then they recommended that the cause would be a mechanical pressure phenomenon. Afterwards, several authors linked constipation to dysfunctional voiding patterns[14-16]. Moreover, higher rates of dilatation of the upper urinary tract and urinary tract infections in constipated patients were showed in some studies[14,15]. These results were ascribed to mechanical compression of the urinary tract by the distended rectum, due the anatomic proximity of the bladder and urethra to the rectum[16]. Also, constipation in children was showed by Fein *et al*[17] as a cause of scrotal or testicular pain, which was ascribed to direct neural stimulation by a fecal mass in the rectum. In this regard, the posterior sacral nerve, ilioinguinal nerve, or genital branch of the genitofemoral nerve has been involved in this neural association[18].

To our knowledge, there is only one study in the literature which investigates the prevalence of chronic constipation in patients with varicocele. According to this study, varicocele was determined in almost 50% of patients with chronic constipation which is suggested to be an important causative factor for the development of left varicocele[19]. The authors demonstrated the statistically significant relationship between the chronic constipation and varicocele, and had proposed a routine ultrasonographic evaluation for the possible development of varicocele in men who have pain or who are otherwise symptomatic[19]. Our study will be the second in the literature demonstrating the relationship between chronic constipation and varicocele.

Generally, the incidence of chronic constipation is reported as 3% in the young population[20]. In the first group of our study, chronic constipation rates were 5% and 11% in the control group and varicocele patients, respectively. These rates may support the relationship between the urinary tract and intestinal system[13-15].

Varicocele may be associated with underlying venous pathological conditions[7]. A study proved that a left varicocele may be two sides disease caused by an inadequacy of the one-way valves in the internal spermatic veins, connected to persistent pathological hydrostatic pressure in the long vertical spermatic veins and venous by passes[21]. Another researcher has been explained that patients with coronary artery ectasia had an increased prevalence of varicocele compared with coronary artery disease[22]. Also, the increased prevalence of peripheral varicose veins has been reported with coronary artery ectasia by Androulakis *et al*[23]. Lately, Koyuncu *et al*[24] have shown that the rate of saphenofemoral insufficiency has been found to be statistically higher in patients with primary varicocele, compared to healthy men. In addition, many of the studies in the literature have shown a close relationship among varicocele, hemorrhoid and venous diseases[25-27]. This relationship suggests that etiopathogenesis of varicocele and hemorrhoids may be a common venous pathology[28]. According to our colonoscopies results, hemorrhoids were found in 25% of patients with varicocele and 18% of patients with no varicocele. As a result, the rational relationship between varicocele and hemorrhoid was similar within the literature. To our knowledge, this is the first study evaluating the relationship between varicocele and chronic constipation by detailing the colonoscopic findings.

Varicocele may be attributable principally to the accompanying distention of the sigmoid colon and distal part of the descending colon, with resultant compression of the left testicular vein in the retroperitoneum, as well as repeated increased intra-abdominal pressure due to chronic straining[19].

In the literature, it has been shown that the incidence of varicocele is 9% at the second decades, 15% at the third decades and 18% at the fourth decades[25]. Contrary to these, we showed that the incidence of varicocele is 24% at the fourth decades in which the chronic constipation is seen more frequently. Therefore, this finding may support the possible relation between the intestinal system and urinary tract.

We anticipate three possible mechanisms for the relation between varicocele and chronic constipation: (1) Anatomic inclination may become more prominent with chronic constipation through the pressure effect to the intra-abdominal organs; (2) Recurrent and abrupt increases in pressure, chronic constipation may cause compression of intra-abdominal organs, eventually contributing to the development of "nutcracker" phenomenon of left renal vein by superior mesenteric artery and aorta; and (3) Chronic constipation may increase oxidative stress with hypoxic attacks and compression of venous return leading to varicocele.

In conclusion, chronic constipation may facilitate the development of varicocele with abrupt recurrent increases in intra-abdominal pressure through mechanisms of compression in intra-abdominal organs and increasing oxidative stress.

However, our study has two limitations. The first is that the number of patients in the study group may be accepted as a limited number and so, in our opinion, these findings should be confirmed with larger series. The other limitation of our study is the difference by means of patient age. The patients group's age should be homogeneous.

In conclusion, there is a rational relationship between varicocele and chronic constipation. However there was no statistical significance between chronic constipation and

varicocele. Therefore, chronic constipation may not be a major predictive factor for the development of varicocele, but it may be a facilitator factor for varicocele. The results of this hypothesis should be confirmed in larger series.

COMMENTS

Background

This study is interesting because there is not much written in literature. Varicocele can cause infertility in men. The etiology of varicocele is still not clear. Chronic constipation is probably a cause of male infertility.

Research frontiers

The study is preliminary working. The duration of constipation associated with varicocele have not been evaluated in this study. This study should be supported in infertile male patients.

Innovations and breakthroughs

To our knowledge, there is only one study in the literature to investigate the prevalence of chronic constipation in patients with varicocele. To our knowledge, this is the first study evaluating the relationship between varicocele and chronic constipation by detailing the colonoscopic findings. Chronic constipation may trigger the development of varicocele. Therefore, defecation habits of patients with male infertility should be asked.

Applications

Scrotum should be examined in male patients with chronic constipation.

Terminology

Specific terminology was not used in this article.

Peer review

This study is interesting because there is not much written in literature.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2646

World J Gastroenterol 2011 June 7; 17(21): 2646-2651
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Secretion of melatonin and 6-sulfatoxymelatonin urinary excretion in functional dyspepsia

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Received: November 25, 2010 Revised: March 5, 2011
Accepted: March 12, 2011
Published online: June 7, 2011

Abstract

AIM: To evaluate blood concentration of melatonin and urinary excretion of its metabolite, 6-sulfatoxymelatonin (6-OHMS), in functional dyspepsia (FD).

METHODS: Ninety individuals were enrolled in the study: 30 in each study group: patients with postprandial distress syndrome (PDS), epigastric pain syndrome (EPS), and controls. Blood samples were drawn at 02:00 and 09:00 h and 24-h urine collection was performed. Serum melatonin and urinary 6-OHMS concentrations were measured by enzyme-linked immunosorbent assay.

RESULTS: Serum melatonin concentration at night and in the morning was significantly ($P < 0.001$) higher in PDS patients [at 02:00 h-93.3 pg/mL, quartile range (QR): 79.8-116.2; at 09.00 h-14.3 pg/mL, QR: 7.06-19.0] than in EPS (57.2 pg/mL, QR: 42.6-73.1; 8.1 pg/mL, QR: 4.1-9.3) and control patients (57.7 pg/mL, QR: 51.2-62.5; 8.1 pg/mL, QR: 5.4-10.3). A similar relationship was observed for urinary 6-OHMS excretion. Patients with severe PDS symptoms had a higher melatonin concentration than these with moderate syndromes, whereas patients with severe EPS had a lower urinary 6-OHMS excretion than patients with moderate symptoms.

CONCLUSION: Evaluation of melatonin serum concentrations and 24-h urinary 6-OHMS excretion are useful methods for differential diagnosis of various clinical forms of FD.

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Key words: Functional dyspepsia; Postprandial distress syndrome; Epigastric pain syndrome; Melatonin; 6-sulfatoxymelatonin

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Chojnacki C, Poplawski T, Klupinska G, Blasiak J, Chojnacki J, Reiter RJ. Secretion of melatonin and 6-sulfatoxymelatonin urinary excretion in functional dyspepsia. *World J Gastroenterol* 2011; 17(21): 2646-2651 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2646.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2646

INTRODUCTION

Melatonin is synthesized by pinealocytes and in the gastrointestinal (GI) tract. Enterochromaffin (EC) cells are widely distributed in the GI tract mucosa, and are a rich source of this hormone, with the total amount of melatonin greatly exceeding that in the pineal gland[1,2]. Melatonin

displays endocrine, paracrine and autocrine properties, which may account in part for its neuroprotective action, and melatonin and its metabolites are powerful antioxidants[3-5]. The hormone also plays a role in the modulation of prostaglandin secretion and nitric oxide generation, as well as stimulation of bicarbonate secretion in the duodenum and pancreas[6-8]. Melatonin exerts an inhibitory effect on gastric acid secretion and myorelaxation effects on the smooth muscles of the GI tract[9,10]. Melatonin anti-inflammatory and immunomodulatory properties may also play a role in its general protective action in the GI tract[11,12].

An obvious question is whether the protective actions of MEL are exerted only in the case of a threat, or whether they are indispensible under physiological conditions. A growing body of evidence suggests the latter possibility and it has become clear that melatonin deficiency plays an important role in the pathogenesis of certain GI diseases[13,14]. Moreover, melatonin may protect gastric mucosa from stress-mediated lesions at a level comparable to or better than ranitidine and omeprazole[15,16].

Patients with duodenal ulcer disease have lower melatonin concentrations in the blood than healthy individuals have. The difference is most pronounced in the autumn and at night, but they it does not depend on the clinical phase (exacerbation or remission) of peptic ulcer disease[17-19]. It has been suggested that fasting and night abdominal pain in ulcer-like dyspepsia could be associated with lower than normal melatonin secretion, but there are some contradictory data[20,21]. Thus, an unequivocal link between melatonin deficiency and occurrence of disease symptoms in the GI tract has not been established. In our previous study, we recommended that patients with GI pain syndromes took 5 mg/d melatonin for 12 wk. In most patients (56.6%), the symptoms resolved after melatonin treatment; in 30% there was some amelioration of symptoms; while only 13.6% reported no clinical effect[22]. These results prompted us to carry out the present study.

The clinical picture of functional dyspepsia (FD) is rather complex. According to the Rome Ⅲ criteria, there are two major forms of this disease: epigastric pain syndrome (EPS) and postprandial distress syndrome (PDS). In patients with EPS, abdominal pain in the epigastrium dominates, but fasting and nocturnal pain also occur. On the other hand, PDS patients rarely suffer from epigastric pain, but they complain of discomfort and distension in the epigastrium after meals, and they often have morning satiety and nausea.

In the present work, we determined the level of melatonin in serum and measured urinary excretion of the main and immediate metabolite of melatonin, 6-sulfatoxymelatonin (6-OHMS), in patients with EPS or PDS.

MATERIALS AND METHODS

Patients

Ninety subjects were enrolled in this study, 58 women and 32 men, aged 19-45 years (mean, 30.9 years). Clinical characteristics of the patients are presented in Table 1. The subjects were divided into three groups: healthy persons with no signs of GI dysfunction; patients with EPS; and patients with PDS without symptoms of irritable bowel syndrome (IBS).

Table 1 Clinical characteristics of the study subjects: controls and patients with postprandial distress syndrome and epigastric pain syndrome

	Controls	PDS	EPS
Number	30	30	30
Age (yr), mean ± SD	28.6 ± 9.4	31.8 ± 12.4	32.3 ± 14.1
Sex (M/F)	12/18	14/16	13/17
Normal gastric mucosa	18/30	17/30	15/30
Superficial gastritis	12/30	13/30	15/30

PDS: Postprandial distress syndrome; EPS: Epigastric pain syndrome.

Diagnosis of FD was based on the Rome Ⅲ Criteria. To exclude other diseases, all the patients underwent upper GI endoscopy with histopathological evaluation of gastric mucosa biopsy specimens, abdominal ultrasound, and laboratory tests including blood cell morphology, C-reactive protein, glucose, electrolytes, bilirubin, urea, creatinine, cholesterol, triglycerides, thyrotropin, and activity of aspartate transaminase, alanine transaminase, γ-glutamyl transpeptidase, alkaline phosphatase, amylase and lipase. To exclude Helicobacter pylori infection, the urea breath test (UBT; FANCI-2 System, Fisher Instruments, Germany) was performed. Patients on any long-term treatment as well as cigarette smokers were not enrolled. Furthermore, Beck Depression Inventory and Hamilton Depression Scale were used to assess mental status and to exclude patients with psychiatric disease.

Methods

Seven days prior to the start of the study, patients were recommended to stop taking any medication and remain on a similar diet, which contained the same amount of tryptophan-rich products. Symptoms reported were graded according to a 10-point scale and the subjects were grouped into categories with moderate (1-5 points, 14 individuals with EPS, 16 with PDS) or exacerbated (6-10 points, 15/15) symptoms. On the day of the study, the patients were in the room with only red light exposure between 21:00 h to 07:00 h, and on the same liquid diet (Nutri Drinks; Nutricia, Warsaw, Poland) that consisted of 3 × 400 mL, which contained 18.9 g carbohydrate, 6.0 g protein and 5.8 g lipid per 100 mL, with a total caloric value of 1800 kcal. They also consumed 1.5 L isotonic gas-free water. Blood samples were drawn from the antecubital vein at 02:00 h and 09:00 h and serum was frozen at -70℃. At the same time, 24-h urine samples were collected and stored at 4℃. At the end of 24-h urine collection, the volume of urine was measured and the samples were frozen at -70℃. Serum melatonin and urinary 6-OHMS were measured by enzyme-linked immunosorbent assay using IBL antibodies (RE-54021 and RE-54031; Nordic Immunological Laboratories, Tilburg, Holland) and Expert 99 MicroWin 2000 Reader (BMG Labtech, Offenburg, Germany).

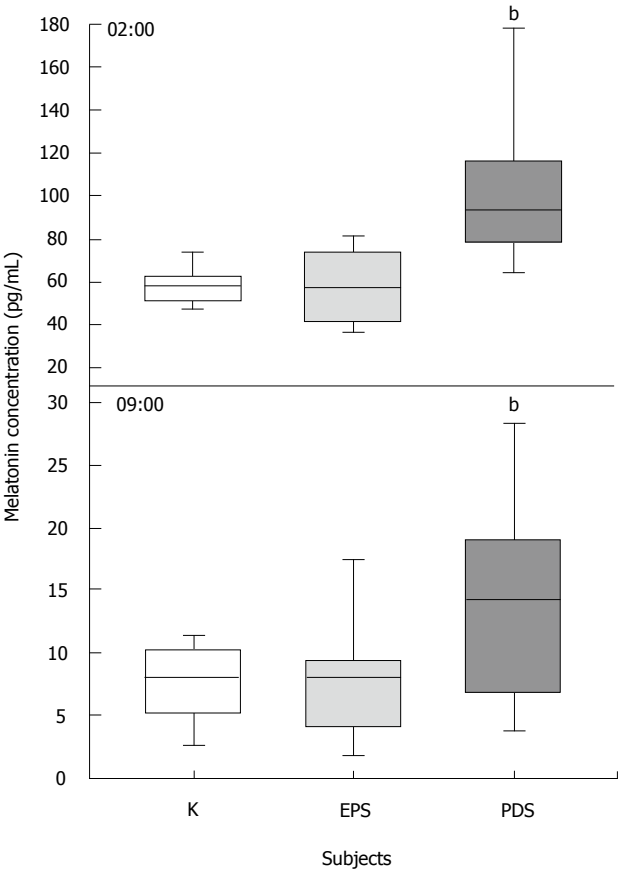

Figure 1 Serum melatonin concentrations at 02:00 h and 09:00 h in healthy subjects (K, clear bar, *n* = 30) and epigastric pain syndrome (grey bar, *n* = 30) and postprandial distress syndrome (dark grey bar, *n* = 30). Box represents median with 25th and 75th percentiles (lower and upper quartiles, respectively). The ends of the error bars represent the smallest and largest measurements in the study groups. $^{b}P < 0.001$. PDS: Postprandial distress syndrome; EPS: Epigastric pain syndrome.

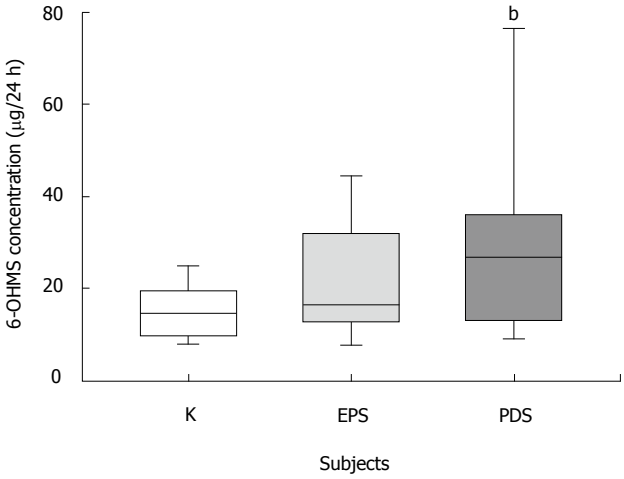

Figure 2 Twenty-four-hour urinary excretion of 6-OHMS in healthy subjects (K, clear bar, *n* = 30) and in patients with epigastric pain syndrome (grey bar, *n* = 30) and postprandial distress syndrome (dark grey, *n* = 30). Box represents median with 25th and 75th percentiles (lower and upper quartiles, respectively). The ends of the error bars represent the smallest and largest measurements in the study groups. $^{b}P < 0.01$. PDS: Postprandial distress syndrome; EPS: Epigastric pain syndrome.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and with the principles of good clinical practice. These studies were approved by the Bioethics Committee of the Medical University of Lodz, Poland (permission no. RNN/26/04/KB). Each patient was acquainted with the aim of the study and gave written informed consent.

Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether data fitted a normal distribution. Differences between groups were evaluated using the Mann-Whitney rank sum test, with $P < 0.05$ regarded as statistically significant.

RESULTS

The median serum melatonin concentration at 02:00 h in patients with PDS [93.3 pg/mL, quartile range (QR): 79.8-116.2] was about two times higher than in the control subjects (57.7 pg/mL, QR: 51.2-62.5, $P < 0.001$) and patients with EPS (57.2 pg/mL, QR: 42.6-73.1, $P < 0.001$) (Figure 1). We observed a similar relationship at 09:00 h when melatonin concentration in the PDS group (14.3 pg/mL, QR: 7.060-19.0) was significantly ($P < 0.001$) higher than in the controls (8.1 pg/mL, QR: 5.4-10.3) and in EPS subjects (8.1 pg/mL, QR: 4.1-9.3) (Figure 1). We also observed higher 24-h urinary 6-OHMS levels in patients with PDS (26.8 μg, QR: 13.4-35.6) as compared with controls (14.6 μg, QR: 10.6-19.4) and patients with EPS (16.4 μg, QR: 13.4-30.7) (Figure 2). The PDS patients with severe symptoms displayed a higher melatonin concentration at 02:00 h (100.0 pg/mL, QR: 91.0-115.0) and 09:00 h (16.1 pg/mL, QR: 12.9-23.4) as compared with patients with moderate symptoms (84.0 pg/mL, QR: 66.1-101.7, $P < 0.05$ and 11.9 pg/mL, QR: 5.2-14.4, $P < 0.001$) (Figure 3). The 24-h urinary excretion of 6-OHMS was also higher in these patients (30.3 μg, QR: 22.9-45.7) in comparison with moderate symptom patients (84.0 pg/mL, QR: 66.1-101.7; 11.9 *vs* 17.5 μg, QR: 9.7-28.9, $P < 0.01$) (Figure 4). We also observed that patients with EPS with severe symptoms had a reduced 24-h 6-OHMS urinary excretion as compared with patients with moderate symptoms (13.2 μg, QR: 10.3-16.6 *vs* 22.8 μg, QR: 16.5-43.3; $P < 0.01$) (Figure 4).

DISCUSSION

Clinical studies of melatonin secretion are usually based on its serum concentrations. These values are relatively low during the daytime and increase significantly at night; especially in the absence of white light. The circadian rhythm of melatonin is a result of secretion by the pineal gland. In pinealectomized animals, no night-time rise in blood melatonin levels is observed, and its concentration throughout a 24-h period remains at a low and relatively constant level[23]. The low residual amounts of melatonin are from sources other than pineal gland, for example, mainly from the GI tract. These sources of melatonin are regulated by mechanisms that do not involve the pi-

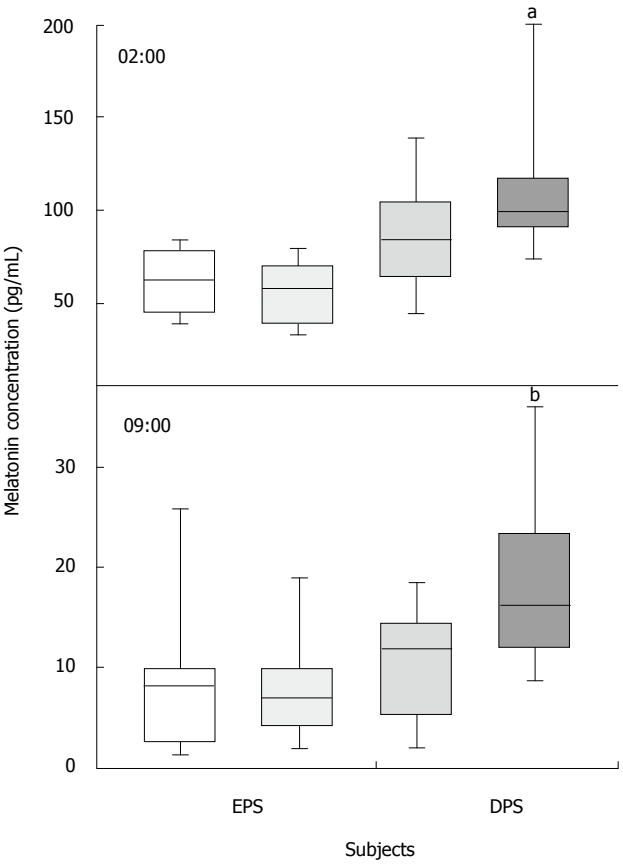

Figure 3 Serum melatonin concentrations at 02:00 h and at 9.00 a.m. in patients with epigastric pain syndrome (two shades of light grey, *n* = 30) and postprandial distress syndrome (two shades of dark grey, *n* = 30). Darker bars represent patients with severe symptoms [*n* = 16 for epigastric pain syndrome (EPS) and *n* = 15 for postprandial distress syndrome (PDS)] and lighter bars, patients with moderate symptoms (*n* = 14 for EPS and *n* = 15 for PDS). Box represents median with 25th and 75th percentiles (lower and upper quartiles, respectively). The ends of the error bars represent the smallest and largest measurements in the study groups. $^{a}P < 0.05$, $^{b}P < 0.01$.

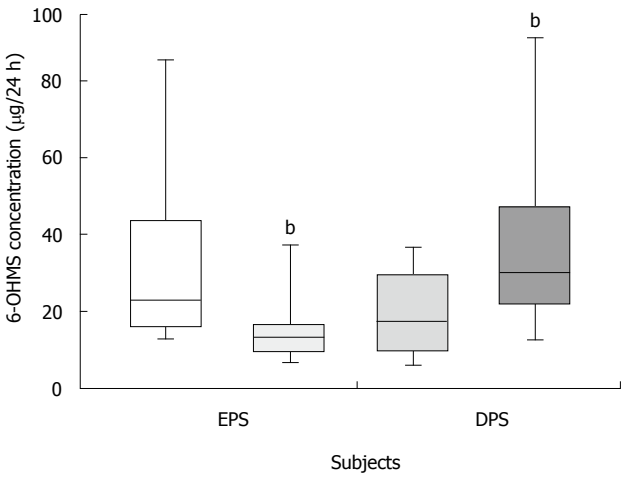

Figure 4 Twenty-four-hour urinary 6-OHMS excretion in patients with epigastric pain syndrome (two shades of light grey, *n* = 30) and postprandial distress syndrome (two shades of dark grey, *n* = 30). Darker bars represent patients with severe symptoms (*n* = 16 for epigastric pain syndrome (EPS) and *n* = 15 for postprandial distress syndrome (PDS)) and lighter bars, patients with moderate symptoms (*n* = 14 for EPS and *n* = 15 for PDS). Box represents median with 25th and 75th percentiles (lower and upper quartiles, respectively). The ends of the error bars represent the smallest and largest measurements in studied groups. $^{b}P < 0.01$.

neal gland. Melatonin secretion from EC cells of the gut increases as a consequence of the activity of muscarinic M3 and β-adrenergic receptors, but also after the intake of food[24]. Large meals rich in L-tryptophan cause satiety and sleepiness in humans and they stimulate the release of melatonin from EC cells. Postprandial melatonin peripheral serum concentrations do not rise markedly because melatonin is absorbed into the portal circulation and transported to liver, where 90% of it is metabolized, mainly to 6-OHMS, which is then excreted by kidneys into the urine[25]. It is accepted that evaluation of urinary 6-OHMS excretion is a useful index of the secretory activity of all cells that produce melatonin[26]. Collecting blood every 1-2 h for the purpose of measuring melatonin concentration is troublesome for patients, disturbs their normal circadian rhythms, and increases emotional stress, which may significantly influence their melatonin levels. As a result, it is widely accepted to perform the measurements twice daily: usually at 02:00 h (in darkness) and at 09:00 h (in daylight)[27].

Melatonin, due to its interaction with receptors[28] and because of its antioxidative properties[29,30], plays an important role in the function of the GI tract and this indoleamine deficiency is likely involved in the pathogenesis of some GI diseases, including gastric and duodenal peptic ulcers[14]. However, the role of melatonin in the etiology of FD has not been established. The diagnosis of FD is still based on patient complaints. Objective indices of this disease are generally lacking. In the current study, the subjects with EPS had melatonin serum concentrations similar to those in healthy controls, but higher urinary 6-OHMS excretion. This suggests enhanced melatonin secretion from the GI tract in these patients. This enhancement might be sufficient to prevent formation of peptic lesions, but not sufficient to prevent the occurrence of dyspeptic symptoms. In this context, increased melatonin release from the GI tract may be a consequence of pathogenic factors such as chronic stress, increased vegetative system tension, and other processes.

We observed a higher concentration of melatonin in PDS patients than in the control subjects; both during the day and at night, but whether this increase was the reason or a consequence of FD is unresolved. A relative melatonin deficiency may have pathogenic implications for the GI tract, as it has in sleep disturbances, depression and in some organic diseases, including cancer. However, elevated melatonin concentrations have been observed in some diseases of the endocrine and reproductive systems[31], liver cirrhosis[32], and anorexia nervosa[33]. The results of the current study show that FD could be one of these diseases. Different melatonin levels in different forms of FD may have diagnostic significance. Melatonin, or its precursor L-tryptophan, could also be administered in cases of their deficiency, or in patients who are receiving gastro toxic drugs. Rapaport *et al*[34] have recommended that melatonin should be taken by patients with duodenal ulcer

disease, after they observed its beneficial clinical effect and ability to heal inflammatory lesions of the gastric antral mucosa. In our previous studies, the therapeutic efficacy of melatonin in patients with ulcer-like dyspepsia has been shown. Moreover, Konturek *et al*[35] have reported a clear gastroprotective effect of L-tryptophan in patients who are taking acetylsalicylic acid. To date, however, melatonin and its analogs have been used mainly in the treatment of sleep disorders and depression[36,37]. Functional diseases of the GI tract are often associated with psycho-emotional disorders. Dyspeptic symptoms are often the main or even the only manifestation of depression, because it is not associated with bad mood. In several cases of sleep disorders, GI complaints and the fear of severe, chronic non-curable disease are markers of depression.

Disturbance of melatonin level is also observed in patients with IBS, which is more likely to share a common background with FD. To date, the role of melatonin in the etiology of IBS has not been established. Melatonin is known to be a regulator of intestinal motility, therefore, it could act by relaxing bowel muscles. It has been observed that melatonin reduces the tone, but not the amplitude or frequency of contractions[38]. This effect is probably due to stimulation of melatonin receptors and regulation of Ca^{2+}/K^{+} channels, and indirectly by the nervous system[39,40]. Other results suggest that melatonin has a peripheral anti-serotonin-like effect[41]. Serotonin (5-hydroxytryptamine; 5-HT) is thought to be a likely contender in the induction and maintenance of visceral hypersensitivity associated with IBS. 5-HT acts mostly at 5-HT3 or 5-HT3-like receptors, and enhances the sensitivity of visceral neurons that project between the gut and central nervous system.

In conclusion, melatonin levels in the peripheral blood are significantly higher in patients with severe symptoms of PDS than in those with EPS. Thus, these levels may be useful in the differential diagnosis of these diseases. Evaluation of 24-h urinary 6-OHMS excretion is a useful method for the estimation of melatonin secretion and may be important in the differentiation of various clinical forms of FD.

COMMENTS

Background

Melatonin is synthesized by pinealocytes and in the gastrointestinal (GI) tract. Melatonin displays endocrine, paracrine and autocrine properties, which may account in part for its neuroprotective action. Moreover, melatonin and its metabolites are powerful antioxidants.

Research frontiers

An obvious question is whether the protective actions of melatonin are exerted only in the case of a threat or whether they are indispensible under physiological conditions. A growing body of evidence suggests the latter possibility, and it has become clear that melatonin deficiency plays an important role in the pathogenesis of certain GI diseases.

Innovations and breakthroughs

This is believed to be the first study to report an association between functional dyspepsia (FD) and melatonin serum level and 6-sulfatoxymelatonin (6-OHMS) urinary excretion.

Applications

By connecting melatonin levels in the peripheral blood with FD, this study may help to establish the new differential diagnosis of various clinical forms of FD. The authors also proved that evaluation of 24-h urinary excretion of 6-OHMS is a useful method for the estimation of melatonin secretion, and together with melatonin levels in the peripheral blood, may be important for the differentiation of various clinical forms of FD.

Terminology

Melatonin is a naturally occurring compound in animals, plants and microbes. Many biological effects of melatonin are produced through the activation of its receptors, while others are due to its role as a pervasive and powerful antioxidant. FD is a disease without organic evidence that is likely to explain the symptoms. These symptoms include: upper abdominal pain, belching, nausea, abdominal bloating and early satiety. FD is estimated to affect about 15% of the general population in western countries.

Peer review

This was a well-designed study that examined the association between FD and melatonin serum level and 6-OHMS urinary excretion.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2652

World J Gastroenterol 2011 June 7; 17(21): 2652-2657
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Endoscopic removal and trimming of distal self-expandable metallic biliary stents

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Received: May 11, 2010 Revised: June 4, 2010
Accepted: June 11, 2010
Published online: June 7, 2011

Abstract

AIM: To evaluate the efficacy and safety of endoscopic removal and trimming of self-expandable metallic stents (SEMS).

METHODS: All SEMS had been placed for distal biliary strictures. Twenty-seven endoscopic procedures were performed in 19 patients in whom SEMS (one uncovered and 18 covered) removal had been attempted, and 8 patients in whom stent trimming using argon plasma coagulation (APC) had been attempted at Tokyo Medical University Hospital. The APC settings were: voltage 60-80 W and gas flow at 1.5 L/min.

RESULTS: The mean stent indwelling period for all patients in whom stent removal had been attempted was 113.7 ± 77.6 d (range, 8-280 d). Of the 19 patients in whom removal of the SEMS had been attempted, the procedure was successful in 14 (73.7%) without procedure-related adverse events. The indwelling period in the stent removable group was shorter than that in the unremovable group (94.9 ± 71.5 d *vs* 166.2 ± 76.2 d, $P = 0.08$). Stent trimming was successful for all patients with one minor adverse event consisting of self-limited hemorrhage. Trimming time ranged from 11 to 16 min.

CONCLUSION: Although further investigations on larger numbers of cases are necessary to accumulate evidence, the present data suggested that stent removal and stent trimming is feasible and effective for stent-related complications.

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Key words: Self-expandable metallic stent; Endoscopic biliary stenting; Endoscopic stent removal; Endoscopic stent trimming

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Ishii K, Itoi T, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Tsuji S, Ikeuchi N, Umeda J, Moriyasu F, Tsuchida A. Endoscopic removal and trimming of distal self-expandable metallic biliary stents. *World J Gastroenterol* 2011; 17(21): 2652-2657 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2652.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2652

INTRODUCTION

Placement of self-expandable metallic stents (SEMS) in the palliation of malignant biliary stricture has been increasingly employed in an attempt to prolong the patency period[1,2]. The recent improvement in multidisciplinary therapy that incorporates treatments such as chemotherapy and radio-

therapy has resulted in an increased number of long-term survivors. This has however been accompanied by an increased chance of experiencing stent-related complications. The majority of stent-related complications involve stent occlusion, leading to cholangitis. Displacement of the stent in the duodenal wall, which is another complication, is not only associated with occlusion but also with the formation of erosions and ulcers from mechanical irritation, and serious bleeding and gastrointestinal tract perforation[3-5].

An intervention for stent occlusion is mechanical cleaning, using devices such as a balloon, but, the occlusion recurs in a comparatively short time in many cases[6]. For patients with repeated stent occlusion, the stent-in-stent procedure with plastic stents or SEMS is used. According to some recent reports[7-9], a procedure whereby SEMS are removed and replaced by new SEMS, has been performed as an alternative method for management of occlusion of SEMS. Furthermore, as an intervention in the displacement of a metallic stent, metallic stent trimming by argon plasma coagulation (APC) has been used in cases of stent migration to the duodenum[4,10-13]. In the present study, we retrospectively evaluated the efficacy and safety of endoscopic removal and trimming of SEMS.

MATERIALS AND METHODS

All SEMS had been placed for distal biliary strictures. Twenty-seven endoscopic procedures were performed in 19 patients in whom SEMS removal had been attempted and in 8 patients in whom APC trimming had been attempted at Tokyo Medical University Hospital between February 2004 and April 2009. The APC trimming group included 3 patients in whom stent removal had been switched to trimming because stent removal was impossible.

The ratio of males to females was 17:10 and the mean age was 68.1 ± 13.7 years (range, 43-91 years) (Table 1). Our policy for managing stent-related accidental symptoms is outlined below. When a stent was occluded, our policy was first to clean the occluded stent using a balloon catheter or basket catheter. If cholangitis due to stent occlusion recurred more than a twice, we attempted stent removal. However, even if the SEMS were positioned appropriately but removal was difficult, mechanical cleaning or a stent-in-stent maneuver was performed. Furthermore, if the end tip of the stent had migrated towards the duodenal wall, only stent trimming was performed. If the stent was occluded even after trimming, mechanical cleaning or a stent-in-stent procedure was performed.

Stent removal

The SEMS used for the 19 cases in whom stent removal was attempted are shown in Table 1. The stents used included one uncovered Wallstent (Boston Scientific Japan, Tokyo, Japan) and 18 covered SEMS. Therapeutic duodenoscope (TJF-240, TJF-260V and JF-260V, Olympus Medical Systems, Tokyo, Japan) were used for the removal of stents. Snare forceps (SD-5L-1, Olympus, Tokyo, Japan) were used to grip the end tip of the stent, which was then removed by pulling it towards the working channel. However, if removal through the working channel was difficult, the stent was removed by endoscopy while holding it. If a Niti-S Biliary covered stent (Taewoong Company, Seoul, Korea) was used with a removal suture, the removal cord was gripped using biopsy forceps and after pulling the stent a distance of about a third from the papilla, it was removed using snare forceps as previously mentioned.

Table 1 Characteristics of patients

	Total	Stent removal	Stent trimming
No. patients	27	19	8[1]
Sex (male/female)	17/10	13/6	4/4[1]
Mean age, yr, (range)	68.1 (43-91)	66.9 (43-91)	70.9 (54-90)
Etiology of biliary strictures			
Pancreatic carcinoma	17	12	5
Biliary ductal carcinoma	3	2	1
IPMC	1	0	1
Lymph node metastasis[2]	1	0	1
Chronic pancreatitis	2	2	0
MFP	2	2	0
AIP	1	1	0
Type of SEMS			
Uncovered Wallstent	2	1	1
Covered SEMS	25	18	7
Covered Wallstent (Partially covered type)	7	4	3
Niti-S Biliary ComVi Stent (Partially covered type)	2	1	1
Niti-S Biliary ComVi Stent (Fully covered type)	14	11	3
Niti-S Biliary Covered Stent with removal suture	2	2	0

[1]Including 3 cases with impossible stent removal; [2]Due to cancer of the uterine body. IPMC: Intraductal papillary mucinous carcinoma; MFP: Mass-forming pancreatitis; AIP: Autoimmune pancreatitis; SEMS: Self-expandable metallic stent.

Stent trimming

An APC system (ENDOPLASMA, PSD-60, Olympus Medical Systems, Tokyo, Japan) was used to perform stent trimming. The settings were: voltage at 60-80 W and gas flow at 1.5 L/min. The APC probe used for all the patients was the front emission elasticity APC probe (MAJ-1011, diameter 2.3 mm, Olympus Medical Systems, Tokyo, Japan). The stent amputation stump margin was set at 5-15 mm from the papilla. A rough distance was maintained so that the tip of the probe would be in minimal contact with the stent. The target line for cutting the stent was a circumferential incision in addition to ablation (Figure 1). In addition, to avoid gut distension due to the retention of excess gas and the accompanying pain, as well as the risk of perforations during the procedure, gas was suctioned periodically. Stent fragments were removed together with the endoscope using a catheter with a retrieval net or by pulling them out while being held by forceps.

RESULTS

Stent removal

The mean stent indwelling period of all SEMS was 113.7

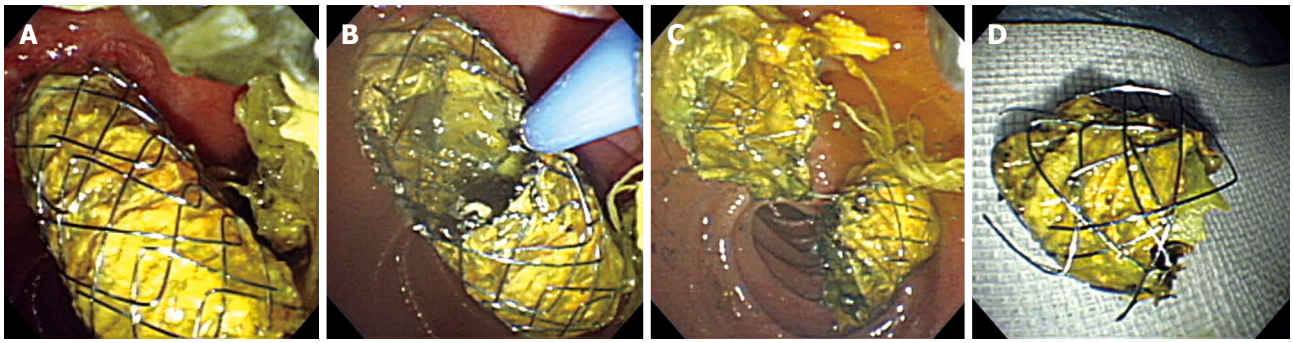

Figure 1 Stent trimming using argon plasma coagulation. A: Endoscopic imaging showing displacement of a Niti-S Biliary ComVi stent to the duodenum; B: Stent trimming was performed using argon plasma coagulation; C: Endoscopic imaging showing the cut down the stent; D: Eventually, the stent fragment was removed from the body.

Table 2 Outcome of removal of metallic stent

No. of case	Disease	Type of SEMS	Duration of stent placement (d)	Outcome	Additional procedures
1	Pancreatic ca.	Uncovered Wallstent	180	Unsuccess	Trimming
2	Pancreatic ca.	Niti-S Biliary ComVi Stent (Partially covered type)	114	Unsuccess	Trimming
3	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	280	Unsuccess	Trimming
4	Bile duct ca.	Niti-S Biliary ComVi Stent (Fully covered type)	81	Unsuccess	Stent in stent
5	Bile duct ca.	Niti-S Biliary ComVi Stent (Fully covered type)	219	Success	New SEMS
6	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	232	Success	New SEMS
7	Pancreatic ca.	Covered Wallstent (Partially covered type)	15	Success	New SEMS
8	Pancreatic ca.	Covered Wallstent (Partially covered type)	78	Success	New SEMS
9	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	155	Success	New SEMS
10	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	32	Success	New SEMS
11	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	98	Success	New SEMS
12	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	50	Success	New SEMS
13	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	157	Success	New SEMS
14	Pancreatic ca.	Niti-S Biliary ComVi Stent (Fully covered type)	75	Success	New SEMS
15	AIP	Niti-S Biliary ComVi Stent (Fully covered type)	8	Success	None
16	MFP	Covered Wallstent (Partially covered type)	100	Success	New SEMS
17	MFP	Covered Wallstent (Partially covered type)	176	Unsuccess	None
18	CP	Niti-S Biliary Covered Stent with removal suture	33	Success	New SEMS
19	CP	Niti-S Biliary Covered Stent with removal suture	77	Success	None

ca.: Carcinoma; CP: Chronic pancreatitis; SEMS: Self-expandable metallic stent; MFP: Mass-forming pancreatitis; AIP: Autoimmune pancreatitis.

± 77.6 d (range, 8-280 d). There was no statistical difference between malignant and benign diseases (126.1 ± 80.0 and 78.8 ± 65.2 d, respectively, $P = 0.25$). Of the 19 patients in whom removal of the SEMS was attempted, the procedure was successful in 14 (73.7%) without complications (Table 2). Stent removal was impossible for one patient with an uncovered stent and 4 patients with a covered stent. The indwelling period in the stent removable group was shorter than that in the unremovable group (94.9 ± 71.5 d *vs* 166.2 ± 76.2 d, $P = 0.08$). Of the 14 patients who underwent successful stent removal, the indwelling period for the 10 patients who had a malignant disease was 111 ± 76.1 d (range, 15-232 d) and the period for the 4 patients who had a benign disease was 54.5 ± 41.6 d (range, 8-100 d). There was no statistical difference between the 2 groups ($P = 0.1$).

Of the 14 patients for whom stent removal was possible, covered SEMS were reinserted in 12, and the remaining 2 patients had a benign biliary stricture without additional stent placement because of successful dilation of the stricture.

In 5 patients for whom stent removal was impossible, a stent-in-stent procedure with a covered Wallstent was used for one patient, and trimming was performed for 3 patients as an alternative method (Table 2). For the one remaining patient, the progress was monitored because the stent was found to be open by imaging.

Stent trimming

Stent trimming was successful for all the patients (Table 3). Trimming time was 12 min for the single patient with the uncovered Wallstent, a mean of 14 min (range, 12-16 min) for 3 patients with the covered Wallstent, a mean of 13 min (range, 11-15 min) for 4 patients with the Niti-S Biliary ComVi Stent. Of the 2 patients for whom stent trimming was successful found to have bile duct stenosis and so a stent-in-stent procedure was performed using the covered Wallstent and Niti-S Biliary ComVi stent.

There was self-limiting hemorrhage due to injury to the esophageal mucosa during removal of the stent fragments in one patient. The patient progressed well with conservative treatment.

Table 3 Outcome of stent trimming

No. of case	Disease	SEMS	Procedural time (min)	Additional procedure after trimming
1	Pancreatic ca.	Uncovered Wallstent	12	None
2	Pancreatic ca.	Niti-S Biliary ComVi Stent	11	Stent in stent
3	Pancreatic ca.	Niti-S Biliary ComVi Stent	14	Stent in stent
4	Pancreatic ca.	Covered Wallstent	14	None
5	Pancreatic ca.	Covered Wallstent	16	None
6	Bile duct ca.	Niti-S Biliary ComVi Stent	12	None
7	IPMC	Covered Wallstent	12	None
8	Lymph node metastasis[1]	Niti-S Biliary ComVi Stent	15	None

Argon plasma coagulation setting: 60-80 W, Coagulation mode 1.5 L/min. [1]Due to cancer of the uterine body. ca.: Carcinoma; IPMC: Intraductal papillary mucinous carcinoma; SEMS: Self-expandable metallic stent.

Table 4 Review of stent removal

Author	Total No. of stent/patient	Type of SEMS	*n*	Disease	Mean duration of indwelling stent (range)	Success rate (%)
Kahaleh *et al*[7]	18/18	Uncovered Wallstent	2	BBS	(1-4 mo)	2/2 (100)
		Uncovered Wallstent	2	MBS	(10-11 mo)	2/2 (100)
		Covered Wallstent	6	BBS	4.7 mo (1-10 mo)	6/6 (100)
		Covered Wallstent	8	MBS	4.5 mo (1-16 mo)	7/8 (87.5)
Familiari *et al*[8]	39/29	Covered Wallstent	12			12/12 (100)
		Shim-Hanarostent	10			9/10 (90)
		Uncovered Wallstent	10	MBS (26)	7.5 mo	5/10 (50)
		Niti-S Biliary Stent	4	BBS (3)	(5 d-16 mo)	3/4 (75)
		Sinus-Superflex Stent	2			0/2 (0)
		Zilver Stent	1			0/1 (0)
Shin *et al*[9]	30/30	Uncovered Wallstent	5	MBS	Uncovered SEMSs	0/5 (0)
		Zilver Stent	3	MBS	121.4 ± 45.4 d	0/3 (0)
		Covered Wallstent	21	MBS	Covered SEMSs	18/21 (85.7)
		Covered Wallstent	1	BBS	129.7 ± 95.4 d	1/1 (100)
Kahaleh *et al*[15]	65/65	Covered Wallstent	65	BBS	4 mo (1-28 mo)	65/65 (100)
Present study	19/19	Covered Wallstent	2	MBS	46.5 d (15-78 d)	2/2 (100)
		Covered Wallstent	2	BBS	(100-176 d)	1/2 (50)
		Uncovered Wallstent	1	MBS	180 d	0/1 (0)
		Niti-S Biliary ComVi Stent	11	MBS	136 d (32-280 d)	8/11 (72.7)
		Niti-S Biliary ComVi Stent	1	BBS	8 d	1/1 (100)
		Niti-S Biliary Covered Stent with removal suture	2	BBS	55 d (33-77 d)	2/2 (100)

SEMS: Self-expandable metallic stent; BBS: Benign biliary strictures; MBS: Malignant biliary strictures.

DISCUSSION

Nowadays, placement of SEMS has become the gold standard for the treatment of malignant distal biliary stricture[1,2]. In addition, SEMS have recently been used for postoperative bile leaks, benign biliary strictures such as in chronic pancreatitis, or postoperative biliary strictures[14-17]. Nevertheless, stent occlusions do occur. Previously, when we encountered a SEMS occlusion, we used the stent-in-stent technique. Recently, stent replacement by stent removal, which is an alternative to the stent-in-stent technique, has been attempted[7-9]. In reports to date, the rate of complete removal of covered SEMS is 95.5% (169/177), which has been described as having a comparatively high rate of success (Table 4). However, an uncovered metallic stent is usually impossible to remove as a result of it being embedded in tissue, regardless of whether the disease is benign or malignant. There are also reports of a stent being removed by breaking it up with forceps[18-20], but this is time consuming and there is also the possibility of the channel being damaged by stent fragments. Therefore, this procedure is unlikely to become popular. In the present study, the rate of success for complete removal of the covered metallic stents was 77.8%,while that of the uncovered metallic stents was 0%, showing a comparatively good result for the rate of complete removal of the covered metallic stent. However, even with covered metallic stents, 22.2% were not removable, showing that in fact, there is a limitation to this technique's total success rate. In reports to date, one of the reasons why it is difficult to remove covered metallic stents is said to be severe tissue growth over the uncovered part[8]. In addition, it has been reported that tumor growth causes duodenal stenosis, making it impossible to approach papillary areas[6,7].

In the present study, although there was no statistical significance, the mean indwelling period of removable SEMS was shorter than those of unremovable SEMS, suggesting that the length of the indwelling period may affect the successful removal of SEMS. Structural properties of

Table 5 Review of biliary stent trimming

Auther	Total No. of cases	SEMS	No. of case	Setting of APC system	Procedural time	Success rate
Demarquay *et al*[4]	3	Uncovered Wallstent	3	85 W, coag. 0.8 L/min	NA	100% (3/3)
Vanbiervliet *et al*[10]	24	Uncovered Wallstent	23	70-80 W, coag. 0.8 L/min	< 30 min	95.7% (22/23)
		Covered Wallstent	1	70-80 W, coag. 0.8 L/min	< 30 min	0% (0/1)
Guda *et al*[11]	2	Uncovered Wallstent	1	80 W, coag. 2.0 L/min	NA	100% (1/1)
		Uncovered Wallstent	1	60 W, coag. 2.0 L/min	NA	100% (1/1)
Rerknimitr *et al*[12]	2	Covered Wallstent	1	60 W, coag. 0.8 L/min	20 min	100% (1/1)
		Uncovered Wallstent	1	70 W, coag. 0.8 L/min	NA	100% (1/1)
Christiaens *et al*[13]	2	Uncovered SEMS (nitinol)	1	60 W, coag. 1.8 L/min	NA	100% (1/1)
		Uncovered SEMS (nitinol)	1	60 W, coag. 1.8 L/min	NA	100% (1/1)
Present study	8	Covered Wallstent	3	60-80 W, coag. 1.5 L/min	< 20 min	100% (3/3)
		Uncovered Wallstent	1	60-80 W, coag. 1.5 L/min	12 min	100% (1/1)
		Niti-S Biliary ComVi Stent	4	60-80 W, coag. 1.5 L/min	< 20 min	100% (4/4)

NA: Not available; SEMS: Self-expandable metallic stent; APC: Argon plasma coagulation; coag.: Coagulation mode.

the stent were also shown to affect the possibility of stent removal. For the covered Wallstent, the entire diameter was found to shrink and straighten when the stent was held and pulled towards the biliary axis. However, for the Niti-S Biliary ComVi stent, only the part that was held was found to shrink and even the straightening was inadequate, making it difficult to transmit force. Therefore, compared to the covered Wallstent, it was our experience that it was slightly difficult to remove. However, these possible correlations are unclear because of the small number of patients.

Another problem with the covered metallic stent is displacement of the stent towards the duodenum. Covered SEMS are easier to remove than uncovered SEMS. However, they migrate more easily than uncovered SEMS[21,22]. In future, the risk of stent displacement due to shrinkage of tumors in patients that respond to chemotherapy and radiotherapy is likely to increase. In cases where the prolapsing part is extremely long and cannot be held with a snare or holding forceps, it is sometimes effective to use recovery methods such as the wire loop technique[23]. Recently, stent trimming has occasionally been used. Basically, the purposes of trimming are to resolve any occlusion as a result of burial of the stent tip towards the duodenal wall, eliminate mechanical irritation of the duodenal wall, and ensure an accessible route to the bile duct. In particular, trimming using APC is known to be inexpensive and simple. Presently, the safety and usefulness of APC trimming have been confirmed in experimental[24] and clinical[4,10-13] studies. However, its indication, method of intervention, and settings have not yet been established because there are no uniform conditions for setting APC in trimming. From various studies, settings for the high frequency wave output ranged from 60 to 85 W, and gas flow from 0.8 L/min to 2.0 L/min, showing notable variation. The main reports to date are presented in Table 5[4,10-13]. In this study, gas flow was set at 1.5 L/min and the output was started at 60 W and gradually increased while observing the results of the trimming. However, at 60 W, the ablation effect was often inadequate; at 80 W, trimming could be done effectively and smoothly. In addition, even at 80 W, the trimming could be done adequately and safely without any major complications. Therefore, setting the output at 80 W initially contributed to a reduction of unnecessary testing time. With regard to the maximum output, there has been histological evaluation in animal models[24] but its status in the human body is unknown. Standardization of a safe and effective setting of the output would be ideal.

There are various types of SEMS in use and these include Elgiloy, which is a cobalt alloy as in the Wallstent (Boston Scientific Co., Tokyo, Japan), other stainless steel stents, and recently, Nitinol, which is made up of a shape-memory alloy as in the Niti-S stent (TaeWoong Medical Co., Seoul, Korea). To date, there are reports on APC trimming with the uncovered metallic stents, Elgiloy and Nitinol, stating that trimming can be done with no major complications[4,10-13]. In the present study, the trimming time was about 15 min (range, 11-16 min) for all the patients, with no significant difference in time. With regard to the differences between the uncovered and covered metallic stents, Vanbiervliet *et al*[10] and Christiaens *et al*[13] were unsuccessful at trimming the covered metallic stents, and the reason given was poor generation of the heat required for trimming due to hindrance of the flow of electricity to the stent by the coated polyurethane resin membrane. However, Chen *et al*[24] reported there was no difference in technique or time required for trimming covered *versus* uncovered stents. This result was different from the results in clinical reports. Presently, no conclusion can be reached regarding the differences. In addition, food residue that attaches to the periphery of stents can also be considered a hindrance to the discharge of electricity[12]. As a pre-trimming intervention, adequate removal of food residue may shorten the time of the procedure.

In conclusion, although further investigations on larger numbers of cases are necessary to accumulate evidence, the present data suggest that stent removal and stent trimming is feasible and effective for stent-related complications.

ACKNOWLEDGMENTS

The authors are indebted to Professor J Patrick Barron of the Department of International Medical Communications Center at Tokyo Medical University for his review of this manuscript.

COMMENTS

Background

Recently, self-expandable metallic stents (SEMS) removal has been performed as an alternative management method of occlusion of SEMS. Furthermore, metallic stent trimming by argon plasma coagulation (APC) has been used in cases of stent displacement or migration to the duodenum.

Research frontiers

In reports to date, the rate of complete removal of covered SEMS was 95.5% (169/177), which has been described as having a comparatively high rate of success. However, an uncovered metallic stent is usually impossible to remove. In the present study, of the 19 patients in whom removal of the SEMS had been attempted, the procedure was successful in 14 (73.7%) without complications. The rate of success for complete removal of the covered metallic stents was 77.8% (14/18), while that of the uncovered metallic stents was 0% (0/1). In addition, stent trimming was successful in all patients. Trimming time ranged from 11 to 16 min. The present data suggested that stent removal and trimming is feasible and effective for stent-related complications.

Innovations and breakthroughs

The present study showed a comparatively good result for the rate of complete removal of the covered metallic stent. However, even with covered metallic stents, 22.2% (4/18) were unremovable, showing that there is a limitation to this technique's total success rate. This report suggests that the indwelling period length and structural properties of the stent may also affect the successful removal of SEMS. There are few reports about stent trimming of biliary covered SEMS. In addition, several investigators have reported no success in trimming the covered metallic stent. Meanwhile, in the present study, stent trimming was successful for all covered SEMS.

Applications

Stent removal and trimming is feasible and effective for stent-related complications.

Terminology

Covered SEMSs can be removed theoretically, because the membrane of SEMS prevents embedding caused by tumor ingrowth or tissue hyperplasia.

Peer review

This is a well written paper on removal and trimming of self expandable metal biliary stents with good results. The technique and setting used are useful for the readers.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2658

World J Gastroenterol 2011 June 7; 17(21): 2658-2662
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Clinical significance of serum expression of GROβ in esophageal squamous cell carcinoma

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Supported by The Grants from International Science & Technology Cooperation and Exchange Programs, No. 2008DFA31130; Joint China/South Africa Science and Technology Agreement; National Natural Science Foundation of China, No. 81021061, No. 0772507 and No. 30700992; State Key Projects for Basic Research of China, No. 2011CB910703
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Received: January 20, 2011 Revised: April 6, 2011
Accepted: April 13, 2011
Published online: June 7, 2011

Abstract

AIM: To determine the association between serum levels of growth-related gene product β (GROβ) and clinical parameters in esophageal squamous cell carcinoma (ESCC).

METHODS: Using enzyme-linked immunosorbent assay, serum GROβ levels were measured in ESCC patients (n = 72) and healthy volunteers (n = 83). The association between serum levels of GROβ and clinical parameters of ESCC was analyzed statistically.

RESULTS: The serum GROβ levels were much higher in ESCC patients than in healthy controls (median: 645 ng/L *vs* 269 ng/L, P < 0.05). Serum GROβ levels were correlated positively with tumor size, lymph node metastasis, and tumor-node-metastasis (TNM) staging, but not with gender or the histological grade of tumors in ESCC patients. The sensitivity and specificity of the assay for serum GROβ were 73.61% and 56.63%, respectively.

CONCLUSION: GROβ may function as an oncogene product and contribute to tumorigenesis and metastasis of ESCC.

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Key words: GROβ; Esophageal squamous cell carcinoma; Metastasis; Cytokine; Tumor markers

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Dong QM, Zhang JQ, Li Q, Bracher JC, Hendricks DT, Zhao XH. Clinical significance of serum expression of GROβ in esophageal squamous cell carcinoma. *World J Gastroenterol* 2011; 17(21): 2658-2662 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2658.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2658

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies in China, South Africa, and some other developing countries[1,2]. In China, patients with ESCC have a poor prognosis, with an average 5-year survival rate of less than 30%[3-5]. It is thus imperative to find new markers, especially serum protein markers, to facilitate the early detection and diagnosis of ESCC.

CXC chemokine receptors are integral membrane proteins that specifically bind and are activated by cytokines

of the CXC chemokine family, a group of small cytokines[6] involved in the response to injury[7] and inflammatory reactions[8] as well tumorigenesis, cancer progression, and metastasis[9,10]. CXCL16 expression is increased in multiple tumor tissues and cell lines, and upregulation is associated with tumor progression and metastasis[11]. The plasma levels of CXCL4 and CXCL6 are much higher in patients with osteosarcoma than in healthy controls, and elevated plasma CXCL4 and CXCL6 levels are associated with poor prognosis[10]. Growth-related gene product (GRO) is a member of the CXC chemokine family, which is composed of GROα, GROβ and GROγ[12,13]. GROα is highly expressed in a variety of tumors and plays an important role in tumor proliferation[14], angiogenesis[15], and metastasis[16]. Compared with GROα, the roles of GROβ in tumors are poorly understood.

Recently, authors from South Africa reported that GROβ is highly expressed in ESCC tissues and cell lines[17]. Although this suggests that GROβ may be secreted from tumor tissues into the extracellular milieu, no report has yet investigated serum GROβ expression levels in human ESCC. In this study, we measured the serum levels of GROβ in ESCC patients and healthy controls by enzyme-linked immunosorbent assay (ELISA), and analyzed the association between clinical parameters of ESCC and serum GROβ levels.

Table 1 Clinical parameters of esophageal squamous cell carcinoma patients and healthy controls *n* (%)

	ESCC patients (*n* = 72)	Healthy controls (*n* = 83)
Age (yr)		
< 50	5 (7)	16 (19)
⩾ 50	67 (93)	67 (81)
Gender		
Male	52 (72)	45 (54)
Female	20 (28)	38 (46)
Histological grade		
Well	16 (22)	-
Well/moderate	2 (3)	-
Moderate	33 (46)	-
Moderate/poor	3 (4)	-
Poor	15 (21)	-
Unknown	3 (4)	-
Tumor size (cm)		
< 5	32 (44)	-
⩾ 5	37 (51)	-
Unknown	3 (5)	-
TNM stage		
Ⅰ	14 (19)	-
Ⅱ	21 (29)	-
Ⅲ	31 (43)	-
Unknown	6 (8)	-
Lymph node metastasis		
Yes	34 (47)	-
No	31 (43)	-
Unknown	7 (10)	-

ESCC: Esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Clinical specimen collection and preparation

Serum samples were collected from 72 ESCC patients [52 males and 20 females; mean age, 61 ± 7.7 years (SD); range 41-76 years] and 83 healthy controls [45 males and 38 females; mean age, 57 ± 8.4 years (SD); range 41-70 years] after the informed consent was obtained. Pathological diagnosis was independently conducted by two senior pathologists. The healthy individuals were negative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. Abdominal ultrasound examination, routine blood tests, and biochemistry tests were performed for the healthy controls, and the results were within normal ranges. Sample preparation was referred to the reference[18]. The clinical characteristics of the serum samples are shown in Table 1.

ELISA for GROβ

A human GROβ ELISA development kit (PeproTech, Rocky Hill, NJ, USA) was used to measure GROβ in human serum. Briefly, 96-well ELISA microplates were coated overnight with 100 μL GROβ antibody (PeproTech, Rocky Hill, NJ, USA) at a final concentration of 0.25 mg/L in PBS. After washing with PBS/0.05% (w/v) Tween-20 (PBST, pH 7.4), the wells were blocked with blocking buffer at room temperature for 1 h. Then, 100 μL diluted serum samples (at 1:5 dilution) were added and incubated at room temperature for 2 h. Similarly, 100 μL PBST lacking antibody was used as a negative control. Following three washes with PBST, 100 μL antibody diluted to a concentration of 0.25 mg/L was added. After incubation at room temperature for 2 h, 100 μL avidin-horseradish peroxidase-conjugated secondary antibody (at 1:2000 dilution) was added, and plates were incubated at room temperature for 30 min. The excess conjugate was removed by washing the plates three times with PBST. The amount of bound conjugate was determined by adding ABTS liquid substrate solution to each well, and plates were incubated at room temperature for color development. The absorbance was measured at 405 nm using a Model 680 microplate reader (Bio-Rad Lab. Inc., Hercules, CA, USA). All analyses were performed in triplicate. The coefficient of variation was lower than 15% between analyses.

Statistical analysis

Statistical significance was determined with the nonparametric Mann-Whitney *U*-test (differences between two groups) and the Kruskal-Wallis nonparametric test (differences among more than two groups) with SPSS 16.0 software (Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Serum GROβ levels in ESCC patients and healthy controls

A total of 155 participants were enrolled in this study. The serum levels of GROβ in ESCC patients ($n = 72$) and healthy controls ($n = 83$) were assessed by ELISA.

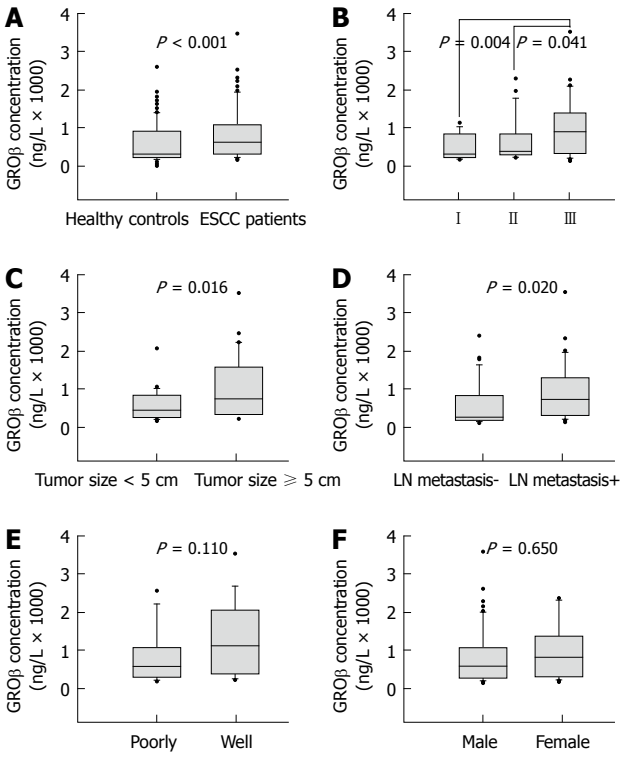

Figure 1 Correlations between serum growth-related gene product β level and clinicopathological parameters in esophageal squamous cell carcinoma. Serum growth-related gene product β (GROβ) levels were measured in esophageal squamous cell carcinoma (ESCC) patients (n = 72) and healthy volunteers (n = 83) using enzyme-linked immunosorbent assay. Serum levels of GROβ were significantly increased in ESCC patients (A). Serum levels of GROβ were correlated positively with tumor-node-metastasis stage (B), tumor size (C), and lymph node (LN) metastasis (D), but not with histological grade (E) or gender (F) in ESCC patients. The boxes represent the distribution of the 25th and 75th percentile data (the line in the box is the median, and the dots are the outlying points).

Serum concentrations of GROβ ranged from 146 ng/L to 3721 ng/L (median = 645 ng/L) in ESCC patients, and from 0 ng/L to 2781 ng/L (median = 269 ng/L) in healthy controls. GROβ levels were significantly higher in ESCC patients than in healthy controls (Mann-Whitney *U*-test, $P < 0.001$, Figure 1A). The sensitivity and specificity of serum GROβ were 73.61% and 56.63%, respectively.

Association between serum concentrations of GROβ and clinical parameters in ESCC

We assessed the potential correlations between serum levels of GROβ and clinical parameters, including tumor-node-metastasis (TNM) stage, tumor size, lymph node metastasis, histological grade, and gender. The median levels of GROβ in the ESCC patients at different stages were: stage I, 284 ng/L (range, 146-1141 ng/L); stage Ⅱ, 360 ng/L (range, 211-2453 ng/L); and stage Ⅲ, 966 ng/L (range, 165-3721 ng/L)". The serum GROβ levels were significantly different (Kruskal-Wallis nonparametric test, $P = 0.006$) among the three TNM stages, and significantly higher in patients at stage Ⅲ than in those at stage Ⅱ (Mann-Whitney *U*-test, $P = 0.041$, Figure 1B) and stage I (Mann-Whitney *U*-test, $P = 0.004$, Figure 1B). However, there was no significant difference between stage I and stage Ⅱ (Mann-Whitney *U*-test, $P = 0.135$). Furthermore, patients with tumor diameters ⩾ 5 cm had higher serum levels of GROβ (median = 786 ng/L; range, 191-3721 ng/L) than those with smaller tumors (median = 456 ng/L; range, 146-2008 ng/L; Mann-Whitney *U*-test, $P = 0.016$; Figure 1C). Serum levels of GROβ in patients with lymph node metastasis (median = 819 ng/L; range, 165-3721 ng/L) were significantly higher than those without lymph node metastasis (median = 330 ng/L; range, 146-2453 ng/L; Mann-Whitney *U*-test, $P = 0.020$; Figure 1D). However, there was no significant difference between serum levels of GROβ in patients with poorly differentiated tumors (median = 607 ng/L; range, 191-2705 ng/L) and well-differentiated tumors (median = 1178 ng/L; range, 239-3721 ng/L; Mann-Whitney *U*-test, $P = 0.110$, Figure 1E). Serum levels of GROβ were not gender dependent (Mann-Whitney *U*-test, $P = 0.650$, Figure 1F).

DISCUSSION

Identification of targets for early detection of ESCC is important to improve the prognosis of the patients with this pernicious disease. Currently, carcinoembryonic antigen, cytokeratin 19 fragments, and squamous cell carcinoma-associated antigen are routinely used as serum markers for detection of ESCC. Due to the low sensitivity and specificity of detection of these markers[19], additional serum markers must be established for early detection and diagnosis of ESCC.

GROβ, also known as the chemokine CXCL2, is a member of the CXC chemokine family and shares the same receptor with interleukin (IL)-8, interleukinIL-8RB/CXCR2. GROβ is widely expressed in the central nervous system[20] and possesses important functions, such as attracting neutrophils to sites of inflammation, mobilizing stem cells[21-25], and modulating neurotransmitter release[26]. The roles of GROβ in tumor formation and development have been investigated[27], and a recent report described significant elevation of GROβ mRNA in colon cancer tissues[28]. Upregulation of GROβ promotes colony formation by melanocytes in soft agar and tumor formation in nude mice[13,29]. Conversely, decreased expression of GROβ inhibits the proliferation and colonization capacity of esophageal cancer cells[30]. Through binding to its receptor CXCR2, GROβ forms an autocrine loop that activates the Ras-Erk1/2 signaling pathway, which is important for cell proliferation[17,25,26,31]. This pathway in turn activates the transcription and expression of the early growth response 1 gene (*egr1*), a transcription factor that regulates the expression of downstream factors related to cell growth and cell cycle regulation (p65, p27), thereby promoting tumor growth[32-34].

Using cDNA microarray analysis, authors from South Africa showed that GROβ was highly expressed in ESCC tissues and cultured cells[17]. As a chemokine, GROβ may be secreted into the extracellular matrix. However, no report has investigated the clinical significance of serum GROβ levels in ESCC. To compare the levels of serum GROβ in patients with ESCC and healthy controls, we measured

serum GROβ concentrations with ELISA. The level of serum GROβ was much higher in patients with ESCC than in healthy controls, which supported the hypothesis that GROβ may function as an oncogene in ESCC.

We assessed the potential correlations between serum levels of GROβ and several clinical parameters of ESCC, including tumor size, TNM stage, lymph node metastasis, histological grade and gender. Serum levels of GROβ were correlated significantly with tumor size and the extent of metastasis. Patients with larger tumors (≥ 5 cm in diameter) had higher levels of serum GROβ than those with smaller tumors ($P < 0.05$). Likewise, serum GROβ levels in patients with lymph node metastasis were significantly higher than those without lymph node metastasis ($P < 0.05$). The elevation of serum GROβ in ESCC patients may therefore reflect the enhanced potential of tumor cells to proliferate and metastasize, which is consistent with TNM staging[35]. Indeed, GROβ serum levels were significantly increased in stage Ⅲ patients compared with stage Ⅱ and stage Ⅰ patients ($P < 0.05$). The histological grade of tumors is also an important indicator associated with the development and prognosis of cancer[36]. However, no significant differences were found in the serum levels of GROβ in ESCC patients with different histological tumor grades ($P > 0.05$) in our study. Because our study involved a relatively small number of ESCC patients and the sensitivity and specificity of our assay to measure serum GROβ were not sufficiently high, further confirmation in a larger sample size is warranted.

In conclusion, we found that serum GROβ levels were increased in ESCC patients and correlated positively with tumor size, TNM stage and lymph node metastasis, but not with gender or the histological grade of ESCC. GROβ may function as an oncogene product and play a role in tumor formation, progression and metastasis. Examining and monitoring serum GROβ levels may be useful in estimating the prognosis of ESCC.

ACKNOWLEDGMENTS

We thank Drs. Yu-Lin Sun, You-Sheng Mao, Fang Liu and Lan-Ping Zhou for critical reading of the manuscript and sample preparations. Additional correspondence authorship: Zhao X, Center of Basic Medical Sciences, Navy General Hospital, Beijing 100048, China.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies in China, South Africa and some other developing countries. In China, prognosis of the patients with ESCC is poor, with a 5-year survival rate of less than 30%. It is important to find new markers, especially serum protein markers to facilitate the early detection and diagnosis of ESCC.

Research frontiers

Growth-related gene product β (GROβ) was reported to be highly expressed in ESCC tissues and cell lines. As a chemokine, GROβ could be secreted from cells to extracellular matrix. But no report has yet investigated serum GROβ expression levels in human ESCC. In the present study, serum level of GROβ between patients with ESCC and healthy controls were firstly measured, and the association between clinical parameters of ESCC and serum GROβ levels were also analyzed.

Innovations and breakthroughs

The serum level of GROβ was increased in ESCC patients and was positively correlated with tumor size, tumor-node-metastasis staging and lymph node metastasis of the patients. GROβ might serve as an oncogene and contribute to tumorigenesis and metastasis of ESCC.

Applications

Examining and monitoring serum GROβ levels are useful in estimating the prognosis of ESCC patients.

Terminology

GROβ, also known as CXCL2 chemokine, is a member of the CXC chemokine family. It is widespread in the central nervous system and involved in many important functions, such as attracting neutrophils to inflammation sites, mobilization of stem cells and neurotransmitter release modulation.

Peer review

GRO, a member of the CXC chemokine subfamily, plays a major role in inflammation and wound healing. CXC chemokines have been found to be associated with tumorigenesis, angiogenesis and metastasis. The roles of GROβ in tumor formation and development were unclear. The papers from tumor cell lines, animal and human studies on this field showed controversial results.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2663

World J Gastroenterol 2011 June 7; 17(21): 2663-2666
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Protective effects of 2,4-dihydroxybenzophenone against acetaminophen-induced hepatotoxicity in mice

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Received: October 28, 2010 Revised: November 21, 2010
Accepted: November 28, 2010
Published online: June 7, 2011

Abstract

AIM: To examine the effects of 2,4-dihydroxybenzophenone (BP-1), a benzophenone derivative used as an ultraviolet light absorbent, on acetaminophen (APAP)-induced hepatotoxicity in C57BL/6J mice.

METHODS: Mice were administered orally with BP-1 at doses of 200, 400 and 800 mg/kg body weight respectively every morning for 4 d before a hepatotoxic dose of APAP (350 mg/kg body weight) was given subcutaneously. Twenty four hours after APAP intoxication, the serum enzyme including serum alaine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) were measured and liver histopathologic changes were examined.

RESULTS: BP-1 administration dramatically reduced serum ALT, AST and LDH levels. Liver histopathological examination showed that BP-1 administration antagonized APAP-induced liver pathological damage in a dose-dependent manner. Further tests showed that APAP-induced hepatic lipid peroxidation was reduced significantly by BP-1 pretreatment, and glutathione depletion was ameliorated obviously.

CONCLUSION: BP-1 can effectively protect C57BL/6J mice from APAP-induced hepatotoxicity, and reduction of oxidative stress might be part of the protection mechanism.

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Key words: 2,4-dihydroxybenzophenone; Acetaminophen; Hepatotoxicity

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He YY, Zhang BX, Jia FL. Protective effects of 2,4-dihydroxybenzophenone against acetaminophen-induced hepatotoxicity in mice. *World J Gastroenterol* 2011; 17(21): 2663-2666 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2663.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2663

INTRODUCTION

Acetaminophen (APAP), which is also named paracetamol, is a widely used antipyretic and analgesic drug. However, it can cause hepatic necrosis and even death in human and experimental animals when taken in overdose[1]. Overdose of APAP can lead to acute liver injury and histopathological changes characterized by centrilobular necrosis[2,3]. Most of APAP is cleared by glucuronic acid or sulphate combination while a part of APAP is metabolized by cytochrome P450 system and generates reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI can be conjugated by glutathione and hence excreted from the body as sulfhydryl adducts. If the formation of NAPQI is beyond the clearing capacity of reduced glutathione (GSH), NAPQI combined with liver cellular protein will cause liver cell injury[4,5]. APAP has been widely used to establish acute liver injury models for liver protective agent research. Since C57BL/6J mice were more susceptible to APAP, C57BL/6J mice were used in the present study.

2,4-dihydroxybenzophenone (BP-1), a benzophenone derivative, is often used as an ultraviolet light absorbent. BP-1 has also been used in some products such as nail polish, polish remover, shaving cream, body cleanser and so on. Previous researches about BP-1 mainly focused on its estrogenic activities. Matsumoto *et al*[6] demonstrated that BP-1 exhibited estrogenic activities by estrogen receptor using MCF-7 (a human breast cancer cell line) cell proliferation assay. In this study, we examined the effects of BP-1 in APAP-induced hepatotoxicity in mice.

MATERIALS AND METHODS

Chemicals

BP-1 was purchased from Huangshi Meifeng Chemical Company (Huangshi, China) and APAP from Jiaozuo Xin'an Science and Technology Company (Jiaozuo, China). BP-1 suspension was prepared with 1% Tween80 from Amresco Company (Solon, USA). TBARS assay kit was purchased from Cell Biolabs (San Diego, USA). Glutathione assay kit was purchased from Calbiochem (Darmstadt, Germany).

Animals and treatment

Healthy and clean male C57BL/6J mice weighing 18-22 g were used in all experiments. They were bought from Peking University Laboratory Animal Department. Mice were housed in a well-ventilated room and fed adaptively for 3 d before experiment. Room temperature was controlled at 21-25℃ with a 12 h/12 h light-dark cycle and humidity at 65%-70%. All mice were allowed free access to water and fed with forage supplied by Laboratory Animal Center of Academy of Military Medical Sciences.

Thirty mice were randomly divided into 5 groups. Group 1 was vehicle control group and Group 2 was APAP alone group, both of which administered intragastrically with 1% Tween80 for 4 d. Groups 3, 4 and 5, administered intragastrically with BP-1 at doses of 200, 400 and 800 mg/kg body weight respectively for 4 d. On the 4th day, all mice except those in vehicle control group were injected with APAP (350 mg/kg body weight) subcutaneously 30 min after the final administration. Twenty-four hours after APAP injection, blood samples were collected from orbital venous plexus. Then all mice were sacrificed, and livers were moved out immediately and washed with saline, dried on a filter paper and weighed. Liver samples were prepared for further tests. The animal experiments and surgical procedures were all performed in compliance with the Guidelines for Animal Care and Use issued by Peking University.

Biochemical test

The serum enzyme levels including serum alaine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined by HITACHI-7170A automatic analyzer. Liver homogenization was carried out in ice-cold KCl solution (0.15 mol/L) or phosphate buffered solution to yield a 5% (w/v) tissue homogenates for malonaldehyde (MDA) and GSH tests. The MDA, GSH and oxidized glutathione (GSSG) levels in liver tissues were determined according to the manufacturer's protocols. GSH/GSSG ratio was calculated.

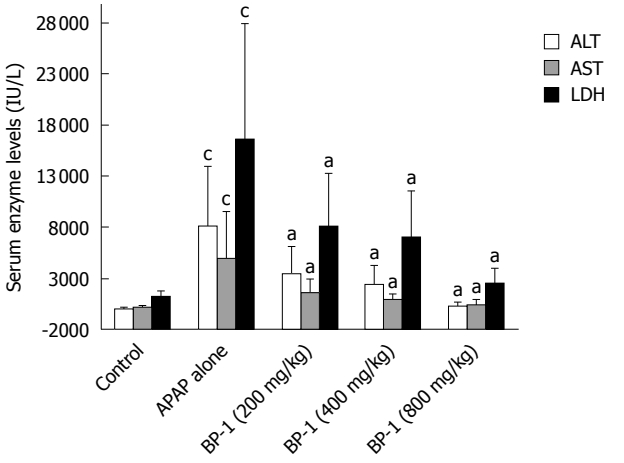

Figure 1 Effects of 2,4-dihydroxybenzophenone administration on serum levels of alaine aminotransferase, aspartate aminotransferase and lactate dehydrogenase in mice. Acetaminophen (APAP) was administered at 350 mg/kg sc and 2,4-dihydroxybenzophenone (BP-1) was administered ig at doses of 200, 400 and 800 mg/kg respectively 4 d before acetaminophen treatment. Alaine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase(LDH) levels were measured 24 h after acetaminophen treatment. Results were expressed as mean ± SE and data was analyzed by one-way analysis of variance using SPSS 13.0 software. $^{a}P < 0.05$ *vs* acetaminophen group; $^{c}P < 0.05$ *vs* control group.

Histological examination

The left liver lobes were cut out and fixed in 10% formalin solution. After pathological sectioning and HE staining, liver histopathologic changes were examined under inverted phase contrast microscope.

Data treatment and statistical analysis

Data were expressed as mean ± SE. Statistical comparison between groups was performed by one-way analysis of variance (ANOVA) with SPSS 13.0 software. Significance was accepted at $P < 0.05$.

RESULTS

BP-1 administration reduced ALT, AST and LDH

Twenty-four hours after a single dose of APAP (350 mg/kg body weight), serum levels of ALT, AST and LDH increased significantly ($P < 0.05$), showing characteristic acute liver injury induced by APAP. Compared with APAP group, BP-1 groups lowered serum levels of ALT, AST and LDH dramatically. At a dose of 200 mg/kg body weight, the difference was not significant; at doses of 400 and 800 mg/kg body weight, there were significant differences between BP-1 groups and APAP group ($P < 0.05$). The effect of BP-1 was dose-dependent and serum levels of ALT, AST and LDH in the highest dose groups were comparable to that of vehicle control group ($P > 0.05$) (Figure 1).

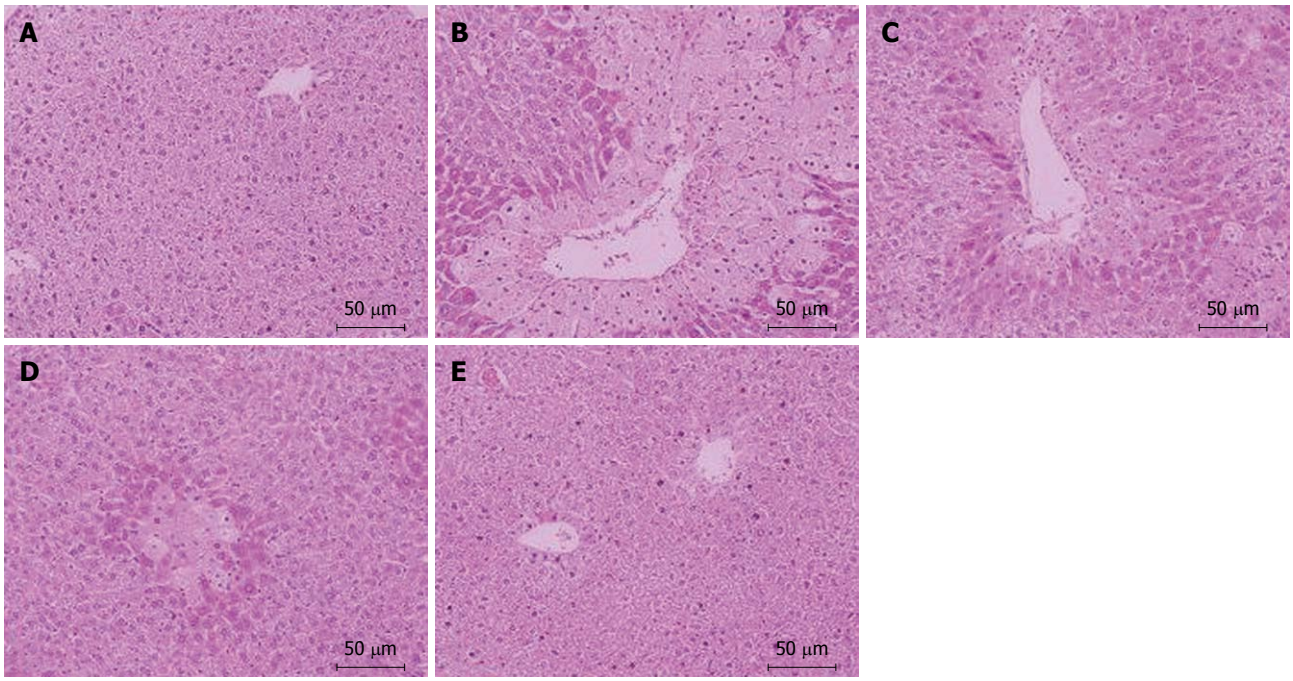

Figure 2 Liver histology of all groups 24 h after acetaminophen treatment (350 mg/kg). Hematoxylin-eosin-stained liver sections displayed representative hepatocellular morphological changes. Original magnification × 200. A: Liver section in the vehicle control group showed a normal lobular structure; B: Liver section in acetaminophen alone group showed large areas of centrilobular necrosis, vacuolar degeneration and inflammatory cell infiltration; C: Liver section in the 200 mg/kg group showed necrosis with the same degree as in acetaminophen group; D: Liver section in the 400 mg/kg group showed a significant alleviation of liver injury; E: Liver section in the 800 mg/kg group showed absence of necrosis and almost normal lobular structure.

Table 1 Effects of 2,4-dihydroxybenzophenone administration on malonaldehyde level and GSH/GSSG ratio in mice

Groups	MDA (μmol/g)	GSH/GSSH ratio
Control	0.30 ± 0.27	10.5 ± 2.7
Acetaminophen	0.90 ± 0.70[c]	7.1 ± 2.1[c]
BP-1 (200 mg/kg)	1.08 ± 0.68	5.1 ± 1.4[a]
BP-1 (400 mg/kg)	0.58 ± 0.33	6.8 ± 1.5
BP-1 (800 mg/kg)	0.50 ± 0.10	9.6 ± 1.9[a]

[a]$P < 0.05$ *vs* acetaminophen alone group; [c]$P < 0.05$ *vs* control group. BP-1: 2,4-dihydroxybenzophenone; MDA: Malonaldehyde.

Effect of BP-1 administration in liver tissue MDA and GSH/GSSG ratio

Compared with vehicle control group, MDA content in liver tissue of APAP alone group increased while the ratio of GSH/GSSG decreased dramatically ($P < 0.05$), indicating an oxidation stress and depletion of GSH. With increasing dose of BP-1, MDA level decreased and GSH/GSSG ratio increased. In the highest dose group (800 mg/kg body weight), the MDA and ratio of GSH/GSSG were close to the normal level as shown in vehicle control group (Table 1).

BP-1 alleviated pathological injury induced by APAP

Observed by naked eyes, the livers of vehicle control group were deep red, moist, glossy and resilient. In APAP group, the livers lost luster and yellow necrosis foci were often found on the surface. Liver injury of BP-1 pretreated mice was attenuated dramatically in a dose-dependent manner.

Under light microscope, liver lobular structures in vehicle control group were clear and regular, and single layer of hepatocytes arranged around the central vein in a radical pattern. There were abundant basophilic granular cytoplasms in the hepatocytes (Figure 2A). In APAP-intoxicated mice, normal liver lobular structures were damaged and collapsed. The hepatocytes showed vacuolization, sinusoidal dilation and congestion. Infiltration of inflammatory cells and loss of cell boundaries were also observed (Figure 2B). Pre-administration of BP-1 showed mild injuries in a dose-related manner. BP-1 at 200 mg/kg could not effectively prevent the damage (Figure 2C). There was a moderate injury in the 400 mg/kg group (Figure 2D) while in the 800 mg/kg group liver lobular structure was well comparable to that in the vehicle control group (Figure 2E).

DISCUSSION

APAP is often used in animal experiments to establish acute liver injury models. Our previous researches found that C57BL/6J mice were more susceptible to APAP[7], so C57BL/6J mice were chosen in the present study as experimental animals. The serum levels of ALT, AST and LDH are the main indexes which reflect liver injury[8]. Our study demonstrated that BP-1 protected mice from APAP-induced acute liver injury as shown by the significant decrease in serum ALT, AST and LDH. BP-1 could also antagonize tetrachloromethane-, cocaine- and thioacetamide-induced acute liver injuries, which was evidenced by reduced serum ALT, AST and LDH levels, as

well as ameliorated pathological changes in the liver (data not shown).

BP-1 showed a broad protective effect in hepatotoxic chemicals-induced acute liver injury, implying that it may target on the oxidative stress, which was the key event in liver injuries caused by these hepatotoxic chemicals. To confirm the hypothesis, we examined the content of MDA in liver tissues which was often used as a biomarker to measure the level of oxidative stress in organisms[9], as well as the GSH/GSSG ratio which indicated the capability to antagonize the oxidative injury[10]. The results demonstrated that, MDA levels in APAP-intoxicated mice increased obviously and GSH/GSSG ratio decreased, implying the existence of oxidative damage and depletion of GSH. However, pre-administration of BP-1 decreased the MDA level and increased GSH/GSSG ratio in a dose-dependent manner. The results indicated that the hepatoprotective effect of BP-1 was associated with its antioxidant activity.

COMMENTS

Background

Acetaminophen (APAP)-induced acute liver injury model has been widely used as hepatoprotective agent screening. The authors have conducted the screening for hepatoprotective agents for a long time. To the best of their knowledge, there has been no report about the hepatoprotective effects of 2,4-dihydroxybenzophenone (BP-1) up to date.

Innovations and breakthroughs

This study shed a light on the hepatoprotective effects of BP-1. The results indicated that the hepatoprotective effect of BP-1 was associated with its antioxidant activity.

Applications

This study provided evidence for the protective effect of BP-1 against APAP-induced liver injury, which implied that BP-1 might be a promising agent for acute liver injury in human. Of course, before possible clinical use, more researches are needed to confirm that BP-1 is both effective and safe for humans.

Peer review

The authors investigated the effect of BP-1 pretreatment on APAP-induced hepatotoxicity in mice. The results demonstrated that: BP-1 pretreatment antagonized APAP-induced liver pathological damage; BP-1 pretreatment reduced APAP-induced hepatic lipid peroxidation; and BP-1 pretreatment ameliorated glutathione depletion.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2667

World J Gastroenterol 2011 June 7; 17(21): 2667-2673
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

PAd-shRNA-PTN reduces pleiotrophin of pancreatic cancer cells and inhibits neurite outgrowth of DRG

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Received: July 12, 2010 Revised: October 19, 2010
Accepted: October 26, 2010
Published online: June 7, 2011

Abstract

AIM: To investigate the silencing effects of pAd-shRNA-pleiotrophin (PTN) on PTN in pancreatic cancer cells, and to observe the inhibition of pAd-shRNA-PTN on neurite outgrowth from dorsal root ganglion (DRG) neurons *in vitro*.

METHODS: PAd-shRNA-PTN was used to infect pancreatic cancer BxPC-3 cells; assays were conducted for knockdown of the *PTN* gene on the 0th, 1st, 3rd, 5th, 7th and 9th d after infection using immunocytochemistry, real-time quantitative polymerase chain reaction (PCR), and Western blotting analysis. The morphologic changes of cultured DRG neurons were observed by mono-culture of DRG neurons and co-culture with BXPC-3 cells *in vitro*.

RESULTS: The real-time quantitative PCR showed that the inhibition rates of PTN mRNA expression in the BxPC-3 cells were 20%, 80%, 50% and 25% on the 1st, 3rd, 5th and 7th d after infection. Immunocytochemistry and Western blotting analysis also revealed the same tendency. In contrast to the control, the DRG neurons co-cultured with the infected BxPC-3 cells shrunk; the number and length of neurites were significantly decreased.

CONCLUSION: Efficient and specific knockdown of PTN in pancreatic cancer cells and the reduction in PTN expression resulted in the inhibition of neurite outgrowth from DRG neurons.

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Key words: Pancreatic cancer; Pleiotrophin; RNA interference; Neurite outgrowth; Dorsal root ganglion

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INTRODUCTION

Pancreatic cancer is one of the most aggressive and intractable human malignant tumors[1,2]. Perineural invasion extending into the pancreatic nerve plexus is a histopathologic characteristic of pancreatic cancer. However, the mechanisms contributing to the invasion of intrapancreatic nerves and spread of cancer cells along extrapancreatic

nerves in the course of pancreatic cancer are still poorly understood. A family of proteins consisting of neurotrophic factors is of interest due to recent experimental data that showed their involvement in the neural invasion of pancreatic cancer[3,4].

Pleiotrophin (PTN) is a type of neurotrophic factor and is also known as a neurite growth-promoting factor. Human, mouse and rat PTN proteins are identical, and they share a 45% amino acid similarity with midkine-another member of this family[5,6]. PTN could promote neurite outgrowth in primary cultures of cortical neurons[7] and neuronal survival[8]. PTN is mainly expressed during early embryogenesis. In human adult tissues, PTN is markedly down-regulated and present only at minimal levels in very few tissues. It is not expressed in normal pancreatic tissues, but highly expressed in pancreatic cancer cells[9]. Kinnunen *et al* found a high expression level of PTN in 78% of the tumor samples from pancreatic cancer patients[10]. PTN and N-syndecan act as a receptor-ligand pair in neurite outgrowth. Anti-N-syndecan antibodies added to the culture media had an inhibitory effect on PTN-induced neurite outgrowth[11]. Therefore, PTN may play an important role in the neural invasion of pancreatic cancer.

RNA interference (RNAi) is a process during which double-stranded RNA induces the homology-dependent degradation of cognate mRNA[12]. In some organisms, the introduction of double-stranded RNA has been proven to be a powerful tool for suppressing gene expression through a process known as RNAi[13]. However, in most mammalian cells, it provokes a strong cytotoxic response[14]. This nonspecific effect could be circumvented using synthetic small interfering RNA (siRNA), which could mediate strong and specific suppression of gene expression[15]. Transfection of chemically synthesized siRNA is routinely used for gene silencing[16]. To circumvent the high cost of synthetic siRNA and establish stable gene knock-down cell lines by siRNA, several adenovirus vector systems, such as the BLOCK-iT™ Adenoviral RNAi Expression System, have been designed to produce siRNA intracellularly. The BLOCK-iT™ Adenoviral RNAi Expression System combines Invitrogen's BLOCK-iT™ RNAi and ViraPower™ Adenoviral technologies to facilitate the creation of a replication-incompetent adenovirus that delivers a synthesized short hairpin RNA (shRNA) of interest to dividing or non-dividing mammalian cells for RNAi analysis. These RNAi expression vectors are highly efficient for *in vitro* and *in vivo* gene transfer into a variety of mammalian cells and tissues and have been used in functional and gene therapy studies[17,18].

In this study, we investigated the silencing effects of pAd-shRNA-PTN on the *PTN* gene in the pancreatic cancer cell line BxPC-3, and observed the inhibition of neurite outgrowth from dorsal root ganglion (DRG) neurons *in vitro*. Our results demonstrated efficient and specific knockdown of *PTN* in the BxPC-3 pancreatic carcinoma cell line, and suggested that PTN is an attractive new target for the study of neural invasion in pancreatic cancer gene therapy.

MATERIALS AND METHODS

Cell lines, medium and reagents

The BLOCK-iT™ Adenoviral RNAi Kits (pAd/BLOCK-iT™-DEST Gateway® Vector Kit; Gateway® LR Clonase™ Enzyme Mix; 293A Cell Line; and BLOCK-iT™ U6 RNAi Entry Vector Kit) and Lipofectamine™ 2000 were purchased from Invitrogen Corp. (Carlsbad, California, USA). Monoclonal mouse antihuman PTN, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, and the secondary antibody (peroxidase-coupled goat anti-mouse IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The human pancreatic cancer cell line BxPC-3 was purchased from the American Type Culture Collection. The BxPC-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) obtained from Life Technologies (Carlsbad, California, USA). Fetal calf serum (FCS) and Ham's F_{12} medium were purchased from Gibco BRL (Carlsbad, California, USA).

Construction of recombinant adenovirus pAd-shRNA-PTN

The construction procedure for recombinant adenovirus pAd-shRNA-PTN has been described previously[19]. Briefly, according to data on the human pleiotrophin mRNA (GenBank accession no. NM002825), four pairs of complementary single-stranded oligonucleotides (ss oligos) were designed and synthesized using the Invitrogen's RNAi Designer online. Then, the ss oligos were annealed to create double-stranded oligonucleotides (ds oligos). The four ds oligos were cloned into the pENTR/U6 vector to produce four shuttle plasmid pENTR/U6-shRNAs that were transduced into the BxPC-3 cells by Lipofectamine™ 2000 after sequencing, to identify and select the shuttle plasmids showing optimal silencing effect. Oligo-3 was proven to have an optimal silencing effect. The sense and antisense strands of oligo-3 were 5'-CACCGCCAGAAGACTGTCACCATCTCGAAAGATGGTGACAGTCTTCTGGC-3' and 5'-AAAAGCCAGAAGACTGTCACCATCTTTCGAGATGGTGACAGTCTTCTGGC-3'. Then, the attL and attR (LR) recombination reaction was performed. Recombinant adenovirus pAd-shRNA-PTN was produced and amplified in HEK 293A cells; the viral titers were determined by TCID50 assays[20]. The construction of pAd-shRNA-PTN was confirmed *via* electron microscopic observation.

Procedure for infecting the BxPC-3 cells

The BxPC-3 cells were placed at a concentration of 1×10^6 cells per well into a 6-well plate, and 2 mL of normal DMEM along with 10% FCS was added into each well. On the day of infection (Day 0), 5 μL adenoviral stock (1×10^{10} pfu/mL) was added into a 2 mL fresh culture medium with 2% FCS (at a multiplicity of infection (MOI), i.e. an MOI of 50). Then, we removed the previous culture medium from the cells, mixed the medium containing virus gently, added it to each cell, and incubated it at 37℃ overnight. The following day (Day 1), we removed

the medium containing virus and replaced it with fresh DMEM containing 2% FCS. The cells were harvested on the 0th, 1st, 3rd, 5th, 7th, and 9th d after infection and assayed for knockdown of the *PTN* gene by immunocytochemistry, real-time quantitative polymerase chain reaction (PCR), and Western blotting analysis. In this study, we regarded the cells harvested on the 0th d after infection as the control.

Immunocytochemistry

Immunostaining of the human pancreatic cancer cell line BxPC-3 was performed on the 0th (control), 3rd, and 5th d after infection. The BxPC-3 cells were grown on glass slides and fixed with acetone; endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol. The sections were washed and incubated overnight with a 1:20 dilution of anti-PTN antibody at 4℃. After subsequent wash in phosphate buffered saline (PBS), the secondary antibody was added and incubated for 1 h at room temperature. After another wash in PBS, the peroxidase activity was localized by staining with diaminobenzidine as the substrate. Then, the sections were rinsed in water, dried, and covered.

Total RNA isolation and real-time quantitative PCR

On the 0th, 1st, 3rd, 5th, 7th, and 9th d after infection, the medium was removed from each well. Total RNA from each plate of BxPC-3 cells was extracted (RNeasy Mini Kit, Qiagen). The extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was verified by calculating the ratio between the absorbance values at 260 and 280 nm. This ratio ranged between 1.83 and 2.00, demonstrating the high quality of the RNA. Reverse transcription reactions were performed at 50℃ for 30 min. The reaction mixtures were heated to 95℃ for 15 min to activate HotStarTaq (Qiagen). The forward and reverse primers of PTN were 5'-TCCTAGTATTTTTTTCCTCAG-3' and 5'-CTTGTTTTCTGCCAATAG-3', respectively; the forward and reverse primers of GAPDH were 5'-TCATCCCTGCCTCTACTG-3' and 5'-TGCTTCACCACCTTCTTG-3', respectively. PCR amplification was performed in a total volume of 20 μL: 4.4 μL PCR master mix (TaKaRa Ex Taq R-PCR Version, TaKaRa), 10 pmol of each primer, 2 mmol $MgCl_2$, 2 μL (1:15000 dilution) SYBr Green I (SYBr Green I Nucleic Acid Gel Stain, Takara), and distilled water. PCR was performed using the ABI PRISM 7700 Sequence Detection System under the following conditions: 95℃ for 30 s and 35 cycles at 95℃ for 0 s for instrument setting to 0 s, 57℃ for 5 s, and 72℃ for 10 s. Cycle threshold (CT) values were obtained from the BIO-RAD iQ5 2.0 Standard Edition Optical System Software (BIO, RAD, USA). Data were analyzed using the ΔΔCT method and GAPDH served as an internal control.

Western blotting analysis

The cells were harvested on the 0th, 1st, 3rd, 5th, 7th, and 9th d after infection, washed once with cold PBS (pH 7.0), and lysed in a lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, and 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). The analysis was performed on all the lysates with equal amounts of protein (20 μg per lane), quantified by colorimetric detection based on the bicinchoninic acid (BCA) test. The samples were heated at 95℃ for 5 min and loaded on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Following electrophoresis, the separated protein fractions were transferred onto a methanol-activated polyvinylidene fluoride (PVDF) membrane and incubated with anti-PTN and anti-GAPDH antibodies, followed by incubation with the corresponding secondary antibodies. The bands were visualized using the enhanced chemiluminescence system.

Monoculture of DRG neurons and their coculture with BxPC-3 cells

DRG neurons were dissociated from a 16-d rat fetus. An average of approximately 300 DRG neurons were plated on the poly-L-lysine (200 μg/mL)-coated glass cover slips (13 mm in diameter) placed in a 6-well plate and maintained overnight at 37℃ in a humidified incubator gassed with 5% CO_2 in air. Following this overnight setting period, co-cultures of the DRG neurons and BxPC-3 cells were prepared. BxPC-3 cells plated at a concentration of 1×10^5 cells per well in a 6-well plate and 2 mL of DMEM/F_{12} medium with 10% FCS were added into each well. On the day of infection (Day 0), 50 μL adenoviral stock and 2 mL fresh DMEM/F_{12} medium containing 2% FCS (at an MOI of 50) were added to the infected groups and 2 mL fresh DMEM/F_{12} medium containing 2% FCS but without adenoviral stock was added to the control (uninfected) group. The previous culture medium was removed from the cells, and the fresh medium was added to each cell. The plate was swirled gently to disperse the medium. After incubating at 37℃ for 6 h, the glass cover slips with 300 DRG neurons were plated on it. The two cell types were cocultured for 7 d in the DMEM/F_{12} medium containing 2% FCS and maintained at 37℃ in a humidified incubator with 5% CO_2 in air.

Statistical analysis

The images obtained from the Western blotting were scanned and analyzed using the Quantity One software. The ratios of PTN to GAPDH were calculated. The statistical significance between the control and infected groups was calculated using one-way ANOVA test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Transmission electron microscopy

Two days after infection, we were able to find characteristic morphological changes. There were lots of scattered virus capsids in the nucleus, next to the nuclear membrane cytoplasm with few virus capsids (Figure 1A). At highest magnifications (× 60000), the capsids showed no

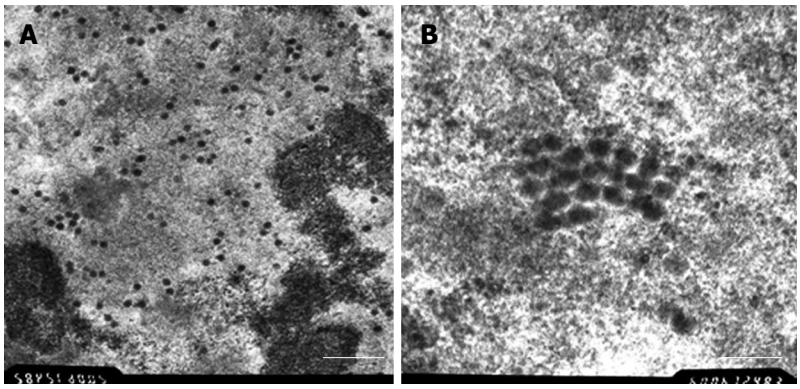

Figure 1 Morphological changes of recombinant adenovirus pAd-shRNA-pleiotrophin in HEK 293A cells. Scale bars: 600 nm (A) and 200 nm (B).

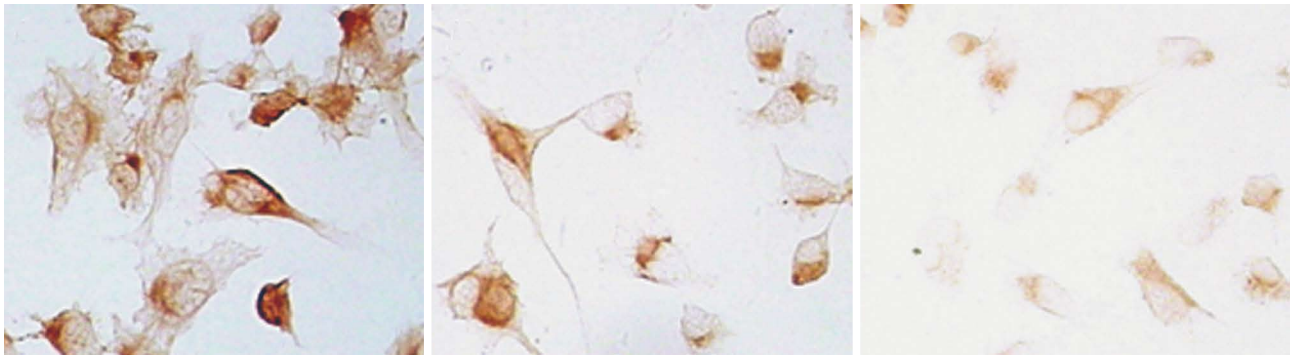

Figure 2 Pleiotrophin immunostaining of BxPC-3 human pancreatic cancer cells (× 400).

connection to each other. Virus capsids were 50-70 nm with an almost round appearance (Figure 1B).

PTN expression in pancreatic cancer cells

Using immunocytochemistry, we found a strong staining pattern for PTN in the cytoplasm of normal BxPC-3 cells (control, Figure 2A). The silencing rate was determined in comparison with the control alone. The expression rate of the PTN protein on the membrane was decreased by 30% (3 d, Figure 2B) and 70% (5 d, Figure 2C).

Down-regulation of PTN mRNA expression in the pancreatic cancer cell line BxPC-3

The efficacy of pAd-shRNA-PTN with regard to knockdown of PTN mRNA was confirmed through real-time quantitative PCR analysis. The melting temperature range of PTN was 86.5-87.5℃, and the melting temperature range of GAPDH was 89.5-90.5℃. No nonspecific amplification was observed. High expression levels of PTN mRNA were observed in the control. The inhibition rates of PTN mRNA expression in the BxPC-3 cells were 20%, 80%, 50%, and 25% on the 1st, 3rd, 5th and 7th d after infection. The PTN mRNA level was decreased on the 1st d, and down-regulation of PTN mRNA expression was most obvious on the 3rd post-infection day. On the 5th d, the PTN mRNA level increased gradually, and the PTN mRNA expression approximately returned to the level of normal BxPC-3 cells on the 9th post-infection day (Figure 3).

Western blotting analysis of the down-regulation of PTN protein expression in the pancreatic cancer cell line BxPC-3

Western blotting analysis of the anti-PTN-specific antibodies revealed that PTN protein expression in BxPC-3 cells infected with pAd-shRNA-PTN was markedly suppressed on days 3-7 compared with the control (0 d). The inhibition rates of PTN protein expression were 47.5%, 80.5% and 20%, respectively, on the 3rd, 5th and 7th d following pAd-shRNA-PTN infection. The maximal knockdown level was observed on the 5th post-infection day. The PTN protein levels began to rise on the 7th post-infection day (Figure 4).

Co-cultures of DRG neurons and BxPC-3 cells

Healthy DRG neurons began to adhere and extend their processes within 3 h of plating. Within 24 h, the neurons were mainly adherent. On the 1st d, the DRG neurons of the monoculture were mostly adherent, and were round or elliptical in shape and approximately 25 μm in diameter. Decades of them gathered together, and no neurite outgrowth was observed from DRG neurons; glial and Schwann cells were also not observed (Figure 5A). During cultivation, morphological changes in the DRG neurons were not obvious. On the 7th d, the number of neurons decreased, and very few neurons extended neurites (Figure 5B); these neurites were significantly shorter than those extending from the neurons co-cultured with normal BxPC-3 cells

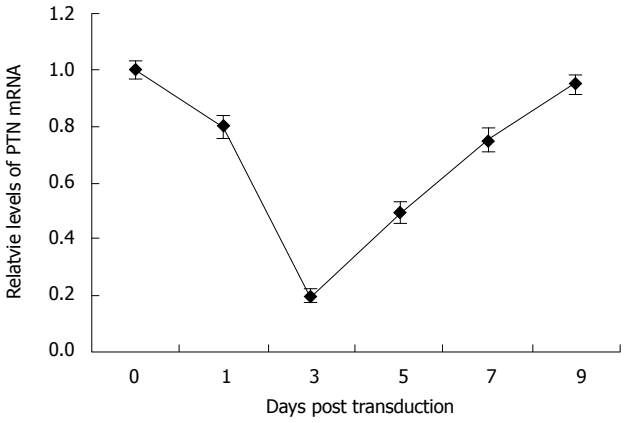

Figure 3 Inhibitory effects on pleiotrophin mRNA expression in the pancreatic cancer cell line BxPC-3. PTN: Pleiotrophin.

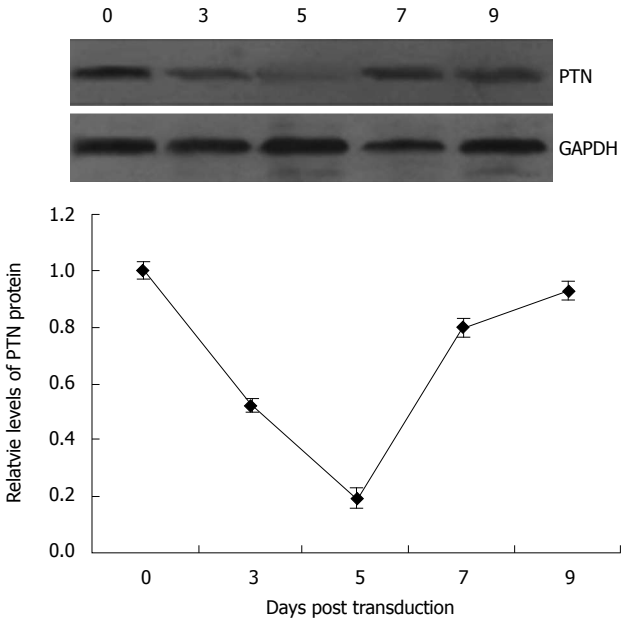

Figure 4 Inhibitory effects on pleiotrophin protein expression in the pancreatic cancer cell line BxPC-3. PTN: Pleiotrophin.

(Figure 5D). On the 7th d after co-culture with BxPC-3 cells, the control (co-cultured with normal BxPC-3 cells) DRG neurons were larger in size and irregular in shape. Robust neurite outgrowth was observed, with extensive outgrowth forming reticulate links and neural networks (Figure 5C). In contrast to the control, the DRG neurons co-cultured with the infected BxPC-3 cells shrunk; the number of neurons that extended neurites was significantly decreased, as was the length of the neurites. No neural networks were formed (Figure 5D).

DISCUSSION

Pancreatic cancer is characterized by perineural invasion[21], early lymph node metastasis, and poor prognosis[22,23]. Perineural invasion is an important cause of local recurrence, but little is known about its mechanism. The perineural invasion originates from not only the extrapancreatic nerve but also the intrapancreatic nerve in pancreatic carcinoma[24]. It is likely that as a neurite growth-promoting factor, PTN acts synergistically to promote the development of perineural invasion in pancreatic cancer. Weber *et al*[9] showed that PTN is expressed in gastrointestinal and, particularly, in pancreatic cancer cells. Using immunocytochemistry, 10 different human pancreatic cancer cell lines were analyzed for PTN expression. Six cell lines were positively stained for PTN: A816-4, BxPC-3, PancTu1, SW850, Panc89, and Colo357[9]. In this study, we also found that PTN was clearly expressed in BxPC-3 cells.

In cancer gene therapy, the success of a strategy depends on its cancer specificity and efficient delivery into mammalian cells. In this study, the RNAi technique and recombinant adenovirus were used to achieve this. The availability of a high virus titer and infection of a broad spectrum of cell types makes adenovirus the vector of choice for siRNA delivery. For example, oncogenic K-ras and other genes could be specifically and stably inactivated in human pancreatic cancer cells using a viral RNA interference (RNAi) vector, leading to loss of tumorigenicity[25]. The construction of recombinant adenovirus was confirmed *via* electron microscopic observation. The pAd/BLOCK-iT™ adenoviral construct is replication-incompetent and does not integrate into the host genome. Therefore, once transduced into mammalian cells, the shRNA of interest will be expressed as long as the viral genome is present. In the actively dividing cells, the adenovirus genome is gradually diluted as cell division occurs, resulting in an overall decrease in shRNA expression over time, i.e. the target protein levels generally return to background levels within 1-2 wk following transduction. For reducing the dilution, we cultured BxPC-3 cells in DMEM/F_{12} medium containing 2% FCS to reduce cell division. Besides, we used an MOI of 50 to obtain the optimal degree of target gene knockdown. In this study, we found that *PTN* gene knockdown in BxPC-3 cells might be detectable (20%) by real-time quantitative PCR on the 1st post-infection day, with maximal knockdown levels (80%) observed on the 3rd post-infection day. On the 5th d, as the adenovirus genome was diluted, the level of PTN mRNA was increased gradually and the PTN mRNA expression returned to the level observed in control BxPC-3 cells on the 9th post-infection day. Immunocytochemistry and Western blotting analysis also revealed the same tendency, except that the maximal knockdown levels of PTN protein were observed two days later; this may be related to the translation process of the protein.

In the nervous system, PTN has been shown to induce neurite outgrowth from dopaminergic neurons[8,26]. PTN and its receptor, N-syndecan, are important in promoting neurite outgrowth. N-syndecan expression was stronger in both the brain and spinal cord during the later developmental period (days 14-16 of gestation); N-syndecan isolated from a 16-d-old rat fetus bound strongly to PTN[27]. Anti-N-syndecan antibodies bound to the surface of the neurites and also perturbed neurite growth[28]. PTN was also involved in the peripheral nerve regeneration following nerve injury and functional recovery following neural transplantation in rats[29,30]. Taken together, the available

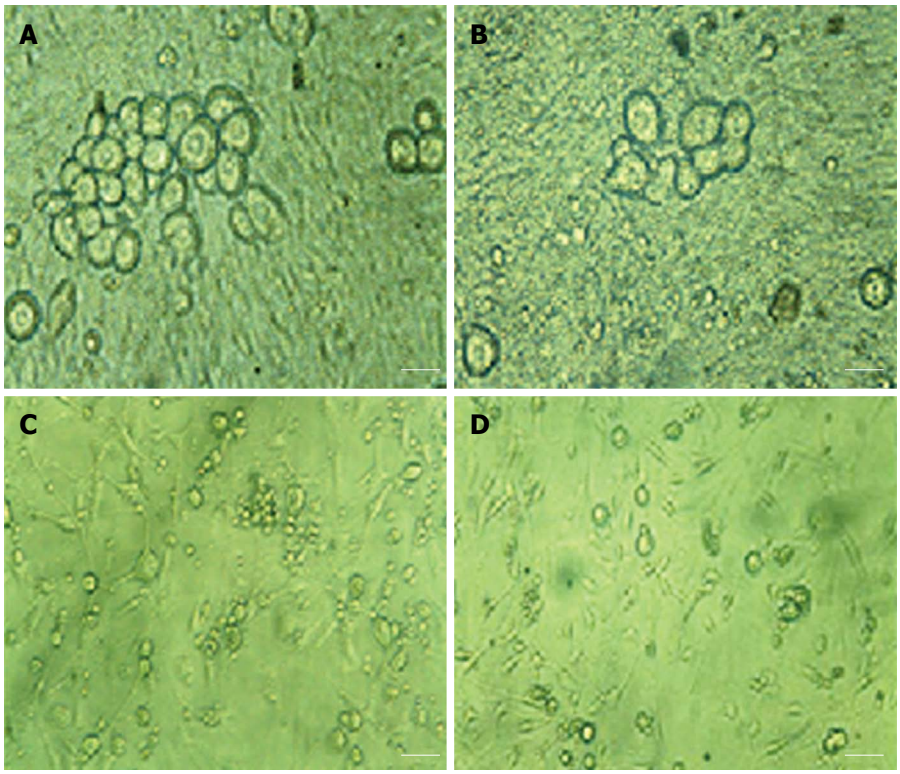

Figure 5 Morphological changes in dorsal root ganglion neurons. A: Monoculture of dorsal root ganglion (DRG) neurons on the 1st d (× 400); B: Monoculture of DRG neurons on the 7th d (× 400); C: Co-culture of DRG neurons and infected BxPC-3 cells (× 200); D: Co-culture of DRG neurons and normal BxPC-3 cells (× 200). Scale bars: 25 μm (A, B) and 50 μm (C, D).

evidence suggests that PTN bound to N-syndecan can promote neurite outgrowth from DRG neurons. In this study, we selected DRG neurons for investigation because they may play a role in the neural invasion of pancreatic cancer[31]. We dissociated DRG neurons from a 16-d-old rat fetus for monoculture or co-culture with BxPC-3 cells. The results indicated that pAd-shRNA-PTN could also be used to infect the BxPC-3 cells and suppress *PTN* gene expression efficiently. In contrast to the number of control cells, the number of DRG neurons co-cultured with infected BxPC-3 cells and extended neuritis significantly decreased. These results were in agreement with those of our previous studies[19].

In conclusion, the adenoviral construct pAd-shRNA-PTN showed efficient and specific knockdown of *PTN* in the pancreatic cancer cell line BxPC-3, and the inhibition of neurite outgrowth from DRG neurons was observed *in vitro*. Therefore, the *PTN* gene and pAd-shRNA-PTN may be very important for the research on the neural invasion of pancreatic cancer. We will set up a model of pancreatic cancer *in situ* in nude mice in the future studies. By injecting pAd-shRNA-PTN stock, we can investigate the effects of the *PTN* gene and adenoviral construct pAd-shRNA-PTN on the neural invasion of pancreatic cancer.

COMMENTS

Background

Pancreatic cancer is still one of the most aggressive and intractable human malignant tumors. Perineural invasion extending into the pancreatic nerve plexus is a histopathologic characteristic of pancreatic cancer. However, the mechanisms contributing to the invasion of intrapancreatic nerves and spread of cancer cells along extrapancreatic nerves in the course of pancreatic cancer are still poorly understood. As a neurite growth-promoting factor, pleiotrophin (PTN) and its receptor, N-syndecan, may play a very important role in tumor growth and neural invasion of pancreatic cancer. Therefore, the authors of this study used recombinant adenovirus pAd-shRNA-PTN to investigate the silencing effects of PTN in pancreatic cancer cells and to observe the inhibition of pAd-shRNA-PTN on the neurite outgrowth from dorsal root ganglion (DRG) neurons *in vitro*.

Research frontiers

Perineural invasion extending into the pancreatic nerve plexus is a histopathologic characteristic of pancreatic cancer. The neural invasion of pancreatic cancer leads to local recurrence, metastasis and poor prognosis, which have been brought difficulty to diagnosis and treatment of pancreatic cancer.

Innovations and breakthroughs

In recent years, researches of pancreatic cancer have focused on the biological characteristics, especially perineural invasion. This study explored the effects of pAd-shRNA-PTN on PTN of pancreatic cancer cells and neurites outgrowth of DRG neurons, which might reveal the relative mechanism of the neural invasion.

Applications

The study indicates that PTN appears to be an attractive target for nerve infiltration of pancreatic cancer gene therapy.

Terminology

PTN is a type of neurotrophic factor and is also known as a neurite growth-promoting factor. N-syndecan is a transmembrane protein and a high- affinity receptor for PTN. PTN and N-syndecan are important in promoting neurite outgrowth. RNA interference (RNAi) is a process during which double-stranded RNA induces the homology-dependent degradation of cognate mRNA. RNAi has been proven to be a powerful tool for suppressing gene expression.

Peer review

The study investigated the silencing effects of recombinant adenovirus pAd-shRNA-PTN on PTN gene in pancreatic cancer cells, and observed the inhibition of pAd-shRNA-PTN on the neurite outgrowth from DRG neurons *in vitro*. The subject is important because very little is known about the molecular mechanism of perineural invasion in pancreatic cancer, that is to say, this study

has revealed the role of *PTN* gene in perineural invasion using pAd-shRNA-PTN. The study is well designed.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2674

World J Gastroenterol 2011 June 7; 17(21): 2674-2680
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Enhanced proliferation, invasion, and epithelial-mesenchymal transition of nicotine-promoted gastric cancer by periostin

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Received: December 21, 2010 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: June 7, 2011

Abstract

AIM: To investigate the contribution of periostin in nicotine-promoted gastric cancer cell proliferation, survival, invasion, drug resistance, and epithelial-mesenchymal transition (EMT).

METHODS: Gastric cancer cells were treated with nicotine and periostin protein expression was determined by immunoblotting. Periostin mRNA in gastric cancer cells was silenced using small interfering RNA (siRNA) techniques and periostin gene expression was evaluated by quantitative reverse transcription-polymerase chain reaction. Gastric cancer cells transfected with control or periostin siRNA plasmid were compared in terms of cell proliferation using the methylthiazolyldiphenyl-tetrazolium bromide assay. Cell apoptosis was compared using annexin V-fluoresceine isothiocyanate and propidium iodine double staining. Tumor invasion was determined using the Boyden chamber invasion assay, and the EMT marker Snail expression was evaluated by immunoblotting.

RESULTS: Nicotine upregulated periostin in gastric cancer cells through a COX-2 dependent pathway, which was blocked by the COX-2-specific inhibitor NS398. Periostin mRNA expression was decreased by ~87.2% by siRNA in gastric cancer cells, and stable periostin-silenced cells were obtained by G418 screening. Periostin-silenced gastric cancer cells exhibited reduced cell proliferation, elevated sensitivity to chemotherapy with 5-fluorouracil, and decreased cell invasion and Snail expression ($P < 0.05$).

CONCLUSION: Periostin is a nicotine target gene in gastric cancer and plays a role in gastric cancer cell growth, invasion, drug resistance, and EMT facilitated by nicotine.

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Key words: Cyclooxygenase-2; Malignant growth; RNA interference; Snail; Smoking

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Liu Y, Liu BA. Enhanced proliferation, invasion, and epithelial-mesenchymal transition of nicotine-promoted gastric cancer by periostin. *World J Gastroenterol* 2011; 17(21): 2674-2680 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2674.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2674

INTRODUCTION

Gastric cancer is malignant and has a high occurrence. It originates from normal gastric epithelial cells that undergo various precancerous diseases, such as chronic atrophic gastritis and intestinal metaplasia, and then eventually cancer[1].

The relationship between smoking and gastric cancer has received increasing interest. A large population epidemiological investigation revealed that previous and current smokers had a 43% and 57% higher incidence of gastric cancer than non-smokers, respectively[2]. Therefore, smok-

ing is the most important behavioral factor leading to gastric cancer[3]. About 60% of tobacco components are carcinogens, including conjugated aromatic chemicals, nitric amines, aromatic amines, trace metals, and nicotine[4]. Nicotine is the most active carcinogen in cigars, and has been shown to promote gastric cancer cell proliferation and neoangiogenesis *in vivo* and *in vitro*[5]. Nicotine activates β-adrenoceptors and the downstream protein kinase C-β, extracellular signal-regulated kinases 1/2, and cyclooxgenase-2 (COX-2) signaling pathways in gastric cancer cells, thus promoting gastric cell mitosis and proliferation[6]. In addition, nicotine contributes to the invasion and metastasis of gastric cancer through the activation of COX-2 and vascular endothelial growth factor receptors in gastric cancer cells[7]. Therefore, COX-2 is an important nicotine-regulated molecule in gastric cancer cells and an important oncogene in the pathogenesis of gastric cancer. Tumor invasion means that malignant cells penetrate the basal membrane, migrate away from primary tumor sites and invade surrounding tissues. Tumor invasion results from the interaction between tumor cells and surrounding mesenchymal cells, leading to further spreading and the malignant advancement of tumors. During the invasion of epithelial-originated malignant tumors, the polarity of the epithelial cells disappears and their migration and mobility increase. Subsequently, epithelial cells are transformed into mesenchymal cells, which is known as the epithelial-mesenchymal transition (EMT).

Periostin, or osteoblast-specific factor 2 (OSF-2), is a newly discovered adhesion molecule that is over-expressed in various tumors, including oral cancer, thyroid cancer, breast cancer, and gastric cancer, and is correlated with tumor invasion, distant metastasis, and angiogenesis[8-11]. Periostin is a mesenchyme-specific protein expressed and secreted by tumor mesenchymal cells. Transfection and over-expression of the periostin gene in 293T cells has been shown to cause EMT as well as cell migration, invasion, and adhesion[12]. Moreover, metastatic loci were formed when recombinant cells over-expressing periostin were transplanted into immune-deficient mice[12].

Although facilitation by nicotine of the invasion and EMT of tumor cells has been confirmed by numerous investigators[5-7], to the best of our knowledge, the relationship between nicotine and the EMT-marker periostin has not been reported. We studied the expression of periostin induced by nicotine treatment in gastric cancer cells *in vitro*, silenced the periostin gene using small interfering RNA techniques and investigated the effects of nicotine on the growth, apoptosis, and invasion of gastric cancer cells. Our study suggested that periostin might be a downstream target gene regulated by nicotine in gastric cancer cells and may significantly contribute to nicotine-induced proliferation, metastasis, and EMT in gastric cancer cells.

MATERIALS AND METHODS

Reagents and equipment

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), annexin V-fluoresceine isothiocyanate (V-FITC), propidium iodine (PI), nicotine, and the COX-2-specific inhibitor NS 398 were from Sigma (St. Louis, MO, USA). The fetal bovine serum was from Sijichun Bioengineering Materials, Inc. (Hangzhou, Zhejiang, China). The DMEM culture media was from Invitrogen-GIBCO (Carlsbad, CA, USA). Vectors for periostin siRNA were constructed by lab co-workers. LipofectAMINE-2000 and TRIzol were from Invitrogen. The reverse transcription-polymerase chain reaction (RT-PCR) kits were from Treasure Biological, Inc. (Dalian, Liaoning, China). The forward and reverse primers were from Shanghai Bioengineering Inc. (Shanghai, China). The flow cytometer (FACS-440) was from BD Biosciences (Franklin Lakes, NJ, USA). The inverted phase contrast microscope (CK-40) was from Olympus (Center Valley, PA, USA). The gastric cancer cell line SGC-7901 was from the cell bank of the Shanghai Biological Institute of the Chinese Institute of Science. The T4 DNA Ligase was from Takara Bio Inc. (Otsu, Shiga, Japan) and the Boyden chamber was from Corning Costar Corp. (Cambridge, MA, USA).

Cell culture

Gastric cancer cell line SGC-7901 was cultured in DMEM media (high glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37℃ and 5% CO_2 in an incubator, and the culture medium was changed every 2-3 d. SGC-7901 cells were treated with nicotine at 200 ng/mL for 24 h before lysing for immunoblotting experiments. To determine if periostin upregulation by nicotine was related to the COX-2 pathway, the COX-2-specific inhibitor NS 398 was applied to SGC-7901 cells 6 h before nicotine treatment.

Construction, transfection, and screening of siRNA plasmids

Targeting sequences (complimentary oligonucleotides) for siRNA of periostin were designed from the human periostin sequence (GenBank accession number NM_006475.2) based on general design principles of siRNA. Forward primers for U6 promoter amplification and reverse primers (at bp 136, 246, and 268) for periostin amplification were used for 2-step PCR to amplify siRNA expression cassettes at 94℃ for 30 s and 72℃ for 90 s over 40 cycles. PCR end products were ligated with precut plasmid pRNAT-U6.1 and T4 DNA ligase at 16℃ overnight. Ligation products were transformed into competent *Escherichia coli* DH5α, followed by blue-white screening, enzyme digestion confirmation, and glycerol storage for gene sequencing at Shen You, Inc. The pRNAT-U6.1-periostin plasmid with the correct sequence was used to prepare and purify a large amount of plasmid DNA.

The gastric cancer cell line SGC-7901 was recovered, subcultured, and plated in 6-well plates at a density of 4×10^5/mL 24 h before transfection, such that the cells were 90% confluent at transfection. SGC-7901 cells were transfected with either pRNAT-U6.1-periostin siRNA plasmid or pRNAT-U6.1 control plasmid. Cells were observed separately for the periostin gene and protein expression by RT-PCR and immunoblots 48 h after transfection. SGC-7901 cells with successful transient pRNAT-U6.1-

periostin siRNA transfection and optimal periostin siRNA were selected, and culture medium with G418 at 400 and 800 μg/mL was used for concentration increase screening.

RT-PCR for detection of periostin mRNA

Total RNA was isolated from SGC-7901 cells with different treatments 48 h after transfection, and RT-PCR was performed to quantify periostin mRNA expression normalized against GAPDH. Five micrograms of RNA was used to synthesize cDNA, followed by PCR.

The periostin forward primer was 5'-GCACTCTGGGCATCGTGGGA-3' and the periostin reverse primer was 5'-AATCCAAGTTGTCCCAAGCC-3'. The GAPDH forward primer was 5'-CTGCACCACCAACTGCTTAG-3' and the GAPDH reverse primer was 5'-TGAAGTCAGAGGAGACCACC-3'.

The amplicons for periostin and GAPDH were 132 and 407 bp, respectively.

The thermal profile of PCR for periostin and GAPDH mRNA detection was 94℃ for 4 min over 1 cycle, 94℃ for 30 s, 57℃ for 30 s, and 72℃ for 1 min over 33 cycles, followed by 72℃ for 7 min over 1 cycle.

The PCR products were electrophorized on 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer. The bands of the PCR products were quantified by grayscale readings using a gel imaging system. The ratios of the grayscale readings of the band for periostin *vs* those for GAPDH using the same samples were calculated as the relative mRNA expression of periostin. Periostin suppression rate (%) = (1 - periostin mRNA relative expression in the pRNAT-U6.1-periostin siRNA group/periostin mRNA relative expression in pRNAT-U6.1 control group) × 100%.

Immunoblots

Proteins were isolated from SGC-7901 cells and the protein concentrations were determined. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (polyacrylamide concentration 100 g/L) and electrophoretically transferred to PVDF membranes. The PVDF membranes were blocked with 3% BSA at 37℃ for 1 h and probed with the primary antibody mouse anti-human periostin, Snail (1:100), or β-actin (1:1000) monoclonal antibody for 2 h. The bound antibody was detected by horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) and enhanced chemiluminescence. The blots were washed with 1 × Tris-buffered saline with Tween buffer for 10 min, 3 times between each step. The density of the targeted bands was quantified using the Developer 100 Plus Imaging Analysis System.

Cell growth assay

The cell growth rate was determined using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and growth curve depictions. Cells with pRNAT-U6.1-periostin siRNA or control plasmid (2×10^5 cell/mL) were seeded onto 96-well plates with 100 μL in each well, cultured with DMEM media supplemented with 10% fetal bovine serum, and observed for cell proliferation at 24, 48, and 72 h after seeding. For the cell viability and growth assay, 10 μL of MTT solution (5 mg/mL) was added into each well and incubated at 37℃ for 4 h, and the reaction terminated with a detergent solution to lyse the cells and solubilize the colored formazan crystals. The supernatant was centrifuged at 3000 r/min for 10 min to obtain a formazan pellet. The supernatant was removed, and the pellet was dissolved completely with 100 μL DMSO and observed at a wavelength of 570 nm using an ELISA plate reader.

Cell apoptosis assay

Cell apoptosis was analyzed by annexin V-FITC and PI double staining. Cells with pRNAT-U6.1-periostin siRNA or control plasmid were evaluated for apoptosis after 24, 48, and 72 h of culture. From each well, 2×10^5 cells were harvested, and the supernatant was collected by centrifugation at 2000 r/min for 5 min. The pellet was washed with PBS buffer once, resuspended in 100 μL 1 × binding buffer, supplied with 2.5 μL annexin V and 5 μL PI (final concentration of 10 μg/mL), incubated for 15 min in the dark, and assayed for cell apoptosis by flow cytometry. The results were analyzed using Lysis software.

Tumor invasion assay

The Boyden chamber invasion assay was used to evaluate the ability of tumor cells to invade. Tissue culture plates with 24 wells and Transwell filter membranes were used for the experiments. Cells with pRNAT-U6.1-periostin siRNA or control plasmid were cultured in the presence or absence of nicotine for 4 days, harvested at 3×10^6/mL, and seeded onto the upper part of the Transwell chamber at 100 μL/well. The basement membrane matrix preparation, Matrigel, served as the matrix barrier and the lower part of the Transwell chamber was filled with 500 μL of culture medium as a chemoattractant. The chamber was kept at 37℃ in a 5% CO_2 incubator for 24 h before regular fixation and Trypan blue staining. The cells on the upper surface of the filter membrane were removed with a cotton swab, and the number of cells migrating through the membrane was determined. Three parallel membranes were counted for each condition. The number of cells migrating through the Matrigel was normalized to those through the non-Matrigel to obtain an index of relative percent migration, indicating the ability of tumor cells to invade *in vitro*.

Statistical analysis

Statistical analysis was performed using commercially available software (SPSS version 14.0). Data are presented as the mean ± SE. Means of 2 groups were compared using the Student's *t* test (unpaired, two tailed). Means of more than 2 groups were compared using one-way analysis of variance (ANOVA) followed by post-hoc testing using the Least-Significant-Difference test. $P < 0.05$ was considered statistically significant.

RESULTS

Nicotine induced periostin upregulation in gastric cancer cells via a COX-2 dependent pathway

Immunoblotting showed that 200 μg/mL nicotine at

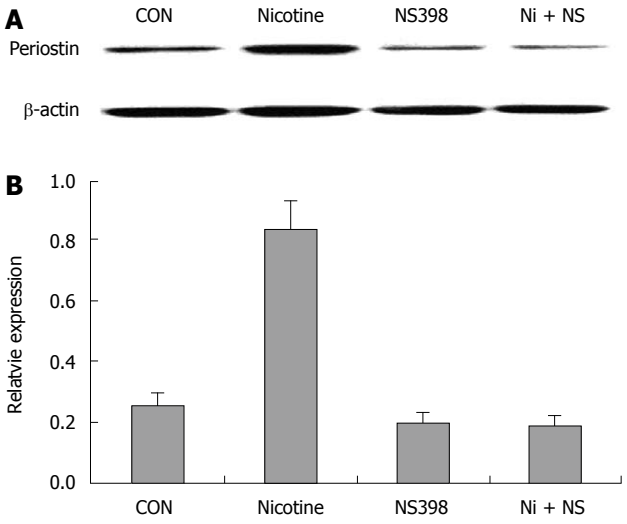

Figure 1 COX-2 inhibitor NS398 showed inhibitory effects on nicotine (200 ng/mL)-induced periostin expression after 24 h of treatment. COX-2-specific inhibitor NS398 (10 μmol/L) was applied to SGC-7901 cells 6 h before nicotine (200 μg/mL) treatment. A: Western blotting bands of periostin protein in four groups of gastric cancer cells; B: Quantification analysis demonstrated significantly higher relative expression of periostin in the nicotine group than in the other three groups ($P < 0.05$). Notes: CON: Control; Nicotine: Treated with nicotine; NS398: Treated with COX-2-specific inhibitor NS398; Ni + NS: Combined treatment with nicotine and NS398.

24 h significantly upregulated periostin protein expression in SGC-7901 cells, suggesting that periostin could be a downstream target of nicotine in gastric cancer cells. Pretreatment with the COX-2-specific inhibitor NS 398 almost completely blocked the upregulation of periostin by nicotine in SGC-7901 cells. NS 398 also reduced periostin protein expression in the absence of nicotine in SGC-7901 cells (Figure 1).

Effects of periostin siRNA on its mRNA expression

The mRNA level of periostin was significantly reduced in all groups (S1-S4) and was most evident in S2 at 87.2% *vs* the plasmid control, suggesting that the periostin gene was successfully silenced by siRNA. SGC-7901 cells with successful transfection of the siRNA S2 plasmid were further screened in culture medium supplemented with increasing concentrations of G418 at 400 or 800 μg/mL. The stably silenced cells were finally maintained in culture media with 400 μg/mL G418 (Figure 2).

Inhibitory effects of periostin siRNA on nicotine-induced gastric cancer cell proliferation

SGC-7901 cells with successful transfection of the control plasmid (siRNA-C) showed slow growth in the MTT assay, but growth was significantly accelerated by nicotine treatment. SGC-7901 cells with successful transfection of the periostin siRNA plasmid (siRNA-P) showed much slower growth *vs* siRNA-C under both the basal and nicotine-stimulated conditions. Additionally, siRNA-P showed increased proliferation in response to nicotine stimulation, indicating that nicotine-induced cell proliferation could not be fully blocked by silencing periostin in SGC-7901 cells (Figure 3).

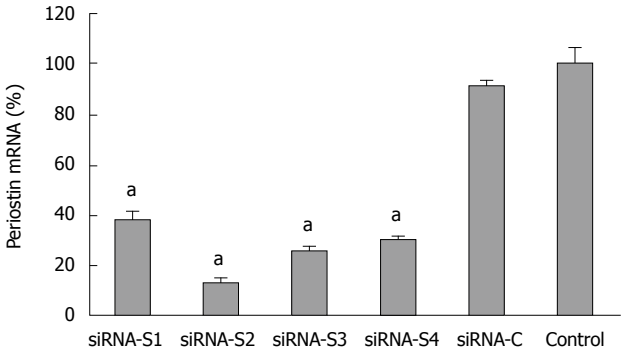

Figure 2 Relative expression of periostin mRNA 48 h after transfection by siRNA plasmid. The silencing effect of the transfection of periostin siRNA plasmid in SGC-7901 cells was confirmed by quantitative reverse transcription-polymerase chain reaction 48 h after transfection. Four representative results are shown (S1-S4). SiRNA-C indicates gastric cancer cells with control plasmid. Control indicates gastric cancer cells without transfection. $^{a}P < 0.05$ *vs* control. The Y ordinate indicates the ratio of periostin mRNA normalized to that of house-keeping genes. Notes: siRNA-S1, siRNA-S2, siRNA-S3, siRNA-S4: four representative results of periostin siRNA transfection; siRNA-C: vacant siRNA-control; control: SGC-7901 cells without transfection.

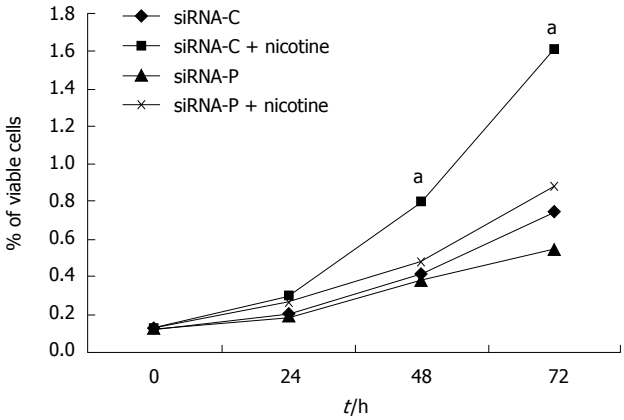

Figure 3 Periostin siRNA inhibited nicotine-promoted cell growth in gastric cancer cells. SGC-7901 cells with stable expression of siRNA-periostin (siRNA-P) and vacant siRNA-control (siRNA-C) were treated with nicotine. Cells were then lysed to analyze cell viability using the methylthiazolyldiphenyl-tetrazolium bromide assay at 0, 24, 48, and 72 h of treatment, and growth curves were constructed. The viability of the siRNA-C+nicotine group was significantly higher than those of the other three groups at the 48 and 72 h time points ($P < 0.05$), which is indicated by "a". Notes: siRNA-C: vacant siRNA-control; siRNA-P transfected with periostin.

Stimulatory effects of periostin siRNA on chemotherapy-induced apoptosis of gastric cancer cells

SiRNA-P showed higher sensitivity to 5-fluorouracil (5-FU, 50 mg/L) treatment than did siRNA-C, as revealed by flow cytometry. After 24 h of 5-FU treatment, the apoptotic rate did not differ between the two groups (4.36% *vs* 6.06%, siRNA-P *vs* -C). However, after 48 and 72 h, siRNA-P had a significantly higher apoptotic rate than did siRNA-C (13.3% *vs* 22.1%, 20.5% *vs* 39.7%) (Figure 4).

Effects of periostin siRNA on invasion and the expression of the EMT marker Snail in gastric cancer cells

SiRNA-P showed a significantly lower index of invasion (21.8% ± 4.11%) than did siRNA-C (55.4% ± 2.64%), in-

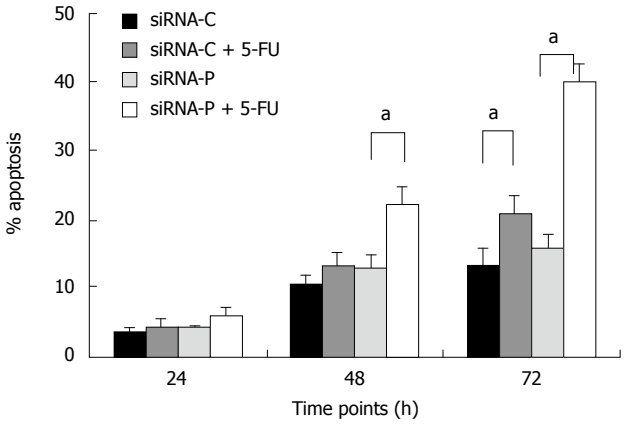

Figure 4 Periostin siRNA increased the sensitivity of gastric cancer cells to 5-fluorouracil-induced apoptosis. SGC-7901 cells with stable expression of siRNA-periostin (siRNA-P) and vacant siRNA-control (siRNA-C) were treated or not with chemotherapy agent 5-FU. siRNA-periostin significantly increased the sensitivity of 5-FU treated gastric cancer cells both at 48 h and 72 h ($^aP < 0.05$). Notes: siRNA-C: Vacant siRNA-control; siRNA-P: Transfected with periostin; 5-FU: 5-fluorouracil (50 mg/L).

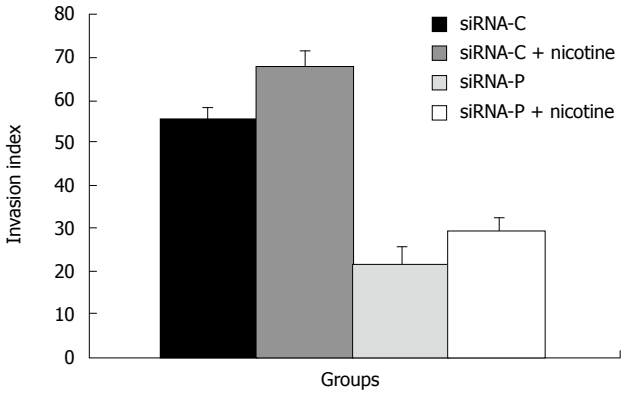

Figure 5 The effect of periostin siRNA on the ability of gastric cancer cells to invade. SGC-7901 cells with stable expression of siRNA-periostin (siRNA-P) and vacant siRNA-control (siRNA-C) were treated with nicotine and evaluated for cell invasion using the Boyden chamber invasion assay.

dicating that silencing of periostin can reduce the capability of gastric cancer cells to invade. Similarly, with nicotine treatment, siRNA-P also showed significantly less invasion (29.5% ± 3.11%) than did siRNA-C (29.5% ± 3.11%) (Figure 5).

The expression of the EMT marker, Snail, was determined in siRNA-P and siRNA-C by immunoblotting. The Snail protein was significantly decreased in siRNA-P compared to the siRNA-C group, both in nicotine-treated and nicotine–untreated groups. Nicotine treatment significantly increased Snail protein expression in siRNA-C, but not in siRNA-P (Figure 6).

DISCUSSION

We observed that nicotine can upregulate periostin protein expression in gastric cancer cells in a COX-2-dependent pathway, which can be blocked by the COX-2-specific inhibitor NS 398. Periostin mRNA expression was signifi-

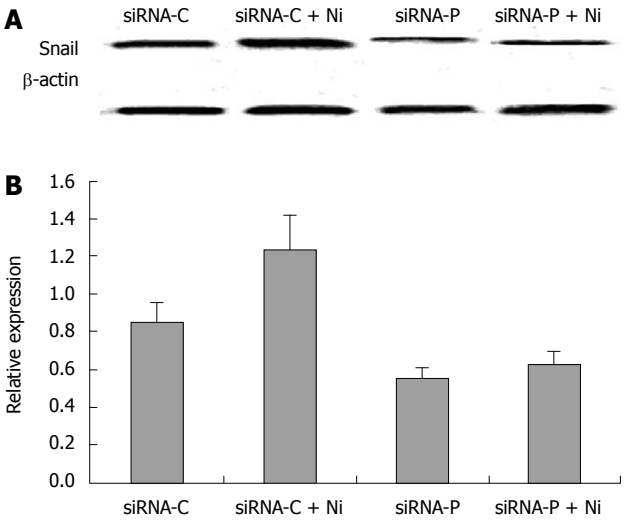

Figure 6 The inhibitory effect of periostin siRNA on nicotine-induced Snail protein expression. A: Western blotting bands of Snail protein in four groups of gastric cancer cells. B: Relative expression of Snail in the above four groups. Nicotine significantly increased the relative expression of Snail protein in gastric cancer cells ($P < 0.05$). However, there were no significant differences in Snail expression between the siRNA-P group and the siRNA-P+nicotine group ($P > 0.05$). Notes: siRNA-C: vacant siRNA-control; siRNA-P: Transfected with periostin; Ni: Treated by 200 ng/mL nicotine.

cantly decreased by 87.2% in siRNA-P, which was stably silenced with G418 screening. Cell proliferation in siRNA-P was significantly inhibited compared to siRNA-C. Nicotine can promote gastric cancer cell growth, which can be partially blocked by periostin siRNA. Furthermore, siRNA-P had a significantly increased sensitivity to apoptosis induced by the chemotherapy reagent, 5-FU, compared to siRNA-C, especially after 48 and 72 h. Nicotine increased the ability of gastric cancer cells to invade *in vitro*, and siRNA-P showed decreased invasion in the presence or absence of nicotine compared to the controls. EMT is an important underlying mechanism used by epithelial cancer cells to invade and metastasize. We found that the EMT marker, Snail protein expression, decreased in siRNA-P *vs* siRNA-C with or without nicotine treatment, indicating that periostin expression could be required for EMT in gastric cancer cells.

Our study revealed that periostin expression was controlled by nicotine. As the most active carcinogen in tobacco, nicotine is well known to promote lung cancer, and its relation to other types of tumors has come under increasing scrutiny. Nicotine has been shown to activate COX-2 in gastric cancer cells, which is related to the growth, survival, invasion, and metastasis of gastric cancer cells[5,7,12]. COX-2 is also an important prognostic factor for gastric cancer. The higher the expression of COX-2 in gastric cancer cells is, the worse the prognosis is[13]. We also found that the regulation of periostin by nicotine was COX-2 dependent. Inhibition of COX-2 almost completely blocked the upregulation of periostin by nicotine, and inhibition of COX-2 itself downregulated periostin expression in gastric cancer cells. These findings indicate that the regulation of periostin by nicotine is completely COX-2 dependent and

that periostin expression is partially dependent on COX-2 in gastric cancer cells. Currently, there are only a few reports on the relationship between COX-2 and periostin. It has been shown that activation of the signaling molecule Wnt can concurrently upregulate COX-2 and periostin in mammary epithelial cells[14]. That both COX-2 and periostin are expressed at the same time in gastric cancer cells, along with the relationship of COX-2 to the prognosis of gastric cancer, warrants further investigation to determine whether periostin can serve as a prognostic indicator for gastric cancer.

Our research indicated that periostin gene silencing could significantly increase the sensitivity of gastric cancer cells to 5-FU. A hypoxic environment has been shown to upregulate periostin expression and promote cell survival *via* activation of the downstream Akt/PKB signaling pathway in non-small cell lung cancer cells[15]. In pancreatic cancer cells, periostin promoted resistance to hypoxia-induced apoptosis through binding to integrin on the cell membrane and activation of the downstream PI3K-AKT signaling pathway, which promotes cell survival[16]. At diagnosis, patients with gastric cancer usually have micro-metastasis of gastric cancer cells into non-gastric areas, such as the liver, abdominal cavity, bone marrow, and peripheral circulation[17-20]. Chemotherapy that does not completely remove these latent cells leads to distant metastasis and the recurrence of tumors. This indicates that tumor cells with metastatic potential may have resistance to apoptosis, and periostin could be an underlying mechanism used to promote invasion, metastasis, and drug resistance in cancer cells *via* a complex intracellular signaling network.

Our findings suggest that periostin expression is required for the EMT of gastric cancer cells. Periostin is a mesenchyme-specific gene[21] expressed and secreted by tumor mesenchymal cells[22] and associated with the EMT of tumor cells. In fact, EMT induced by nicotine has been the subject of numerous publications. Nicotine-induced cell invasion and EMT has been shown to relate to the nicotinic acetylcholine receptors on the surface of lung cancer cells[23,24]. Nicotine was recently shown to induce EMT in gastric cancer cell lines through the activation of 5-lipoxygenase[25]. During EMT, epithelial markers decrease, mesenchymal markers increase, and periostin mainly resides in mesenchymal cells[26]. It could be that nicotine-induced periostin upregulation results from EMT, leading to fewer epithelial cells and more mesenchymal cells. However, our results showed that in gastric cancer cells with the periostin gene downregulated by siRNA, there was significant downregulation of the EMT marker gene, Snail, with nicotine treatment, along with the inhibition of cell growth, drug resistance, and tumor invasion. This suggests that periostin changes in gastric cancer cells are not a result of EMT and that, instead, periostin contributes to EMT *via* a specific pathway.

In this *in vitro* study, the concentration of nicotine was 200 ng/mL, this was chosen as the most appropriate concentration from our preliminary results. In smokers, venous nicotine levels range from 5 to 15 ng/mL, which is much lower than the level used in our *in vitro* study[27]. Nicotine, which can be absorbed *via* respiratory tissues, the skin, and the gastrointestinal tract, takes part in the initiation and promotion of carcinogenesis in the gastrointestinal tract. Because this is a gradual process, a lower *in vivo* nicotine concentration could enhance the detrimental effects of other carcinogens over a long period of time. In fact, the *in vitro* effects of nicotine on periostin expression were only observed up to 24 h in this study. Whether this effect is longer lasting will be investigated in subsequent *in vivo* animal experiments.

In summary, we showed that periostin is a nicotine-regulated gene that contributes to nicotine-induced cell growth, drug resistance, invasion, and EMT in gastric cancer cells. These findings may lead to a new target for the prevention of nicotine-induced gastric cancer.

ACKNOWLEDGMENTS

The authors would like to thank the Department of Pathophysiology, Division of Basic Medicine, Central South University Xiangya School of Medicine for their generous funding and technical support.

COMMENTS

Background

Gastric cancer has a high occurrence and its relationship with smoking has been a focus of recent research. As the most active carcinogen in cigars, nicotine has been shown to promote gastric cancer cell proliferation and neoangiogenesis *in vivo* and *in vitro*. Periostin is a mesenchyme-specific protein that is over-expressed in gastric cancer cells and is related to invasion, distant metastasis and angiogenesis in many tumors.

Research frontiers

During the invasion of epithelial-originated malignant cancers, the polarity of epithelial cells disappears along with an increase in migration and mobility, which is known as the epithelial-mesenchymal transition (EMT). Nicotine can enhance invasion and EMT in various tumor cells; however, the relationship between nicotine and EMT-marker periostin has not been unequivocally addressed. In this study, the authors demonstrate that the expression of periostin can be induced by nicotine treatment in gastric cancer cells and that proliferation, drug resistance and invasiveness in gastric cancer cells is induced by nicotine.

Innovations and breakthroughs

Recent reports have highlighted the importance of the EMT and its related molecules, including Snail, in gastrointestinal carcinogenesis and progression. In gastric cancers, EMT is enhanced. This is the first study to report that EMT-related periostin is upregulated by nicotine in gastric cancer cells. Furthermore, The authors' *in vitro* studies suggest that this protein may be the cause of the proliferation, drug resistance and invasiveness induced by nicotine in this cancer.

Applications

By understanding how periostin is induced and by silencing its expression, this study may represent a future strategy for therapeutic intervention in patients with nicotine-associated gastric cancer.

Terminology

Periostin and Snail are proteins involved in a process called the EMT. EMT occurs when epithelial cells lose polarity and increase migration and mobility. Such a mechanism is crucial in the invasion and metastasis of many epithelial-originated malignant tumors.

Peer review

The study is entitled "Enhanced proliferation, invasion, and epithelial-mesenchymal transition of nicotine-promoted gastric cancer cells by periostin" presents convincing evidence for the effect of nicotine on the induction of periostin and its effect on EMT of gastric cancerous cell lines.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i21.2681

World J Gastroenterol 2011 June 7; 17(21): 2681-2682
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Lower body weight and female gender: Hyperphosphatemia risk factors after sodium phosphate preparations

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Received: September 19, 2010 Revised: February 27, 2011
Accepted: March 6, 2011
Published online: June 7, 2011

Abstract

Casais *et al* have reported an inverse correlation between serum phosphate and body weight after administration of sodium phosphate at a dose of 60 g. Our group has already described the relationship between body weight and hyperphosphatemia with these preparations, although our study was not quoted by Casais. We performed a pharmacokinetic study involving 13 volunteers who were divided into two groups on the basis of body weight: group Ⅰ consisting of seven women with a median weight of 60 kg and group Ⅱ consisting of five men and one woman with a median weight of 119.2 kg. Group Ⅰ developed higher peak phosphate levels and maintained these levels above the subjects in Group Ⅱ for a prolonged time period despite adequate hydration being ensured with frequent monitoring of weight, fluid intake and total body weight. Our study demonstrated that adequate hydration does not protect against the secondary effects of hyperphosphatemia. In the study by Casais *et al*, 66% of the study subjects were women, the correlation between serum phosphate and gender in their data also appears to be important. Women are at higher risk of acute phosphate nephropathy due to a diminished volume of distribution of the high dose of ingested phosphate. Decreased volume of distribution in women is due to diminished body weight. This is further compounded by decreased creatinine clearance in females.

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Key words: Colonoscopy bowel preparation; Lower body weight; Hyperphosphatemia; Sodium phosphate; Female

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Deepak P, Ehrenpreis ED. Lower body weight and female gender: Hyperphosphatemia risk factors after sodium phosphate preparations. *World J Gastroenterol* 2011; 17(21): 2681-2682 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i21/2681.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i21.2681

TO THE EDITOR

Casais *et al*[1] have described an inverse relationship between serum phosphate and body weight after administration of sodium phosphate at a dose of 60 g (90 mL) in 100 consecutive patients. Significantly, adequate hydration was provided to their patients with ingestion of 4 L of clear liquids. Our group has already described the relationship between body weight and hyperphosphatemia after so-

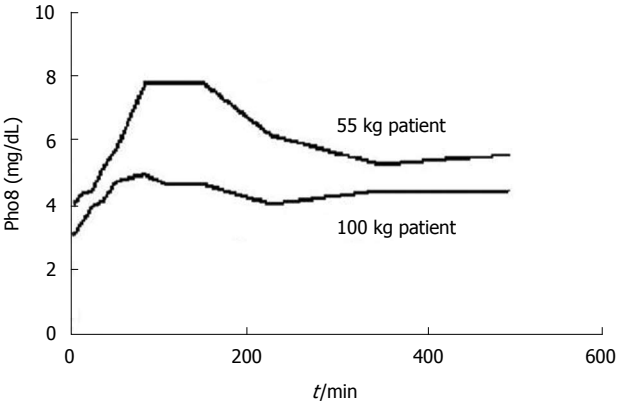

Figure 1 Comparison of serum phosphate levels in a 55 kg from Group Ⅰ and a 100 kg patient from Group Ⅱ.

dium phosphate preparations and determined the pharmacokinetic basis for this observation[2-4], although our studies were not referenced by Casais *et al*[1]. In our study, we administered a single half dose (45 mL) of Fleet Phospho-Soda containing 30 g of sodium phosphate to 13 normal volunteers consisting of two groups. Group Ⅰ had seven women with a median weight of 60 kg, and Group Ⅱ had five men and one woman with a median weight of 119.2 kg. Multiple serum and urine levels of phosphate, calcium, ionized calcium and other electrolytes were measured for 12 h. Hydration was maintained throughout the study by monitoring the weight, fluid intake, and total body water, with increased intake promoted for declines in any value.

The subjects in Group Ⅰ developed higher peak phosphate levels and maintained these levels above the subjects in Group Ⅱ for a prolonged time period (Figure 1). The normalized area under the phosphate *vs* time curve was much higher in group Ⅰ (1120 ± 190 mg/dL per min) than in group Ⅱ (685 ± 136 mg/dL per min), $P < 0.001$. Urinary excretion of calcium was significantly lower in group Ⅰ (mean 16.4 ± 7.6 mg) than in group Ⅱ (mean 39.2 ± 7.8 mg), $P < 0.001$, and most subjects in Group Ⅰ had a prolonged time of abnormal ionized calcium levels during the study. Our study demonstrated that individuals, especially women, develop high serum phosphate levels for prolonged periods of time after ingesting sodium phosphate, even under the idealized condition of continuous monitoring of fluids and weight as done in this study. This suggests that adequate hydration does not protect against the secondary effects of hyperphosphatemia, as others have proposed[5,6]. Markowitz *et al*[7] has published 37 cases of acute phosphate nephropathy (APN), 30 (81%) of them were females. In the study by Casais *et al*[1] 66% of the study group were women, the correlation between serum phosphate and gender in their data also appears to be important. Pharmacokinetics demonstrates that women of diminished body weight become extremely hyperphosphatemic based on their diminished volume of distribution for the high dose of ingested phosphate. This is further compounded by decreased creatinine clearance in this group (a function dependent in part on body weight). Despite adequate fluid intake, these subjects are at very high risk for renal damage and acute phosphate nephropathy. Females, especially those of lower body weight should avoid using sodium phosphate laxatives for colonoscopic preparation.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 June 7; 17(21): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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World J Gastroenterol 2011 June 7; 17(21): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, 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 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 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 J Gastroenterol 2011 June 7; 17(21): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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

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World Journal of Gastroenterology

ISSN and EISSN
ISSN 1007-9327 (print)
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Indexed and Abstracted in
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Organization as author

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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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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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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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Volume 17 Number 22 Jun 14 2011

ISSN 1007-9327 (print)
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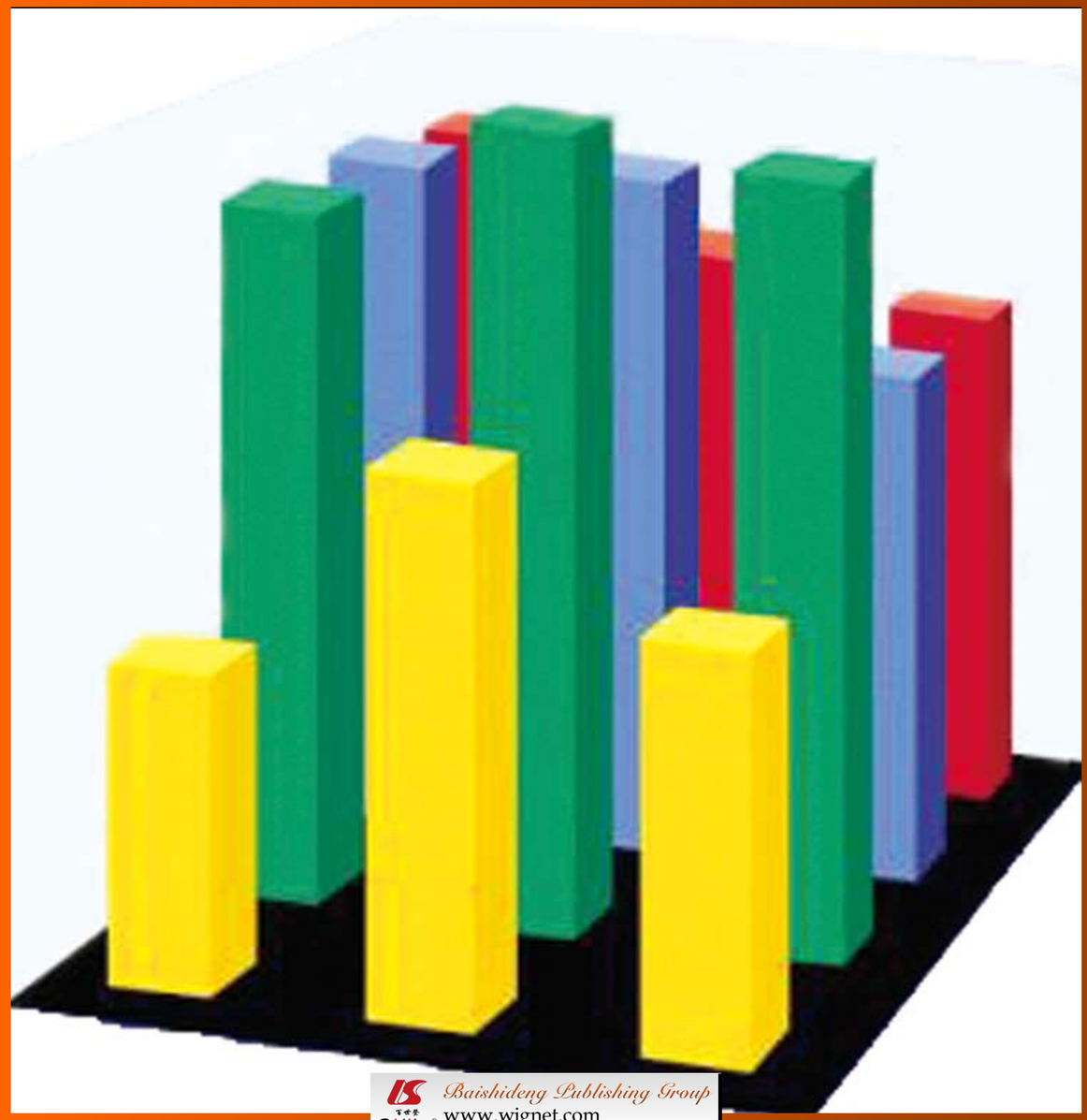
World Journal of *Gastroenterology*

World J Gastroenterol 2011 June 14; 17(22): 2683-2780



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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 22 June 14, 2011

EDITORIAL

2683 Interplay among cellular polarization, lipoprotein metabolism and hepatitis C virus entry
Benedicto I, Molina-Jiménez F, Moreno-Otero R, López-Cabrera M, Majano PL

TOPIC HIGHLIGHT

2691 Inflammatory bowel disease in adolescents: What problems does it pose?
Lu Y, Markowitz J

2696 Inflammatory bowel disease in pregnancy
Beaulieu DB, Kane S

2702 Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome?
Veloso FT

2708 Inflammatory bowel disease in travelers: Choosing the right vaccines and check-ups
Esteve M, Loras C, García-Planella E

2715 Familial aggregation in inflammatory bowel disease: Is it genes or environment?
Nunes T, Fiorino G, Danese S, Sans M

2723 Comorbidity in inflammatory bowel disease
San Román AL, Muñoz F

2734 Old-age inflammatory bowel disease onset: A different problem?
Hinojosa del Val J

2740 Ulcerative colitis in smokers, non-smokers and ex-smokers
Bastida G, Beltrán B

ORIGINAL ARTICLE

2748 miRNA studies in *in vitro* and *in vivo* activated hepatic stellate cells
Maubach G, Lim MCC, Chen J, Yang H, Zhuo L

2774 Influence of chitosan nanofiber scaffold on porcine endogenous retroviral expression and infectivity in pig hepatocytes

Han B, Shi XL, Xiao JQ, Zhang Y, Chu XH, Gu JY, Tan JJ, Gu ZZ, Ding YT.

World Journal of Gastroenterology
Volume 17 Number 22 June 14, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Veloso FT. Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome? *World J Gastroenterol* 2011; 17(22): 2702-2707 http://www.wjgnet.com/1007-9327/full/v17/i22/2702.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

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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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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
June 14, 2011

ISSN and EISSN
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.

ONLINE SUBMISSION
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2683

World J Gastroenterol 2011 June 14; 17(22): 2683-2690
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Interplay among cellular polarization, lipoprotein metabolism and hepatitis C virus entry

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Author contributions: Benedicto I and Majano PL conceived, designed, and wrote the manuscript; Molina-Jiménez F, Moreno-Otero R and López-Cabrera M contributed to the design and critical revision of the article.
Supported by CIBERehd to Moreno-Otero R, López-Cabrera M and Majano PL; SAF2007-61201 (Ministerio de Educación y Ciencia) to López-Cabrera M; CP03/0020 (Instituto de Salud Carlos Ⅲ), SAF2007-60677 (Ministerio de Educación y Ciencia) and PI10/00101 (Ministerio de Ciencia e Innovación, Instituto de Salud Carlos Ⅲ, FEDER) to Majano PL. Benedicto I was financially supported by CIBERehd and Molina-Jiménez F by Instituto de Salud Carlos Ⅲ and FIB Hospital de la Princesa
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Received: October 21, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: June 14, 2011

Abstract

Hepatitis C virus (HCV) infects more than three million new individuals worldwide each year. In a high percentage of patients, acute infections become chronic, eventually progressing to fibrosis, cirrhosis, and hepatocellular carcinoma. Given the lack of effective prophylactic or therapeutic vaccines, and the limited sustained virological response rates to current therapies, new approaches are needed to prevent, control, and clear HCV infection. Entry into the host cell, being the first step of the viral cycle, is a potential target for the design of new antiviral compounds. Despite the recent discovery of the tight junction-associated proteins claudin-1 and occludin as HCV co-receptors, which is an important step towards the understanding of HCV entry, the precise mechanisms are still largely unknown. In addition, increasing evidence indicates that tools that are broadly employed to study HCV infection do not accurately reflect the real process in terms of viral particle composition and host cell phenotype. Thus, systems that more closely mimic natural infection are urgently required to elucidate the mechanisms of HCV entry, which will in turn help to design antiviral strategies against this part of the infection process.

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Key words: Cellular polarization; Tight junctions; Lipoprotein metabolism; Hepatitis C virus

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Benedicto I, Molina-Jiménez F, Moreno-Otero R, López-Cabrera M, Majano PL. Interplay among cellular polarization, lipoprotein metabolism and hepatitis C virus entry. *World J Gastroenterol* 2011; 17(22): 2683-2690 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2683.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2683

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-polarity RNA virus that belongs to the *Flaviviridae* family and infects mainly hepatocytes[1]. The HCV genome encodes a polyprotein that is processed by host and viral proteases to yield

ten mature products, which include three structural proteins [the capsid protein (core) and two envelope glycoproteins (E1 and E2)], the p7 protein, and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer harboring the two envelope glycoproteins, which heterodimerize and play a major role in HCV entry[1]. Cellular infection begins with the attachment of the viral particle to the host cell and, after interacting with cell surface molecules, the virus is subjected to clathrin-mediated endocytosis and its envelope is fused with the endosomal membrane, releasing the viral genome into the cytosol[1]. These steps involve a set of attachment factors and cellular co-receptors, including highly sulfated heparan sulfate, the low-density lipoprotein receptor (LDL-R), the scavenger receptor class B type I (SR-BI), the tetraspanin CD81, and the tight junction (TJ) proteins, claudin-1 and occludin[2-4]. These molecules are not exclusively present in hepatocytes; therefore, HCV hepatotropism may be determined by other factors, such as the absence of inhibitory proteins[5].

In early reports, the soluble E2 envelope protein was employed as an approach to search for host cell HCV binding factors[6,7]. However, the conformation and function of the soluble glycoprotein may differ considerably from HCV envelope-anchored E2. In addition, envelope-anchored E2 is associated with E1[8], which may have additional implications in terms of receptor recognition and binding. The study of HCV entry was boosted by the use of HCV pseudotyped particles (HCVpp)[9], providing an infection system exclusively relying on the envelope glycoproteins. Since the establishment of a cell culture-derived HCV (HCVcc)[10-12], it has become the most powerful tool for studying HCV because it reflects the complete viral cycle. The use of both HCVpp and HCVcc has been of crucial importance in the discovery of new receptors, and has enabled high-throughput assays to test molecules for their ability to inhibit HCV entry.

INHIBITION OF HCV ENTRY AS A THERAPEUTIC ALTERNATIVE

HCV infection is the most frequent cause of liver failure worldwide[13,14]. Current therapies based on PEGylated interferon-alpha and ribavirin often fail to clear the infection and present a wide spectrum of systemic side effects[15]; therefore, alternative therapeutic options need to be developed. Among the different steps of HCV cycle, viral entry could be considered as a clinical target, especially in the context of orthotopic liver transplantation, where allograft reinfection occurs within hours after reperfusion and is followed by an accelerated chronic disease progression[16]. To date, several molecules have been found to inhibit HCV entry. Matsumura *et al*[17] showed that phosphorothioate oligonucleotides, previously described as HIV entry inhibitors[18], blocked HCV entry *in vivo*. Other inhibitors of HIV entry, such as cyanovirin-N, have also proved effective[19]. Furthermore, serum amyloid A, an acute phase protein mainly produced by the liver in response to different stimuli, including infections, has been demonstrated to inhibit HCV entry[20,21]. Moreover, the milk thistle (*Silybum marianum*)-derived silymarin and its purified flavonolignans have been recently shown to inhibit HCV infection both *in vitro* and in non-responder patients[22,23], blocking viral entry and transmission[24]. Therefore, increasing evidence suggests that blocking the entry step of HCV infection may be a good therapeutic alternative.

The genetic diversity of HCV contributes to its evasion from the host immune response[25], challenging the development of effective vaccines and virus-targeted inhibitors[26-28]. Nonetheless, this problem could be overcome by developing antiviral strategies aimed at blocking essential host factors for viral infection. To this end, multiple strategies have been pursued to inhibit HCV entry at different levels, including viral attachment, post-binding events, and fusion with the endosomal membrane[4,29]. One of these approaches consists of interfering with the interaction between the viral particle and cell surface co-receptors by the use of glycosaminoglycans, natural ligands, recombinant proteins, or blocking antibodies (Table 1). Notably, it has been demonstrated that antibodies against CD81 can prevent HCV infection of human liver-uPA-SCID mice[16], probably by inhibiting the E2-CD81 binding process[6,16,30]. As a more realistic and economical alternative, small molecules with similar properties could be used instead of blocking antibodies. In search of these compounds, several high-throughput screenings have been performed recently to identify molecules with the ability to inhibit HCV infection at the entry step[31-33]. Importantly, the possible cytopathic effects of these inhibitors should be assayed prior to starting clinical trials and considering them as potential therapeutic options. Chockalingam *et al*[32] developed a cell protection screen where cytotoxicity and inhibition of infection were evaluated simultaneously. As a more practical approach, Gastaminza *et al*[31] performed the screening with a set of drugs that had already been clinically approved.

The study of HCV infection and the search for inhibitory molecules are usually carried out with the use of HCVpp or HCVcc, and a highly permissive cell line, such as Huh7 and its derivatives. However, several conflicting results have arisen when attempting to validate the data in a more pathophysiologically relevant context (Table 1). A soluble form of CD81 was shown to prevent infection of Huh7.5 cells by HCVcc; however, it was not effective when primary human hepatocytes (PHH) were challenged with serum-derived virus[34]. Moreover, infection of hepatoma-derived cell lines with HCVpp and HCVcc does not seem to depend on LDL-R[35-37], whereas it has been demonstrated to participate in the infection of PHH with HCV from human plasma[38]. These facts stress the importance of being cautious with results obtained from HCV surrogates and cell lines, which should be validated *in vivo* whenever possible or at least in systems that more closely mimic real infection.

CELL POLARIZATION, TJ-ASSOCIATED PROTEINS, AND HCV ENTRY

In contrast to "simple" polarized cells, which present the

Table 1 Inhibition of infectivity by the blockade of hepatitis C virus co-receptors in different systems

		Host			Viral particle			Ref.
Co-receptor	Blocking agent	Cell lines	PHH	*In vivo*	HCVpp	HCVcc	Serum	
Heparan sulfate	Heparin	x			Y			[87]
		x				Y		[30,88,89]
			x[1]			Y		[90]
LDL-R	Anti-LDL-R	x			N			[36]
			x				Y	[38]
	Soluble LDL-R		x				Y	[38]
	LDLs/VLDLs	x			N			[9,37,91]
		x				N		[37]
			x				Y	[38]
CD81	Anti-CD81	x			Y			[9,36,92]
		x				Y		[30,88,89]
			x		Y			[9,92]
			x			Y		[34]
			x				Y	[34]
				x		Y		[16]
	CD81-LEL	x			Y			[9,36]
			x			Y		[34]
			x				N	[34]
	Knockdown	x			Y			[37]
		x				Y		[89]
			x				Y	[34]
SR-BI	Anti-SR-BI	x			N			[36]
		x			Y			[35,93]
		x				Y		[30,89]
	BLT-4	x			N/Y[2]			[91,93]
	ITX 5061/7650	x				Y		[94]
			x		Y			[94]
	oxLDLs	x			Y			[37]
		x				Y		[37]
	Knockdown	x			N/Y[2]			[37]
		x				Y		[89]
Claudin-1	Anti-claudin-1	x			Y			[30,56]
		x				Y		[30,56]
			x		Y			[56]
			x				Y	[56]
	Knockdown	x			Y			[42,43,95]
		x				Y		[42,43,95]
Occludin	Knockdown	x			Y			[43-45]
		x				Y		[43-45]

x: Experimental system employed; Y: Inhibition of infection; N: No inhibition of infection; [1]HCV-core immortalized PHH; [2]Only in the presence of high density lipoproteins. HCVpp: Hepatitis C virus pseudotyped particles; HCVcc: Cell culture-derived hepatitis C virus.

typical epithelial columnar phenotype with individual basolateral and apical domains, hepatocytic polarity is very peculiar and complex[39]. The plasma membrane of polarized hepatocytes is divided into several basolateral and apical poles, the latter forming a continuous network of bile canaliculi (BC) into which bile is secreted[40]. BC are delimited by TJs, which maintain cell polarity by separating apical from basolateral domains and form the intercellular barrier between bile and blood[41]. Claudin-1 was the first TJ-associated protein to be described as a HCV co-receptor[42]. Soon after, occludin was also shown to participate in HCV entry[43-45]. Despite these discoveries clearly pointing to a role of TJs in HCV cell entry, recent works have reported conflicting data about how cell junctions and polarity influence HCV infection. Perturbation of cellular junctions by calcium depletion promotes opposing effects depending on the system employed, e.g. it decreases viral entry in Huh7 cells[46] but increases it in "simply" polarized Caco-2 cells[47]. Furthermore, junctional accumulation of claudin-1 has been shown to either improve[48] or hinder[49] infection of Huh7.5 and polarized HepG2 cells, respectively. Collectively, these data suggest that the HCV entry process may vary considerably depending on the polarization state of the target cells.

Several studies have questioned the importance of TJ integrity in the function of claudin-1 and occludin as HCV co-receptors. For example, HCV infection is not affected after knocking down other TJ-associated proteins, such as ZO-1 and JAM-A[45], and claudin-1's association with CD81 at the basolateral membrane of HepG2 cells, but not at the TJ, defines HCV entry[50,51]. Furthermore, fluorescent HCV particle internalization generally occurs outside of cell-cell junctions[52], and VEGF induces a reduction of junctional occludin concomitant with an increase of HCV

infectivity[53]. Moreover, claudin-1 and occludin mutants lacking domains that are important for their correct junctional localization and function are still capable of rendering cells susceptible to HCVpp entry[42,54,55]. Finally, HCV infection of HepG2 cells is negatively regulated by cell polarity[49,53], but is not affected by TNF-α- and IFN-γ-mediated TJ disruption[49], and claudin-1 blocking antibodies inhibit HCV infection without perturbing TJs[30,56]. Taken together, these data strongly suggest that the role of claudin-1 and occludin in viral entry is relevant, but not necessarily when these proteins are part of functional TJs, which may indeed be a barrier for HCV infection. Interestingly, it has recently been demonstrated that hepatitis A virus infects HepG2-derived cells from the basolateral domain and that TJ-dependent polarization restricts infection[57].

The mechanisms by which claudin-1 and occludin participate in HCV entry have not been clearly established. In both cases, an extracellular loop of the protein has been shown to be indispensable for infection[42,44,55,58]. Several reports have shown that occludin precipitates with HCV E2 in infected, transfected, or replicon-containing cells[43,55,59], but its direct interaction with viral particles or envelope glycoproteins has not been demonstrated. Additionally, it has been shown that occludin interacts with dynamin Ⅱ, a well known regulator of endocytosis[55]. This observation, along with data obtained from cell-cell fusion experiments[45], suggests that occludin might participate in late steps of the HCV entry process. Interestingly, occludin endocytosis has been implicated in group B coxsackievirus infection, although not by directly interacting with the virus[60]. On the other hand, kinetic studies with blocking antibodies have shown that claudin-1 mediates an HCV entry step closely linked to CD81[30]. Indeed, it has been described that basolateral pools of claudin-1 are associated with CD81 in polarized HepG2 cells[50,51], and that disrupting this interaction, either by site directed mutagenesis or claudin-1 blocking antibodies, neutralizes HCV infection by reducing E2 association with the cell surface[30,51]. However, Cukierman *et al*[54] generated a mutant version of claudin-1 which, in spite of maintaining its interaction with CD81, no longer localized to cell-cell contacts and lost HCV receptor properties. This result suggests that, besides favoring E2 binding to the host cell, additional mechanisms involving claudin-1 participation in HCV entry may exist. Nevertheless, as these experiments were carried out in the non-hepatic, non-polarized HEK cell line, data should be carefully interpreted. This is a good example of how cell polarity may influence the results obtained, especially when studying features of TJ-associated proteins in a hepatocellular context.

LIPID METABOLISM AND HCV ENTRY

Cell polarization is crucial for the correct localization and function of TJ-associated proteins with HCV receptor activity, which could in turn be important for viral entry[41,61]. In addition, polarization may affect other steps of the HCV cycle, such as assembly and egress. Indeed, it has been shown that assembly of RNA enveloped viruses in MDCK cells is closely related to cell polarization[62]. It is also noteworthy that polarization is tightly linked to lipoprotein secretion[63,64], especially because some low density natural HCV particles have been found to be complexed with ApoB and/or ApoE-positive triglyceride-rich lipoproteins[65-67]. This association is believed to take place during viral egress[68] because HCVcc virions were found to be secreted in a manner that parallels the formation of VLDLs[69-71]. Thus, cell polarization may influence lipoprotein secretion, which is important for the generation of correctly assembled HCV progeny. Indeed, non-polarized Huh7-derived cells have been shown to be unable to secrete authentic, ApoB-containing VLDLs[72]. In addition, when HCVcc generated in these cells was subjected to isopycnic gradient ultracentrifugation, it was found to have higher average buoyant density than viral particles obtained from PHH[72]. Interestingly, the density profile of serum-derived viral particles obtained from HCVcc-infected animals was significantly lower than that of the initial HCVcc inoculum[73]. In both reports, specific infectivity of PHH and serum-derived HCV particles was shown to be greatly increased compared to standard HCVcc, and these properties were lost after passaging the virus again in Huh7.5 cells. These findings strongly suggest that HCVcc generated in Huh7-derived cells probably presents a defective lipoprotein association, which may in turn affect viral infection.

The lipoprotein composition and distribution within the viral particle may thus be important for the mechanisms underlying HCV entry[74]. Indeed, several reports have shown that lipoprotein lipase and hepatic triglyceride lipase alter both the physiological characteristics and the infectivity of HCV[75-77]. This notion is supported by the fact that LDL-R participates in the first steps of the entry process[38], and that viral particle density is inversely correlated to infectivity *in vivo*[66]. Additionally, the virus-host interaction could be affected in a context of a lipoprotein-defective viral particle because of changes in the exposure and accessibility of E2 to cellular co-receptors. In fact, this could explain the differences observed between HCVcc and serum-derived HCV infection in terms of inhibition by the soluble CD81 large extracellular loop[34]. Moreover, in contrast to serum-derived HCV[38], infection with HCVcc and HCVpp seems to be LDL-R independent[35-37], probably because of differences in lipoprotein composition.

Therefore, it is necessary to study HCV entry in systems that more accurately mimic the viral cycle in a pathophysiological context, from viral particle generation to target cell infection. The interplay between HCV and hepatocytes seems to be closely related to their particular phenotype (Figure 1); therefore, data obtained from models that do not reflect hepatocellular polarization and lipoprotein secretion should be cautiously interpreted.

TOWARDS A MORE PHYSIOLOGICALLY RELEVANT MODEL

The use of HCVpp and HCVcc has meant a great advance in HCV research. Their adaptability to high-throughput

analysis has enabled genome-wide screening for host proteins involved in the HCV cycle at different stages. In addition, they provide a valuable tool for testing the possible antiviral activity of large chemical libraries of compounds. However, it is important to bear in mind the limitations of these *in vitro* models, as both viral particles and target cells differ notably from serum-derived HCV and polarized hepatocytes, respectively. Thus, results obtained with HCVpp and HCVcc should be validated in systems that more closely mimic real HCV infection before establishing firm conclusions. To this end, significant work is being done by Molina and colleagues, who have already confirmed a role for LDL-R and CD81 in serum-derived HCV infection of PHH[34,38]. This model is considered an accurate *in vitro* system, as cells can be maintained in a differentiated phenotype to retain their polarity and drug metabolizing capacities. In addition, they can be infected with serum-derived HCV of any genotype and, in contrast to what is observed in hepatoma cell lines (e.g. Huh7 cells and derivatives), the innate immune response is fully preserved[78]. However, production of measurable titers of progeny virus in this system has not been achieved[79], indicating that the model fails to reflect the entire HCV cycle. Recently, it has been shown that HCVcc infection of PHH results in the robust production of infectious viral particles, which were in turn able to efficiently infect naïve PHH[72]. This primary cultured-derived HCV (termed HCVpc), compared to HCVcc, exhibited lower average buoyant density and higher specific infectivity, reminiscent of what is seen for virus recovered from the blood of animals infected with HCVcc[72,73]. Therefore, HCVpc infection of PHH emerges as a valuable tool for studying the complete HCV cycle in a more relevant context. Nevertheless, this system presents the drawbacks of working with PHH, such as restricted availability, the difficulty of studying long-term infections, and the heterogeneity of samples and results. Interestingly, Matrigel-embedded 3D cultures of Huh7 cells display a hepatocyte-like polarization and are susceptible to HCVcc infection. Progeny viruses generated by these cultures, similarly to HCVpc, also present a shift towards lower densities (our unpublished observations). This result suggests that if hepatocellular polarity is achieved, it is possible to generate viral particles that more closely mimic real HCV, even when cell lines are used as the source of virus.

Animal models are essential to validate *in vitro* data, because not only hepatocytes, but also the liver as a whole, may determine the mechanisms of HCV cell entry. Indeed, the liver sinusoidal endothelial cell-expressed protein, L-SIGN, has been shown to bind serum-derived HCV[80] and mediate trans-infection of Huh7 cells by HCVpp[81,82]. Additionally, co-culture of PHH with liver sinusoidal endothelial cells significantly increases the expression of the HCV co-receptor LDL-R[83]. Regarding *in vivo* obtained data, CD81 is the only co-receptor that has been demonstrated to participate in HCV infection using the human liver-uPA-SCID mouse model[16]. This system, albeit constituting a useful tool, is limited by the fact that the animals lack a functional immune system. This may be important, not only for the outcome of the infection, but also for the entry process itself, because DC-SIGN, expressed on dendritic cells, has been shown to capture and transmit HCVpp to Huh7 cells[81,82]. More recently, peripheral blood B cells have been shown to exert a similar function[84]. To date, chimpanzees are the only immunocompetent *in vivo* system for studying HCV infection, but their use is limited by ethical concerns, restricted availability, requirement of special facilities, and very high costs[85]. In search of a small, HCV susceptible and immunocompetent animal model, it has been proposed to combine human liver chimeric models with mice harboring a human hematolymphoid system[85], although this approach depends on the availability of human primary cells. Thus, the ideal model would be an immunocompetent mouse susceptible to HCV infection without the need of harboring human cells. Given that HCV species tropism is restricted to human and primates[58,85], an alternative strategy consists of using an HCV variant able to infect murine cells. Bitzegeio et al. adapted HCVcc to mouse CD81 and identified three envelope glycoprotein mutations which together enhanced infection of cells with mouse or other rodent receptors by approximately 100-fold[86], thus overcoming the species-specific restriction of HCV cell entry. Another possible approach would be to employ genetically engineered mice bearing the human entry factors that confer species specificity[44]. However, mouse hepatocytes fail to initiate viral replication[85]; therefore, these models would not be able to mimic the entire HCV cycle.

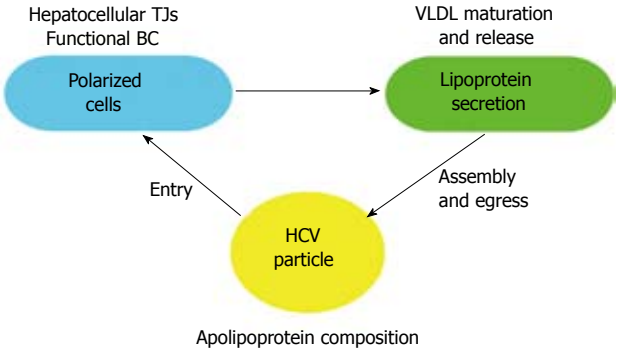

Figure 1 Interplay among cell polarization, lipoprotein secretion, and hepatitis C virus particle assembly, release, and entry into host cells. The correct polarization of hepatocytes, implying the presence of functional bile canaliculi delimited by TJs, may be important for the proper maturation and secretion of lipoproteins. This process is tightly associated with the composition and assembly of hepatitis C virus (HCV) lipoviroparticles and their exit from infected cells. Finally, HCV entry may be affected by the lipoprotein composition of the viral particle, the hepatocellular polarization of target cells, and the localization of the TJ-associated proteins claudin-1 and occludin.

CONCLUSION

During HCV infection, hepatocytes almost exclusively constitute both the target and the virus-producer cells. Thus, it is mandatory to perform HCV studies in systems that closely mimic the complex nature of hepatocyte pheno-

type. These models should enable the generation of viral particles that resemble the ones found *in vivo*, and reproduce the hepatocyte physiology as accurately as possible. Only the combination of these two factors will provide the necessary information to establish firm conclusions. Nevertheless, the choice of employing HCVpp, HCVcc, HCVpc, or serum-derived HCV to infect cell lines, PHH, or animals should depend on the stage of the research process: the *in vitro* systems are more adequate for high throughput screenings and the *in vivo* models are essential for validating the data. Once the mechanisms of HCV entry are deciphered in detail, this step of the viral cycle could be an effective target for the development of antiviral compounds. These inhibitors should ideally be effective for a broad range of HCV genotypes and subtypes, and even for other viruses, such as HIV, that might share some entry mechanisms and co-infect some patients. Thus, blocking cellular factors might be a good therapeutic alternative in the fight against viral genetic variability. However, targeting host molecules could alter their physiological functions and result in harmful side effects. In addition, this approach does not rule out the possible emergence of viral variants that would be able to circumvent the specific effect of the entry inhibitor. Moreover, HCV cell-to-cell transmission may bypass the inhibition of cell-free virus entry and allow viral spread. Therefore, clinical strategies based on broad-spectrum compounds or the combination of different therapeutic molecules should be developed to simultaneously interfere with several steps of the viral cycle to efficiently control infection with the minimal side effects.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2691

World J Gastroenterol 2011 June 14; 17(22): 2691-2695
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Inflammatory bowel disease in adolescents: What problems does it pose?

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Received: February 27, 2010 Revised: April 2, 2010
Accepted: April 9, 2010
Published online: June 14, 2011

Abstract

Adolescents with inflammatory bowel disease face daily and long-term challenges that may be difficult for teenagers to manage. The developmental and psychosocial changes unique to this age group include becoming more autonomous and being more vulnerable to peer influence. These changes may lead to problems in medical management such as poor medication adherence and risky behavior. Being aware of these issues will help the medical team provide anticipatory guidance to address these concerns.

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Key words: Inflammatory bowel disease; Adolescent; Patient compliance; Growth and development; Nutrition; Peer group; Risk behavior

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Lu Y, Markowitz J. Inflammatory bowel disease in adolescents: What problems does it pose? *World J Gastroenterol* 2011; 17(10): 2691-2695 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2691.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2691

INTRODUCTION

Inflammatory bowel disease (IBD) is one of the more common chronic illnesses afflicting adolescents, with an estimated prevalence in those younger than 20 years old of 71 per 100 000[1]. Both Crohn's disease (CD) and ulcerative colitis (UC) present day-to-day as well as long-term challenges that can be particularly difficult for the adolescent patient. The developmental and psychosocial changes unique to adolescence, including establishing autonomy, risk-taking behavior, and undue susceptibility to peer influence make managing IBD in this age group more difficult.

MEDICATION ADHERENCE

Adhering to a prescribed medication regimen is difficult for teenagers. Adolescents need to remember to take medications amidst a busy lifestyle with school and extracurricular activities, and to accurately recall the types and number of medications to be taken at various times of the day. They can be particularly resistant to taking medications that cause cosmetic changes, choosing a better appearance over persistent IBD symptoms. The concept of maintenance therapy can also be challenging for teens who, once acute symptoms subside, often decide to stop taking medications "because I don't need them anymore".

A study by Hommel *et al*[2] examined the problem of medication nonadherence in 42 adolescents with IBD. In addition to recording an objective measure of adherence

using pill count, they further evaluated the problem by determining the adolescents' subjective assessments of their adherence through standardized interviews. Subjects reported that they missed 6% of their 6-mercaptopurine/azathioprine (6-MP/AZA) doses and only 3% of the 5-aminosalicylate (5-ASA) medications. However, pill counts revealed that 38% of 6-MP/AZA and 49% of 5-ASA doses had been missed. Only 14% of patients had therapeutic levels of thiopurine metabolites, further confirming their poor adherence to the prescribed 6-MP/AZA regimen. The subjects who were nonadherent to 6-MP/AZA were also likely to miss 5-ASA doses. The frequent dosing and large quantity of pills per dose were factors associated with poor adherence to 5-ASA treatment.

A survey study by Greenley *et al*[3] of 64 adolescents and their parents found that 48% of teenage patients took one IBD medication or supplement, 36% took two, and 11% took three. Only 65% of adolescents reported perfect adherence. Common barriers to medication adherence included lack of time, medication side effects, feeling well, or belief that the medication was ineffective. Patients on polytherapy had more barriers to successful medication-taking than those on monotherapy. A similar study by Ingerski *et al*[4] found that the most common barriers to medication nonadherence included forgetting, being away from home, and interference with an activity.

Based upon these studies, physicians may help increase medication adherence by minimizing the frequency of doses and number of pills. When prescribing a new treatment, physicians need to carefully explain to adolescents why they need to take their medications as prescribed, especially when patients have low expectations that the therapeutic regimen will be effective, or are likely to experience side effects[3]. Physicians must also clearly communicate the concept of maintenance therapy, both when medications are first prescribed, and more importantly, during routine follow-up visits so that teenagers understand why they need to continue to take medications even when they are feeling well. Suggestions to help teens remember to take their medications include using a pill box, carrying an extra supply of pills, and setting an alarm as a reminder on a watch, cell phone, or portable electronic device.

GROWTH AND DEVELOPMENT

IBD may lead to growth failure and delayed puberty during adolescence. Pfefferkorn *et al*[5] examined growth outcomes in children with CD at diagnosis, 1 year, and 2 years using data from the Pediatric IBD Registry, a prospective, multicenter observational database established in 2002. The study found that the mean height z-scores were approximately -0.50 standard deviations (SD) at each of the three time points despite improvement in disease activity over the course of the observation period. Although the mean height velocity z-score increased between the first and second years, the proportion of patients with a height velocity z-score less than -1 SD was similar and substantial (45% at 1 year and 38% at 2 years). Furthermore, all subjects in the study were Tanner Ⅰ to Ⅲ at diagnosis and at 1 year. At 2 years, 84% (92/110) of patients did not progress to Tanner Ⅳ. These data are disturbingly similar to those derived from patients diagnosed in the 1970s and 1980s, in whom periods of significant growth failure were demonstrated in 37% of young adults whose CD had been diagnosed before their adolescent growth spurt[6]. Despite advances in nutritional and anti-inflammatory therapy, promoting normal growth continues to be a significant challenge for the physician caring for adolescents with IBD.

Not only is height adversely affected in children with IBD, but bone mineral density (BMD) is as well. Sylvester *et al*[7] prospectively followed Caucasian children with IBD over a 2-year period from diagnosis. The mean total body BMD z-score at diagnosis was significantly lower for CD patients compared to healthy controls of similar age (-0.78 for CD, -0.46 for UC, and -0.17 for controls). CD patients with a BMD z-score lower than -1 tended to have a lower body mass index (BMI) and higher serum interleukin-6. Despite clinical improvement in the CD and UC patients over the follow-up period, the mean BMD z-score was unchanged for CD. Similarly, the mean BMD z-score for UC patients was unchanged from diagnosis to 1 year, but increased from the first to second year.

Sylvester's study[7] also found that both CD and UC patients had decreased serum concentrations of biochemical markers of bone formation at diagnosis compared to controls. In addition, CD patients had a lower concentration of N-telopeptide of collagen, a marker of bone resorption, compared to controls. Over the course of the study, improvement in clinical status and nutrition was associated with increased concentrations of markers of bone formation but not of bone resorption. However, mineralization rates did not significantly improve, particularly in CD patients.

Dubner *et al*[8] evaluated the bone and muscle of the left tibia using peripheral quantitative computed tomography (pQCT) in pediatric CD patients. pQCT provides a 3-dimensional assessment of trabecular and cortical volumetric BMD (vBMD) and geometry, and a cross-sectional area of muscle and fat. Patients with CD had musculoskeletal deficits (in trabecular vBMD, cortical bone geometry, and muscle mass) at diagnosis compared to healthy controls of similar age. Six months after diagnosis, there was improvement in trabecular vBMD and muscle mass, but worsening of cortical bone geometry. One year after diagnosis, all 3 were still low compared to controls.

In addition to physical appearance, growth failure and delayed puberty may also affect psychosocial aspects of adolescent life. Looking different from peers may render teens to feel self-conscious or "abnormal". Teens may have difficulty "fitting in" with peers or dating because they appear less mature. Having short stature may also affect their ability to participate in or excel at sports, or at least give adolescents this perception. All these

factors may lead teens to become repeatedly frustrated, have low self-esteem, or seclude themselves.

Poor nutrition may contribute to poor growth. Patients may not meet their nutritional requirements because of inadequate caloric intake, malabsorption, or increased metabolic needs from chronic inflammation. Patients with CD are more likely to be malnourished than patients with UC. It is important to monitor nutritional status by anthropometrics, and checking blood for albumin and micronutrient levels (e.g. iron, folate, and B12). Patients who are undernourished or have micronutrient deficiencies should receive supplementation with oral or nasogastric tube feeds, or with specific vitamins or minerals. In addition, it may be beneficial for patients to take a daily multivitamin. Patients receiving sulfasalazine and/or methotrexate should be given folic acid supplements[9]. Patients may also be referred to a nutritionist for evaluation of dietary intake and guidance on how to eat a healthy and appropriate diet.

PEER INFLUENCE

Peer influence is strong during adolescent years. This period is filled with the desire to be accepted, identity formation, intense introspection, and internal conflict. Teenagers may feel embarrassed or self-conscious about their body as a result of IBD, its complications, or side effects from medications. Patients may feel uncomfortable about their short stature, delayed puberty, body habitus (thin from malnutrition or weight gain from corticosteroids), surgical scars, or ostomy. Patients missing school or limiting extracurricular activities due to IBD flares or medical appointments may feel isolated from peers. Teenagers may not want to appear different from friends or raise suspicion about having a medical condition, especially one that involves potentially embarrassing topics such as diarrhea.

When adolescents are ready to share their medical condition with friends or someone they are dating, they must decide when is the appropriate time, and how to do so. Patients may want to let peers realize that disclosing this information demonstrates that patients trust their peers and that their relationship has reached a certain level. Patients may also explain that the disease does not define them as a person. In addition, adolescents should understand that they have the option of sharing only information which they are comfortable disclosing.

RISKY BEHAVIOR

Adolescents may participate in risky behaviors such as practicing unprotected sex, and abuse of alcohol and illicit drugs[10,11]. It is common to hear adolescents explain their risky behavior because they think that bad consequences "won't happen to me." Adolescents with IBD are no exception, but their risky behavior can also take the form of discontinuing their medications without consulting their physician. Therefore, it is important for the physician caring for adolescents with IBD to address risky behavior and provide anticipatory guidance. For example, adolescent IBD patients should be counseled about the importance of using protection if they decide to have sexual intercourse. If the female adolescent patient decides to use oral contraceptive pills, she should be aware of the possible increased risk of thrombosis[12]. It is also important to explain that, although sulfasalazine can lower sperm counts, it should not be considered a method of contraception[13].

If the teenage patient is pregnant, she may want to seek family planning to decide whether to continue the pregnancy. If the decision is to continue the pregnancy, it may be beneficial to transfer her to an adult gastroenterologist because internists have more experience with pregnancy and high-risk obstetrics than pediatricians. Patients should be encouraged to tell their physicians that they are pregnant because some IBD medications such as methotrexate are known teratogens and others may need to be monitored carefully during pregnancy. Conversely, some medications are safe during pregnancy but there may be a popular misconception that they are not (such as infliximab until 28 wk gestation)[14].

Toxic ingestion (including alcohol consumption) and illicit drug use are other risky behaviors that are important to address. Teens may abuse alcohol and illicit drugs for various reasons, such as peer pressure, recreational use, emotional escape, pain control, or appetite stimulation. Regardless of their reason, alcohol and illicit drug use may have multiple adverse health effects including damage to the liver, an organ vital in metabolizing some medications used to treat IBD. In addition, intravenous drug use, unprotected sex, and having tattoos or body piercing are risk factors for contracting hepatitis B and C[15-18]. Adolescents should also be informed that metronidazole must not be taken with alcohol, as it can cause a disulfiram-like reaction[19].

TRANSITION TO AN ADULT GASTROENTEROLOGIST

Transition to an adult gastroenterologist provides older teenagers and young adults with a hospital or clinic environment that is appropriate for their age. In addition, adult gastroenterologists are more experienced than their pediatric counterparts regarding issues frequently encountered in adults such as pregnancy, fertility, and cancer surveillance. In addition, the process of transition promotes independence and better adherence with therapy because patients must learn to take care of themselves, be proactive in their medical care, form decisions, communicate with the medical team, and be a self-advocate[20,21].

The transition may be stressful for everyone involved in the process: patients, parents, as well as pediatric and adult gastroenterologists. Patients and families must relinquish the familiarity of the pediatric medical team and institution to face a new one that may have a different practicing style and expect patients to be independent. The

adult gastroenterologist may subsequently be perceived as less concerned about the needs of the patients and their families. Pediatric gastroenterologists may be reluctant to transfer patients to an adult provider whose medical management may be different or who may be less familiar with the issues of adolescent patients. Adult gastroenterologists may see young adult patients as less mature or self-reliant, and their families as overly involved[20].

Hait *et al*[21] proposed a timeline consisting of competencies patients should achieve and tasks the medical team should implement based on patients' age, to help promote a successful transition. Pre-adolescents (ages 11-13 years) should learn to identify their disease and medications (names, dosages, and major side effects). The medical team should present the idea of independent visits in the future and discuss the effects of exercise, sexual intercourse, and substance abuse on IBD. Young adolescents (ages 14-16 years) should learn to identify their medical team and be knowledgeable about their medical history (including prior procedures and tests), relevant family history, and the risk of medication nonadherence. The medical team should start to help patients to become more independent by addressing patients first and having the family step out of the room for part of the visit. However, it is important to explain that the medical team has a legal obligation to share certain aspects with parents. Patients should be taught how to contact the medical team, schedule appointments, and fill prescriptions. In addition, physicians should inquire about their patients' plans after high school and introduce the idea of transition. Older adolescents (ages 17-19 years) should be able to manage their medical needs, which include scheduling appointments and having a plan to attend them, asking and answering questions during their private conversation with the gastroenterologist, filling prescriptions and picking them up at the pharmacy, and being knowledgeable about their insurance. Physicians should discuss possible difficulties encountered in the transition process and provide names of prospective adult gastroenterologists.

When patients are ready to transfer care, transition is easier when there is stability in their disease and life. Transition is more successful when patients in college are transferred to an adult gastroenterologist after graduation and have acquired a job or started graduate school. Patients who do not plan to attend college may be transferred after securing housing and a job. A medical summary should be provided at the time of transfer[21].

A survey by Hait *et al*[22] illustrated adult gastroenterologists' perspectives of the transition process. The survey of 363 adult gastroenterologists revealed that factors they believed were most important for a smooth transition were often lacking amongst their young adult patients. These factors included patients' knowledge about their medications (name, dose, and major side effects), medical history, and health effects of smoking, drugs, and alcohol. Adult gastroenterologists also considered it helpful that pediatric gastroenterologists should provide a medical summary of their patients. The vast majority of adult gastroenterologists thought it was important that adult providers be familiar with adolescent medical and developmental issues, but a significantly low proportion felt proficient in this area.

CONCLUSION

In summary, adolescents have unique issues such as poor medication adherence, growth failure, peer influence, and risky behavior, which make managing teenage IBD patients more challenging. It is important for physicians to recognize these issues so that they are prepared to address patients and parents early to help improve adherence to medications, nutritional status, complications of IBD, social well-being, and the transition process.

ACKNOWLEDGMENTS

The authors thank Janis H Arnold, LICSW for the helpful suggestions she provided towards this manuscript.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2696

World J Gastroenterol 2011 June 14; 17(22): 2696-2701
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Inflammatory bowel disease in pregnancy

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Received: April 27, 2010 Revised: June 15, 2010
Accepted: June 22, 2010
Published online: June 14, 2011

Abstract

Crohn's disease and ulcerative colitis affect women in their child-bearing years. Family planning has come to be a common discussion between the gastroenterologist and the inflammatory bowel disease (IBD) patient. Disease control prior to desired conception and throughout pregnancy is the most important thing to keep in mind when caring for the IBD patient. Continued medical management during pregnancy is crucial in optimizing outcomes. Studies indicate that quiescent disease prior to conception infer the best pregnancy outcomes, similar to those in the general population. Active disease prior to and during pregnancy, can lead to complications such as pre-term labor, low birth weight, and small for gestational age infants. Although there are no definitive long term effects of pregnancy on IBD, there are some limited studies that suggest that it may alter the disease course. Understanding the literature and its limitations is important in the modern era of IBD care. Educating the patient and taking a team approach with the obstetrician will help achieve successful outcomes for mother and baby.

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Key words: Inflammatory bowel disease; Pregnancy; Crohn's disease; Ulcerative colitis; Breastfeeding

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Beaulieu DB, Kane S. Inflammatory bowel disease in pregnancy. *World J Gastroenterol* 2011; 17(22): 2696-2701 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2696.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2696

INTRODUCTION

Approximately fifty percent of patients are less than 35 years of age at the time of diagnosis and twenty five percent conceive for the first time after their diagnosis of inflammatory bowel disease (IBD)[1-3]. Advances in the field of IBD have made successful pregnancy outcomes a reality for many women. It is very important that as a gastroenterologist you have input into the conversation regarding management during pregnancy. It is important to understand the effect of pregnancy on IBD and the effect of IBD on a pregnancy. This review discusses the evidence for the important questions that female patients have in regards to this topic, and recommendations based on clinical experience of the authors has also been included.

HOW DOES IBD AFFECT THE OUTCOMES OF PREGNANCY?

The general consensus agrees that the impact of IBD

on pregnancy depends on disease activity at conception. Studies suggest that quiescent disease throughout a pregnancy leads to similar risks to those of the general population in regards to spontaneous abortion, pregnancy related complications, and adverse perinatal outcomes[4-6]. Disease activity at conception has been associated with preterm births, low birth weight (LBW), and fetal loss[4,7-10]. In addition, active disease during pregnancy results in the greatest risk of adverse perinatal outcomes[3,4,11]. This risk appears to be higher in women with Crohn's disease (CD) than in those with ulcerative colitis (UC). However, severe disease relapses during pregnancy in UC are associated with shorter gestation periods and lower birth weights[12]. In the study by Reddy *et al*[12] there was a higher risk of preterm births among women hospitalized for severe UC, with the mean gestational age being 35 wk *vs* 38.7 wk in the control group (without disease relapse).

In 1997, Kornfeld *et al*[13] studied 756 women with IBD and found that IBD was an independent risk factor for LBW and premature infants. However, they did not differentiate between CD and UC. Studies in the US and Denmark have demonstrated an increased risk of preterm delivery, small for gestational age (SGA), and LBW in infants to CD mothers[8,14,15]. In a study of Danish UC women, 1531 infants were included and there was no difference in rates of pre-term delivery, LBW, or SGA compared to controls[16]. The rate of healthy offspring to UC women was similar to the general population[3,8]. Despite a consistent trend of preterm delivery, most of the deliveries occurred after 35 wk with favorable outcomes[14].

Khosla *et al*[17] examined a cohort of 54 pregnant CD patients, and found that those with active disease at conception had rates of miscarriage up to 35% higher than those of patients who were in remission. In 2007, Nørgård *et al*[18] examined the impact of disease activity on birth outcomes in CD, and reported that activity during pregnancy only increased the risk of preterm birth. Furthermore, Moser *et al*[19] concluded that the presence of ileal disease in CD women was a strong predictor for LBW.

In a study by Dominitz *et al*[14] a greater risk of congenital abnormalities was seen in UC women compared to controls (7.9% *vs* 1.7%, $P < 0.001$). This study, however, failed to take into consideration disease activity or medication use, and this finding has not been replicated by other investigators. Indeed, most studies have found no greater risk of malformations in UC or CD[8,10,20]. For example, Lamah *et al*[21] found that there was no increased risk of spontaneous abortions, perinatal mortality, or congenital malformations in their UC cohort.

Using the 2005 Nationwide Inpatient Sample, Nguyen *et al*[22] examined 2372 CD deliveries and 1368 UC deliveries. In this population-based study, the adjusted odds of a cesarean section were higher in women with CD (aOR 1.72) and UC (aOR 1.29) compared to non-IBD controls. The risk of a venous thromboembolism was also substantially higher in IBD pregnancies. Interestingly, protein calorie malnutrition occurred more frequently in IBD women, as did blood transfusions in CD deliveries (aOR 2.82). To date, there are no associated complications of hypertension or proteinuria with IBD pregnancy[5].

Beniada *et al*[11] reported a series of 76 cases of women with their first IBD flare during pregnancy, and observed that they were at increased risk for pre-term delivery and/or LBW. The largest study to date on this topic is the meta-analysis by Cornish *et al*[23] that evaluated 12 studies in regard to the impact of IBD on pregnancy. This study comprised a total of 3907 patients with IBD and 320 531 controls. Based on this analysis, women with IBD were more likely to experience adverse pregnancy outcomes, such as premature birth and LBW. In fact, premature delivery was almost twice as likely compared to the general population. Women with IBD were also 1.5 times more likely to undergo cesarean section. Unfortunately, neither medication use nor disease activity was analyzed as a cofounder, which makes it difficult to put the results into proper perspective. The meta-analysis reported a 2.37 fold greater risk of congenital abnormalities (95% CI: 1.47-3.82, $P < 0.001$), but most of the studies included did not differentiate between minor and major malformations[23].

In 2006, a Spanish study of 124 pregnant CD women looked at pregnancies before and after diagnosis. They concluded that the course of IBD did not adversely affect pregnancy or the postpartum time period. The study determined that diagnosis prior to pregnancy did not influence the number of cesarean sections performed or increase the presence of LBW infants[24].

In 2007, Mahadevan *et al*[25] compared pregnancy outcomes between women affected with IBD and those unaffected. The study comprised of 461 pregnant IBD patients and a randomly selected cohort of age matched controls, and represents the largest US study to date. Women with IBD were more likely to have an adverse pregnancy complication compared to those women without IBD, but there was no difference in adverse newborn outcomes or congenital abnormalities. This difference was seen irrespective of the disease activity. The use of IBD medications was not found to be predictive of adverse outcome in this large, non-referral population. There was no statistically significant difference in newborn outcomes between the IBD and control pregnancies.

In the general population, smoking is a known risk factor for LBW infants and for disease activity in CD women[2]. Pregnant CD patients who smoke are at a substantially increase risk for LBW and preterm delivery[2,21]. Conversely, smoking in UC women does not increase their risk of preterm delivery[26]. However, given the known risk of smoking on the individual and the baby, smoking cessation should be encouraged in all scenarios.

Numerous studies continue to associate preterm birth and IBD; however the majority of these "preterm" deliveries occurred after 35 wk of gestation[14,26]. In UC, if resection is needed during pregnancy, Nielsen *et al*[8] found an increased risk of preterm delivery. Many theories have been put forth for this observation, but the etiology is unclear. One hypothesis is that an increase in circulating

prostaglandin levels during a flare could initiate pre-term labor with the induction of smooth muscle contraction[12,27]. Another theory is that the role of increased gut permeability during increased inflammation could alter nutritional and immunological factors affecting labor[12]. In addition, safety concerns for the mother and baby might prompt induction of early delivery, which would bias the end result of an LBW infant. Although each theory is plausible, more data are needed to clarify IBD and preterm infants.

While the data are conflicting at times, it appears that the disease activity is the main impetus of adverse pregnancy outcomes[28], as miscarriages are seen more frequently with active disease[13-15]. In our practice, we encourage women to be in remission before considering conception, and for those who become pregnant, we monitor closely and treat disease activity aggressively.

HOW DOES PREGNANCY AFFECT THE DISEASE COURSE?

A consistent finding in more recent literature is that the rate of disease flare during pregnancy (26%-34%) is similar to non-pregnant flare rates[7,10,29,30]. An exacerbation rate of 34% per year during pregnancy in UC women and 32% per year when not pregnant was observed by Nielsen *et al*[8]. These rates of relapse were similar in the CD population[10]. The Kaiser cohort, previously discussed, included women with inactive disease throughout their pregnancy, with no sudden increase in activity post-partum[25,29].

UC

When conception occurs during a quiescent state, 70%-80% of UC patients will remain in remission[2,8]. The rate of relapse is thus similar to a non-pregnant UC patient. Unfortunately, when flares do occur, the data is unreliable in relation to the stage of pregnancy. It was initially believed that an increase of disease flare occurred in both the first trimester and post-partum, but timing of a flare appears to more related to disease activity at conception and at term. Moreover, disease flare is often related to discontinuation of medical therapy (first trimester) or resuming smoking after delivery (post-partum)[2,11,31]. Active disease at conception can be associated with a worse prognosis. In a cohort of UC patients, Willoughby *et al*[32] noted that active disease during these times was more resistant to treatment.

The patient who has undergone an ileoanal anastomosis procedure presents a special situation. Ravid *et al*[33] examined 67 pregnancies in 38 UC women with ileal pouch anal anastomosis surgery. It was determined that pregnancy was safe with some alteration of pouch function, almost exclusively during the third trimester. For most of the women, the pouch function returned to its pre-pregnancy state. However, there was a small proportion of women that suffered long term disturbance of pouch function. This long- term effect was not related to the method of delivery. Although the mode of delivery in a pouch patient remains disputed, the method of delivery should not be determined by the presence of a pouch, but by patient and obstetric decisions. There are no long term data on pouch function after vaginal delivery but short-term data showed that pouch function, continence and quality of life are not affected by uncomplicated vaginal delivery[2,34].

CD

CD during pregnancy is similar to patients with UC. As with UC, the key to a good outcome is the disease state at conception and delivery. If the CD is quiescent at conception, 70% of pregnant CD patients will remain quiescent compared to the non-pregnant CD patient[2,17,35]. There has been some suggestion that CD symptoms might improve during gestation and that relapse is more common in the first trimester[2,17,26]. When disease is active at the time of conception, we follow "the rule of thirds". One third of women will get better, one third will stay the same, and one third will worsen. The biological mechanism of this finding has yet to be fully explained, but several studies have suggested that the immune disparity between mother and fetus might play a role in immune regulation, thereby altering immune function and pathology[36].

The recommended mode of delivery in CD patients is still controversial. In comparison to the general population, CD patients undergo cesarean sections more frequently, with the rate of cesareans increasing after diagnosis[37,38]. There are conflicting data on vaginal deliveries and perianal disease. The current recommendation, based on small observational studies, is to avoid vaginal deliveries in the setting of active perianal disease. CD patients with uncomplicated disease should be treated like the general population when deciding on delivery, but episiotomy should be avoided. The presence of a colostomy or ileostomy should not designate delivery choice. Despite the limited data, choice of delivery should be a collaborative decision between the patient, gastroenterologist, and the high-risk obstetrician.

Nursing

Any detriment to maternal health secondary to nursing after delivery is controversial. There have been some reported associations between nursing and increased disease activity, but this is unclear whether this is related to disease course or cessation of medication. Kane *et al*[31] found the odds ratio (OR) of disease flare for women who breastfeed was 2.2 (95% CI: 1.2-2.7) compared to those who did not breastfeed. However, once medication discontinuation was factored in, the OR became non-significant. Moffatt *et al*[39] published a population based study of breastfeeding and found no increased risk of flare in the postpartum period, and a possible protective effect once the discontinuation of medications was taken into account.

Long term effects of pregnancy on IBD

There have not been any data to suggest a long term detrimental effect of pregnancy on IBD and there is

never a role for elective termination. In the specific cases where methotrexate or thalidomide are involved, the decision for a therapeutic abortion may need to be addressed, given that it is a category X drug with known association with fetal abnormalities. Pregnancy has not been shown to definitively alter disease phenotype[38]. Riis *et al*[38] demonstrated that parous IBD patients experienced a reduction in relapse rate in the three years following pregnancy when compared to the three preceding years[2,40]. The rate of relapse decreased in the years following pregnancy in both UC and CD. Riis *et al*[38] looked at 580 IBD pregnancies in a European cohort. The pregnancy itself did not influence disease phenotype or surgery rates, but it was associated with a reduced number of flares in the following years (UC 0.34 flares/year *vs* 0.18 flares/year, $P = 0.008$ and CD 0.76 flares/year *vs* 0.12 flares/year, $P = 0.004$). Nwokolo *et al*[41] demonstrated a negative correlation between increasing parity and number of resections[2]. They found that in parous women with CD, the need for surgical resection was inversely correlated with increasing parity. Castiglione *et al*[42] studied parous IBD women and found that the incidence of relapses in the first three years after pregnancy was lower than that prior to pregnancy. Hormonal changes during and after pregnancy might account for a change in fibrosis and stricture formation[5]. Some studies suggest a down regulation of the immune system with maternal fetal HLA disparity[36]. Maternal immune response to paternal HLA antigens might result in immunosuppression that can in turn affect the maternal immune-mediated response. Kane *et al*[36] looked at 50 pregnancies in 38 women and found 42 disparate (84%) at the DRB1 locus, 34 (68%) at the DQ locus, and 31 (62%) at both loci. A significant difference was found in IBD activity between women mismatched at both loci *vs* only 1 or neither locus (OR 8.4, $P = 0.01$) Improvement of IBD symptoms during pregnancy was associated with disparity in HLA class Ⅱ antigens between mother and fetus. When logistic regression was performed, pre-partum disease activity and disparity at both DRB1 and DQ were significant predictors of overall disease activity during pregnancy. Pregnancy should never be discouraged or terminated in a patient with IBD, but instead, the goal of care should be early counseling and appropriate medical management.

IBD medications and pregnancy outcomes (Table 1)

It is believed that the greatest risk to IBD pregnancy is active disease and not active therapy. In addition to the effect on disease activity during pregnancy, the fear of a medication's effect on the fetus often prompts physician and patient to discontinue all medications. Pregnancy data on outcomes and disease course are complicated by cessation of drugs. One of the earliest available drugs for the treatment of colitis, sulfasalazine, readily crosses the placenta, but has not been linked to any fetal abnormalities in several large studies. However, patients taking sulfasalazine should be supplemented with folic acid to decrease the risk of neural tube defects. A dose of one milligram twice daily is appropriate.

Table 1 Use of medications during pregnancy in inflammatory bowel disease

Benefit clearly outweighs risk	Limited data	Contraindicated
5-ASA, oral and topical	Olsalazine	Methotrexate
Corticosteroids	Natalizumab	Thalidomide
Metronidazole, amoxicillin		
Azathioprine/6-MP		
Anti-TNF agents		

TNF: Tumor necrosis factor.

Aminosalicylates: The safety of 5-ASA compounds during pregnancy has been demonstrated in a number of trials, despite the fact that mesalamine and its metabolite, acetyl-5-aminosalicylic acid are found in cord plasma[43,44]. In two separate studies, women taking 2-3 g/d for either UC or CD had no increased incidence of fetal abnormalities compared to normal healthy women.

Immunomodulators: In the retrospective chart review by Francella *et al*[45], there were 79 women with 325 pregnancies. The compared patients on 6-MP during conception, those that stopped prior to conception, and patients never exposed to 6-MP were compared. Although they did not look at prematurity or LBW, there were no statistical differences in spontaneous abortions, major congenital abnormalities, neoplasia, or increased infection [RR 0.85 (0.47-1.55), $P = 0.59$]. Moskovitz *et al*[46] looked at IBD medications (including 6-MP and azathioprine) taken during pregnancy. In this age-controlled multivariate analysis of 113 patients with 207 conceptions, there was no evidence that medications affected pregnancy outcomes (abortions, premature birth, healthy full-term birth, ectopic pregnancy, congenital abnormalities, birth weight, or type of delivery). Nørgård *et al*[47] combined two large national data registries with a national prescription database to look at therapeutic drug use in women with CD and birth outcomes. Among the women that were exposed to 6-MP/azathioprine throughout their pregnancies, the risk of preterm birth and congenital abnormalities was 4.2 (95% CI: 1.4-12.5) and 2.9 (95% CI: 0.9-8.9), respectively. Preterm births were more prevalent among steroid and 6-MP/azathioprine exposed women compared to the reference group. However, they were unable to stratify disease activity to adverse birth outcomes due to the low number of hospital admissions. Due to model fitting issues, the authors could not adjust for the "disease activity" when looking at the LBW infants at term[48].

In has been our practice to continue immunomodulator therapy through pregnancy. While classified as an FDA category D medication, it received this designation in the 1950s when originally approved to treat leukemia. The doses used to treat IBD are much smaller and the above cited literature suggests this to be a low risk therapy at these doses. Stopping therapy once pregnancy is diagnosed only puts the mother's disease at risk of flaring, as organogenesis has already occurred at this point and the fetus has been exposed at its most vulnerable-

cessation for safety to the fetus has to be 6-8 wk before conception, not after.

Biologics: Biologics are now more commonly used for more aggressive disease, and sometimes are used as first line in the "top-down" therapeutic approach. The first series of intentional infliximab use throughout pregnancy by Mahadevan *et al*[49] examined outcomes in ten women with active CD during pregnancy. All ten pregnancies resulted in live births, with no congenital malformations. There were three pre-term births and one LBW infant, but these were not unexpected in a population of women with CD significant enough to require biologic therapy. Infliximab has been detected in the infants born to mothers receiving infliximab during the third trimester of pregnancy; however, to date, the long term effect of this placental transfer is unknown[50].

A published report for the successful use of adalimumab in pregnancy describes a patient with severely active disease at conception[51]. She was placed on adalimumab one month prior to conception and delivered of a normal growth infant without visible congenital anomalies. A case series recently presented suggested its safety in fetal outcomes in women treated for CD[52]. More recent data presented in abstract form showed that patients exposed to natalizumab during pregnancy had a spontaneous abortion rate comparable to what is expected in the general population[53]. However, the number of exposed patients was too low to draw any definitive conclusions. Therefore, it is important for the physician to discuss with each patient the risk to benefit ratio of biologic therapy to control disease in pregnancy.

It has been our practice to continue biologics at least through the second trimester of pregnancy. Placental transport of IgG begins around week 20 and increases thereafter. Theoretically therefore, no therapy is reaching the fetus until administrations after week 20. If the last infusion of infliximab is timed to be around week 32, then the next infusion can be after delivery. Adalimumab injections are held after week 36.

Other agents: Corticosteroids have not been associated with teratogenicity in humans and can be used as required to control disease activity. Prednisolone crosses the placenta less efficiently than other steroid formulations, such as betamethasone or dexamethasone. Only limited data are available regarding the safety of antibiotics as treatment for CD. Currently, ampicillin, cephalosporins, and erythromycin are believed low risk, as is ciprofloxacin. Metronidazole has been used to treat vaginitis in women during the first trimester of pregnancy but no controlled trials have definitively demonstrated its safety[54].

CONCLUSION

Over twenty years ago it was recognized that IBD patients flaring at the time of conception had a higher chance of spontaneous abortion, still birth, and premature delivery. The classic Miller[6] paper from 1986 is often referenced. For patients with active disease, one third will improve, one third will stay the same, and one third will worsen. This leaves two thirds of patients having to live with active disease during pregnancy[48]. Despite conflicting and sometimes confusing data, it is clear that there are potential risks involved with IBD pregnancies. It is crucial to understand the literature and also recognize its limitations. Ideally, conversations with the patient should occur before conception. Furthermore, decisions should be made with an informed approach with a team effort between the patient, the obstetrician, and her gastroenterologist. The key is continued monitoring and aggressive control of the disease prior to conception and throughout, to achieve optimal outcome for mother and baby.

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S- Editor Wang YR **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2702

World J Gastroenterol 2011 June 14; 17(22): 2702-2707
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome?

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Received: April 27, 2010 Revised: August 11, 2010
Accepted: August 18, 2010
Published online: June 14, 2011

Peer reviewer: Dr. Stephan Johannes Ott, 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

Veloso FT. Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome? *World J Gastroenterol* 2011; 17(22): 2702-2707 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2702.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2702

Abstract

Crohn's disease and ulcerative colitis are chronic inflammatory bowel diseases that often involve organs other than those of the gastrointestinal tract. Immune-related extraintestinal manifestations (EIMs) are usually related to disease activity, but sometimes may take an independent course. Globally, about one third of patients develop these systemic manifestations. Phenotypic classification shows that certain subsets of patients are more susceptible to developing EIMs, which frequently occur simultaneously in the same patient overlapping joints, skin, mouth, and eyes. The clinical spectrum of these manifestations varies from mild transitory to very severe lesions, sometimes more incapacitating than the intestinal disease itself. The great majority of these EIMs accompany the activity of intestinal disease and patients run a higher risk of a severe clinical course. For most of the inflammatory EIMs, the primary therapeutic target remains the bowel. Early aggressive therapy can minimize severe complications and maintenance treatment has the potential to prevent some devastating consequences.

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Key words: Inflammatory bowel disease; Immune-related extraintestinal manifestations; Treatment

INTRODUCTION

Inflammatory bowel diseases (IBDs) are clinically heterogeneous disorders with the potential for systemic involvement. The clinical spectrum of these extraintestinal manifestations (EIMs) varies from mild transitory to very severe lesions, sometimes more incapacitating than the intestinal disease itself.

Most of the EIMs, such as those that occur in the joints, skin, mouth and eyes, are related to the activity of the bowel disease and, consequently, have been referred to as "inflammatory". Others are associated with autoimmunity, while further manifestations result from nutritional or metabolic dysfunction.

Patients with ulcerative colitis (UC) are considered a more homogeneous group than patients with Crohn's disease (CD). The most important significant variables in UC are the extent of colonic involvement and the activity of disease.

The current clinical practice for management of CD patients should take into account a major number of events, such as age at diagnosis, location, behavior, intestinal complications, EIM symptoms and previous medical or surgical treatment.

The aim of the present manuscript is to review the common immune-related EIMs and to evaluate whether they are important targets of treatment or just an expression of disease activity.

PREVALENCE OF EIMS IN IBD

Globally, about one third of patients with IBD develop inflammatory systemic manifestations. The frequency of these manifestations in previous major studies ranged from 21% to 41%, depending on the patient population and criteria used[1-5]. In our series they were observed in 26% of the patients and were more common in CD than in UC patients. However, during the follow-up period the cumulative probability of developing EIMs increased from 12% at diagnosis up to 30%[2].

We have studied the course of CD according to the Vienna classification and clinical activity[6]. More recently, in this cohort of 480 patients followed from diagnosis, we investigated the cumulative probability of inflammatory EIMs, which varied from 22% at diagnosis to 40% 10 years after diagnosis. Here we showed that a single manifestation occurred in 80 patients, whereas multiple manifestations overlapping joints, skin, mouth, and eyes occurred in 89 patients (Figure 1).

The specific disease entities considered were peripheral and axial arthritis (joints), erythema nodosum and pyoderma gangrenosum (skin), aphthous stomatitis (mouth), and episcleritis and uveitis (eye). Thus, it was confirmed that two or more manifestations in the same patient occurred more frequently than would be expected by chance alone ($P < 0.001$)[2,4].

Patients with one of these EIMs not only run a high risk of a repetition of the same manifestation, but also of having any of the other associated manifestations. In our patients the cumulative probability of a second manifestation was about 70% at 10 years of follow-up.

Moreover, we have also studied the appearance of various EIMs throughout the clinical course and it was observed that some types of manifestations are more likely to appear during the early stages. This was particularly evident for skin manifestations, which tend to occur in most of the patients for the first time during the first 2 years of follow-up (Figure 2).

We have also studied the probability of EIMs in different subsets of CD patients[7]. The location of disease was significantly different between patients who had and who did not have EIMs and was higher in those with colonic involvement ($P < 0.005$) (Figure 3).

To calculate the clinical course, we constructed a Markov model to study the probability of maintaining a similar state during the following year. Patients were divided into two exclusive groups, those who had and those who did not have manifestations. Patients with EIMs ran a higher risk of a severe clinical course ($P < 0.005$)[7].

In UC it is still controversial whether EIMs are correlated with the extent of colonic involvement, although they are usually related to the activity of the disease.

DESCRIPTION OF EIMS

Joint manifestations

Peripheral involvement of the joints is the most common of the EIMs and it affects between 5% and 20% of IBD patients[8-11]. In our series, arthritis occurred frequently, twice as often in CD as in UC (20% *vs* 11%)[2]. The joint involvement is mostly monoarticular, asymmetrical, transitory and migrating. Generally, it is of short duration and is unaccompanied by residual deformity or radiological change.

The diagnosis is based on clinical signs of synovitis, eventually associated with enthesitis. However, using articular scintigraphy, a sensitive indicator of active joint disease, asymptomatic and mild forms of synovitis could be detectable. We have evaluated articular involvement, clinically and by scintigraphy, in forty-two IBD patients. More affected joints have been diagnosed scintigraphically than

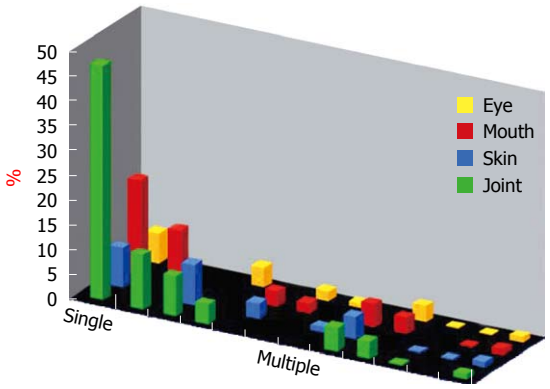

Figure 1 Associations of the four major inflammatory extraintestinal manifestations in 169 of 480 Crohn's disease patients.

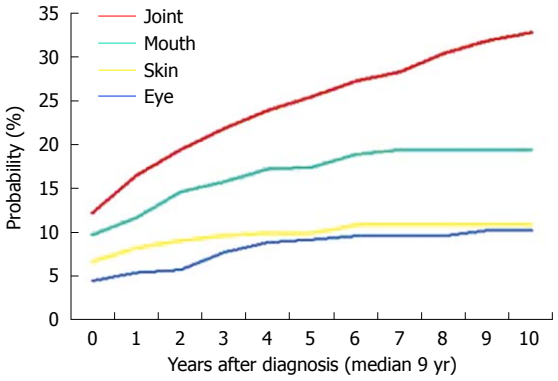

Figure 2 Cumulative probability of having a repetition of the same inflammatory extraintestinal manifestations.

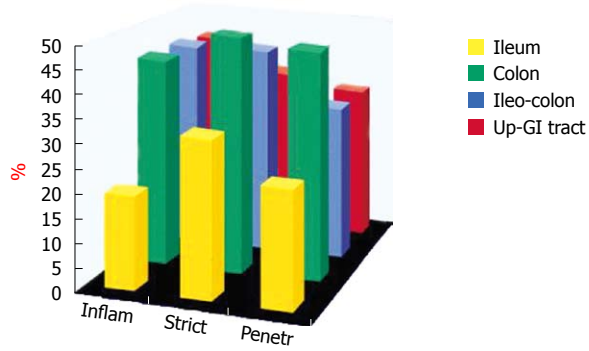

Figure 3 Distribution of inflammatory extraintestinal manifestations according to the Vienna classification in 169 Crohn's disease patients.

clinically, indicating that the prevalence of this complication could be much higher than generally believed[11].

The clinical activity of synovitis tends to parallel that of the intestinal disease. In most instances, the synovitis symptoms follow rather than precede IBD and recurrence is common, frequently coinciding with a flare-up of intestinal disease. Also, they are more common in patients with extensive colitis.

Regarding treatment, the main issue should be to control the clinical activity of the underlying bowel disease. Thereafter, there is agreement that this manifestation generally responds well to medical or surgical treatment targeted to the bowel disease. Conventional treatments for mild to moderate inflamed joints consist of acetaminophen, sulfasalazine, corticosteroids, and if necessary nonsteroidal anti-inflammatory drugs (NSAIDs). Furthermore, the majority of IBD patients with active intestinal disease and peripheral arthritis will notice an improvement of joint symptoms after receiving infliximab[12].

Ankylosing spondylitis, as defined by the Rome criteria, is associated with IBD in 5% of cases and it runs an independent course to the IBD[8-10]. In fact, the onset of ankylosing spondylitis is not related to the onset of IBD and usually precedes it. Also, there is no association between the severity of IBD and axial arthritis.

The proven efficacy of infliximab therapy in CD and rheumatoid arthritis has led to the suggestion that anti-tumor necrosis factor-α treatment might also be a useful agent in the management of spondyloarthropathies associated with CD.

In several open studies, infliximab has been used in patients with ankylosing spondylitis, leading to clinical, biologic and imaging improvement[12-15].

In our clinical practice the use of infliximab in patients with spondyloarthropaties associated with CD has resulted in an impressive improvement in clinical and laboratory abnormalities. The same has been reported in other uncontrolled studies[12,16]. In a multicentric, randomized, double-blind international trial to evaluate the efficacy of infliximab maintenance therapy in patients with moderate to severe active CD (ACCENT I), it has been reported that maintenance therapy with infliximab is superior to a single dose in resolving arthritis/arthralgia[17].

Clinical data suggest that intensive therapeutic strategies using biologic drugs should be tailored to the individual with severe or refractory articular disease. However, long-term studies with larger numbers of patients will be necessary to determine the patients who are most likely to benefit from these specific treatments. Furthermore, careful attention should be paid to the potential adverse events; mainly infections and malignancy.

Skin manifestations

There is a wide range of dermatological manifestations reported in patients with IBD, of which erythema nodosum is the most common form of reactive eruption. The prevalence of erythema nodosum has been reported to occur in up to 15% of patients[1], although other, more recent studies have reported that the prevalence may be considerably lower[10,18]. In fact, in a previous report from our group it was seen in 8% and 3% of CD and UC patients, respectively[2]. Clinically, erythema nodosum appears in conjunction with symptoms of active bowel disease and is most common in females, patients with large intestine involvement and peripheral arthritis[2].

Figure 4 Severe lesions of erythema nodosum treated with infliximab.

Erythema nodosum lesions usually respond to treatment with corticosteroids used to control the bowel flare-up. In refractory cases, potassium iodide, colchicine, hydroxychloroquine, and thalidomide have been used[19]. More recently a successful response to infliximab in severe erythema nodosum has also been reported[20]. We have used this treatment in a patient with colonic CD complicated with severe lesions of erythema nodosum and arthritis, refractory to high doses of steroids, which resulted in a rapid and complete resolution of skin and articular manifestations (Figure 4).

Pyoderma gangrenosum is a severe and debilitating skin disorder, characterized by the occurrence of nodules and pustules that rapidly enlarge and ulcerate. Lesions typically occur on the extensor surfaces of the lower extremities, but may also be found elsewhere. Since there is no specific test, the diagnosis can be difficult, only being established when the clinical and histopathological pictures are consistent with pyoderma gangrenosum, and other pustular or ulcerative dermatoses have been excluded.

This chronic ulcerating skin disorder can be found in 0.4%-2% of patients with IBD[10,19]. Patients with severe disease and colonic involvement are most likely to develop this complication. Pyoderma gangrenosum may run a course independent of the IBD, but sometimes coincides with an exacerbation of the underlying intestinal disease.

Management of pyoderma gangrenosum continues to be a therapeutic challenge and usually requires aggressive local and systemic therapy. High doses of oral prednisone and/or intralesional injections of corticosteroids are generally effective in the management[21]. However, the best form of treatment, particularly in patients with inactive intestinal disease, has not been established yet. Several clinical cases have been successfully treated with intravenous cyclosporine, oral and topical tacrolimus, or mycophenolate mofetil and granulocytapheresis[22-27].

In several reports patients treated with infliximab have shown rapid healing of their lesions[28]. We have successfully treated one patient in this way (Figure 5). In a recent controlled study, 6 of 13 patients with pyoderma

Figure 5 Pyoderma gangrenosum before, during and after treatment with infliximab.

Figure 6 Aphthous ulcers in the buccal mucosa and on the tongue.

gangrenosum were successfully treated with infliximab[29]. Thus, infliximab treatment is highly effective in healing refractory lesions and has become the first choice for the treatment of pyoderma gangrenosum.

Oral ulceration

Aphthous ulcers are the most common oral lesions in IBD; they occur in about 10% of patients. Aphthae are shallow, round ulcers with a central fibrinous membrane and erythematous halo (Figure 6). Their onset is usually sudden, coinciding with the flare-up of intestinal disease and occurring simultaneously with other EIMs[2,30].

Many similarities exist between CD and intestinal Behçet's disease. Both diseases have similar EIMs, particularly aphthous ulcers, which are an initial symptom in the majority of patients with Behçet' disease[31].

Oral ulcers usually respond to the treatment of the underlying bowel disease, but sometimes they may be resistant to conventional therapy. In these situations topical applications of steroids or tacrolimus may be of use[32]. Oral thalidomide has been reported to be beneficial in the treatment of aphthous stomatitis related to HIV infection and could be tried for lesions in patients with IBD. Infliximab has also been used in the treatment of patients with CD complicated with gigantic oral ulcers[7].

Eye manifestations

Ophthalmologic complications have been reported to occur in up to 12% of patients according to the published literature and these complications seem to occur more frequently in CD patients[33]. The most common complications are episcleritis, scleritis, and uveitis. Uveitis is the most serious complication and may be the cause of significant morbidity. Often it occurs independently of the bowel disease and is particularly associated with the musculoskeletal EIMs[34] (Figure 7). Specific treatment may include topical and systemic steroids. In the management of ophthalmologic manifestations, immunosuppression is often required. Infliximab has been reported to have a beneficial effect in a few patients suffering from CD with uveitis and spondyloarthropathy, and is increasingly being used in both acute and chronic ocular inflammatory manifestations of IBD[12,35-37].

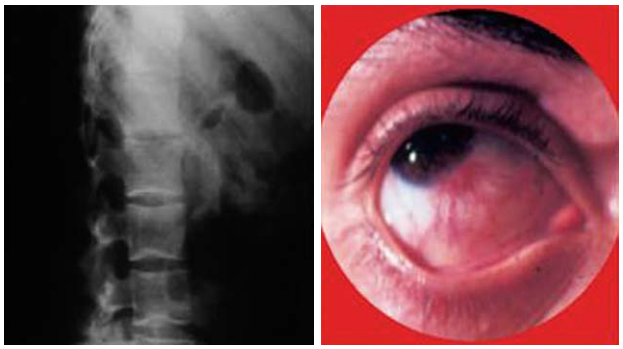

Figure 7 Ankylosing spondylitis and uveitis in the same patient.

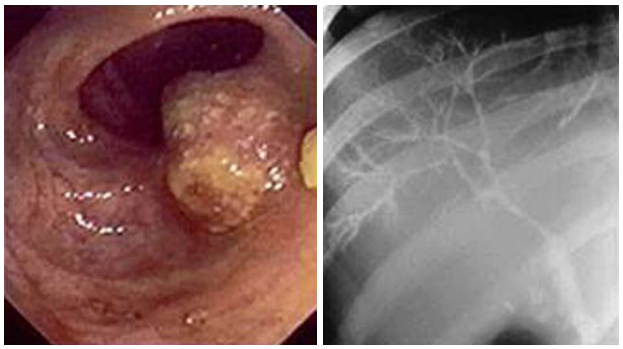

Figure 8 ERCP showing sclerosing cholangitis and endoscopic view of carcinoma of the colon observed in the same patient.

Hepatobiliary manifestations

Primary sclerosing cholangitis (PSC) is the most common immune-mediated hepatobiliary disease. It is a chronic, progressive, cholestatic disorder characterized by inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts. The prevalence of IBD (mostly UC) is about 75% and PSC complicates approximately 2%-5% of cases of UC and 0.7%-3.4% of cases of CD[10,38].

Most patients with PSC are diagnosed when asymptomatic with abnormal liver function tests performed in routine follow-up. Symptoms of PSC usually consist of fatigue, pruritus, right upper quadrant pain, fever, jaundice, and weight loss. Magnetic resonance (MR) cholangiography is often used for diagnosis of PSC and avoids the complication of endoscopic retrograde cholangiopancreatography. Liver biopsy is not always necessary where typical features are present on MR cholangiography[39].

IBD-PSC patients have a specific phenotype, characterized by extensive, mild disease with a particularly high risk of colonic dysplasia and carcinoma (Figure 8), as well as an increased risk of bile duct cancer. Yearly surveillance

colonoscopy is recommended for those patients with colonic involvement[40,41].

The medical management of PSC remains controversial. Ursodeoxycholic acid is widely used for this indication, and the best results have been obtained with high dose (up to 22-25 mg/kg). Moreover, the evidence that this medication prevents progression of PSC and that it may reduce the high risk of colonic cancer in these patients may become an indication for its use[38]. Liver transplant is the only treatment for patients with advanced PSC, with a 5-year survival rate of approximately 80%. However, the optimum timing for transplantation remains a difficult decision.

CONCLUSION

During the course of IBD, EIMs related to the activity of intestinal inflammation are quite common. Phenotypic classification shows that certain subsets of patients are more susceptible to developing EIMs. They also have a higher probability of maintaining active disease during the clinical course. For most of the inflammatory EIMs, the primary therapeutic target remains the bowel. Early aggressive therapy with biologic drugs may be required for several EIMs and maintenance treatment has the potential to prevent some devastating consequences. Long-term studies with larger numbers of patients will be necessary to determine the patients who are most likely to benefit from specific treatments.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2708

World J Gastroenterol 2011 June 14; 17(22): 2708-2714
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Belén Beltrán, MD, PhD, *Series Editor*

Inflammatory bowel disease in travelers: Choosing the right vaccines and check-ups

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Received: April 27, 2010 Revised: June 14, 2010
Accepted: June 21, 2010
Published online: June 14, 2011

Abstract

The majority of patients with inflammatory bowel disease (IBD) achieve good control of the inflammatory activity using available therapies. When remission is achieved and quality of life recovered, a considerable proportion of IBD patients express their desire to travel abroad, be it for business, academic or leisure purposes. Their physicians should help and encourage them whenever possible. However, preventive measures are warranted to minimize the risk, since IBD patients are exposed to the same infections affecting the general population, plus opportunistic infections (OI) related to the immunosuppression. There are a large number of potential OI that might affect patients with IBD. The true prevalence of these infections is unknown, and can vary from country to country. Therefore, reactivation or *de novo* acquisition of infections such as tuberculosis, malaria, and viral hepatitis will be much more frequent in endemic areas. Therefore, physicians should be aware of these aspects when planning specific preventive measures for patients traveling to a particular country. This includes good control of environmental exposure, chemoprophylaxis when indicated, and the use of a specific vaccination program to prevent endemic infections. In addition, it should be noted that, though the risk of acquiring an infectious disease is probably greater for IBD patients traveling from a developed to a developing country, the inverse situation can also occur; it depends on the previous acquired immunity of the host against infections in any particular environment.

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Key words: Inflammatory bowel disease; Vaccination; Opportunistic infections

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Esteve M, Loras C, García-Planella E. Inflammatory bowel disease in travelers: Choosing the right vaccines and check-ups. *World J Gastroenterol* 2011; 17(22): 2708-2714 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2708.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2708

PREVENTIVE MEASURES AND CHECK-UPS BEFORE THE TRIP

Patients traveling abroad should adhere to a specific vaccination program depending on the country to which they are traveling. Specific advice by specialists in tropical medicine might be required at least 4-6 wk before the trip, to allow time for vaccines to take effect and to start taking chemoprophylaxis to prevent malaria when needed. Requirements for visiting/staying in any particu-

lar country can be found on the websites of the World Health Organization (WHO) (http://apps.who.int/tools/geoserver/www/ith/index.html) and of the Centers for Disease Control and Prevention (CDC) (http://wwwnc.cdc.gov/travel/destinations/list.aspx). An interactive map provides information on health risks for travelers to specific countries by clicking on any part of the country or a nearby country.

The vaccination status according to current recommended vaccination program for adult inflammatory bowel disease (IBD) patients[1-3] (Table 1) should be periodically monitored, particularly before the start of a trip. Concerns about the safety of live-attenuated vaccines in immunosuppressed patients have arisen due to the possibility of fatal reactivation of the infectious agent contained in the vaccine. However, there are no data regarding the required time free of immunomodulators for administering these vaccines with confidence. The risk of reactivation, which also occurs with opportunistic infections (OI), probably depends on the type, dose, and duration of immunosuppressants added to a particular host response, for example the existence or not of leucopenia and lymphopenia[4]. Thus, an immunosuppressant washing-out period of 2 mo before and after live attenuated vaccine administration seems to be prudent. Live attenuated vaccines include, among others, varicella and yellow fever vaccines (Tables 1 and 2). Additionally, there is limited information as to whether IBD patients, mainly those under immunosuppressants, acquire adequate levels of protection using the present recommended vaccination schedule. For example, in one study including adult patients with rheumatological diseases and Crohn's disease vaccinated against two strains of influenza (A/H3N3 and B), the majority achieved protective titers $\geqslant$ 40, irrespective of whether or not they were taking anti-tumor necrosis factor (TNF) therapy[5]. However, impaired response against a single dose of 23-valent pneumococcal polysaccharide vaccine[6] and against standard three doses of hepatitis B vaccine[7] was reported, mainly in those patients treated with combined immunosuppressants and biologics. Therefore, it remains to be established whether higher doses of vaccine and booster administration might afford better immunization against specific infections.

For IBD travelers coming from countries with well-developed immunization programs, vaccination against tetanus, diphtheria, and inactivated poliomyelitis should be administered every 10 years and a booster dose is recommended before traveling. A booster dose against pertussis is also advisable in combination with the former, as a resurgence of pertussis has been documented in many industrialized countries, such as the United States, Australia, and Canada. Estimating rates of pertussis in developing countries is difficult, because of a lack of access to diagnostic methods and under-reporting. However, epidemiologic estimates by the WHO from Asia, Africa, and South America demonstrate that these areas have the highest disease burden, and there is a public health problem in all age groups[8].

Antibodies against hepatitis B should be checked when planning a trip to high prevalence areas (China, Southeast Asia, and tropical Africa) or intermediate prevalence areas (Eastern Europe, the Mediterranean, Russia, and Central and South America). For immunocompromised patients, regular testing and booster administrations, when anti-HBs antibody levels fall below 10 mIU/mL, are recommended[9]. This is important because not only can the response to standard dose of vaccination be impaired, but antibody titters can decrease with time in immunosuppressed patients who have achieved an adequate previous immunization. There is a combined Hepatitis A inactivated and hepatitis B recombinant vaccine for the immunization of adults. The effect of hepatitis A immunization in IBD patients is not known, but it seems to be severely impaired in immunosuppressed patients for other disease conditions[4].

Influenza viruses change rapidly from season to season, consequently Influenza vaccine must be manufactured and administered every year. This vaccine includes the three strains that cause the most illness in the upcoming season based on virus samples and patterns collected from around the world. Influenza viruses circulate worldwide and IBD travelers should be aware of the outbreaks of specific influenza viruses when they plan a trip to a particular country. They should be vaccinated for the specific strain affecting this particular area at a given moment. In this sense, the pandemic caused by the H1N1 swine-derived influenza strain, or the previously emerged avian influenza strains still circulating, constitute good examples of the need for specific protection[10].

One of the biggest problems for IBD patients traveling to tropical areas of South America and Sub-Saharan Africa is how to prevent yellow fever. It is caused by an RNA hepatotropic virus that causes a pansystemic disease, with fever, hepatic, renal and myocardial injury, hemorrhage, shock, and mortality as high as 50%. Vaccination against yellow fever is mandatory when visiting 16 countries and strongly recommended for all endemic countries. Although limited studies indicate that vaccine immunity lasts for at least 45 years, the WHO requires booster immunizations every 10 years to maintain adequate protection. The yellow fever vaccine 17D contains live attenuated virus and is contraindicated for IBD patients who cannot stop immunosuppressants for at least 4 mo. Hence, traveling to endemic areas should be discouraged for patients requiring continuous immunosuppression to keep their IBD under control. However, the long-lasting immunization effect of yellow fever vaccine allows its administration at any time that is convenient for immunosuppressant discontinuation when a trip to endemic areas is expected in the future. Risk of serious adverse events following yellow fever vaccination is very low, but increases with age (4 per 100 000 doses for people aged 60-69 and 7.5 per 100 000 doses for people 70 and older)[11].

Neisseria meningitidis causes endemic meningococcal disease worldwide, with a specific serotype distribution

Table 1 Current recommended vaccination program for adult inflammatory bowel disease patients[1-3]

Illness	Vaccine	Recommendation	Schedule
Tetanus Diphteria	Purified anatoxin	Recommended	Every 10 yr
Poliomyelitis	Injectable: inactivated	Recommended	Every 10 yr
Pertussis	Acellular antigen	Authorized	Every 10 yr
Hepatitis B	Recombinant peptide	Recommended	Single/double doses? Booster?
Pneumococcal disease	23-valent purified antigen	Recommended	Every 5 yr Single/double doses? Booster?
Influenza	Inactivated virus	Recommended	Annually
Human papillomavirus infection	Recombinant L1 protein	Authorized	??
Measles,mumps and rubella	Live attenuated	Contraindicated during immunosuppression	??
Varicella	Live attenuated	Contraindicated during immunosuppression	Double dose (4 wk interval)
Haemophilus influenzae B disease	Conjugated capsular polyosidique antigen	Authorized	Single dose

Table 2 World distribution of travel-related preventable illnesses and current recommended vaccination program for adult inflammatory bowel disease patients

Illness	Regions with high and intermediate endemicity	Vaccine/schedule	Recommendation
Hepatitis A	High: Africa South America, Middle East Southeast Asia, China Intermediate: Southern and Eastern Europe	Inactivated virus (every 10 yr)	Authorized
Yellow fever	Africa: Sub-Saharan Africa America: Central and South America	Live attenuated (every 10 yr) 17D strain (17D-204 /17DD)	Contraindicated during immunosuppression
Meningococcal disease	Europe: Serogroups B, C	Conjugate polysaccharide C	Authorized
	Americas: Serogroups B, C, Y	Polysaccharide combined A+C	Authorized
	Africa and Asia: serogroups A, C, W135	Polysaccharide combined A+C+W+Y (single dose among persons aged 11-55)	Authorized
Typhoid	High: Southern Africa Western, Eastern South central and Southeastern Asia Intermediate: Eastern, Middle and Northern Africa, Western Asia, Latin America/Caribbean, Oceania	Vi Capsular polysaccharide (single dose IM. Booster dose every 2-3 yr for those at risk)	Authorized
Cholera	Africa: Congo, Kenya, Mozambique, Uganda, Tanzania and West Africa	Oral Killed (2 doses at 1-6 wk interval with a buffer to protect the B-subunit against stomach acidity)	Authorized
	South and Central America: Peru, Ecuador, Guatemala, Nicaragua Asia: Afghanistan, India, Cambodia, Malaysia, Nepal, Sri Lanka	Oral live	Contraindicated during immunosuppression
Rabies	High: Africa, Asia, parts of Central and South America Intermediate: Eastern Europe, parts of central and South America (Chile, Argentina)	Cell culture-derived vaccine (travellers, not handling animals: 2 doses, at days 0-28. If risk continues booster dose at 6-12 mo)	Authorized
Japanese encephalitis	Southeast Asia Far East	Cell culture-derived vaccine (2 doses, at days 0-28 booster dose?)	Authorized
Tick-borne encephalitis	Europe: Central and Eastern Europe, Russia Asia: China, Siberia, Russian Fareast	Inactivated virus (3 doses at 0,1 and 12 mo)	Use with caution

per continent (Table 2). Polysaccharide vaccines against meningococcal serogroups A, C, Y, and W135 have been available for several decades, but have been little used due to poor immunogenicity and minimal effects on nasopharyngeal carriage. The recent advent of a quadrivalent conjugate vaccine including A, C, Y, and W135 ensures a broad coverage for travelers and should always be considered before the polysaccharide vaccine[12]. However, effective global prevention of meningococcal disease will not be achievable without the availability of a vaccine against Group B meningitis (predominant in Europe and America), for which outer membrane protein vaccines are under development[13].

Travel to the Indian subcontinent is associated with the highest risk of contracting enteric fever. There are two available vaccines against *S. typhi*: the live attenuated oral vaccine containing the *S. typhi* strain Ty21a (Ty21a vaccine) and the parenteral capsular polysaccharide vaccine based on the *S. typhi* Vi antigen (Vi vaccine). Thus, the Vi vaccine is recommended for IBD patients. It is

available for children ≥ 2 years old, conferring protection 7 d after injection with a maximum neutralizing antibody concentration demonstrated 28 d after vaccination[14]. Percentages of efficacy in immunocompetent individuals range from 55% to 72%, but the figure is unknown for IBD patients taking immunosuppressants. The gastroenterologist should discuss with the patient the efficacy of the vaccine and reinforce the necessity of strict food and water precautions. The same control measures are required to prevent cholera and all diarrheal illnesses. However, when access to clean water and sanitation are not guaranteed, cholera vaccine should be administered, conferring 85% short-term protection, and 60% protection up to 3 years following vaccination. IBD patients taking immunosuppressants should receive the oral-killed vaccine licensed in more than 20 countries, including the European Union (Dukoral®). Another available oral-killed vaccine (Vabiotech, ORC-Vax®) was initially licensed only in Vietnam[15].

Rabies is a viral zoonosis, almost invariably fatal in humans. Rabies is widely distributed throughout the world and present in all continents. However, the probability of rabies exposure is directly related to the incidence of rabies in the area and the probability of contact with infected animals. In areas of high-risk exposure, such as most parts of Africa, Asia, and Latin America, human rabies occurs from the bite of domestic and stray dogs and cats without owners. By contrast, in low-risk areas (North America, southern Africa, parts of the Caribbean, and Europe), the principal mammalian reservoir species are wild carnivores[16]. Currently, cell-culture derived vaccines are used, and these are authorized for use by IBD patients traveling to high risk areas, particularly where there is limited access to medical care[1,3].

Japanese encephalitis (JE) is a leading cause of viral meningoencephalitis transmitted by *Aedes* mosquitoes in large parts of Asia. It is mainly a problem in rural rice growing and pig farming regions, but can also be found at the outskirts of cities. It occurs more commonly in the rainy season (roughly May-September), when the mosquitoes are most active[17]. Individuals under chronic conditions and under anti-TNF therapy are particularly considered candidates for JE vaccine[18]. A novel inactivated cell culture-derived vaccine (IXIARO®) has recently been licensed in the United States and Europe and can be safely administered to IBD patients.

Tick-borne encephalitis (TBE) is a disease of the central nervous system caused by a tick-borne viral infection. A recent systematic review has demonstrated that the three currently licensed TBE vaccines (Encepur children®, Encepur Adults®, and FSME-IMMUN"new"®) gave seroconversion rates of over 87%[19]. However, the relationship between seroconversion and clinical protection has not been established. As all the vaccines may produce commonly but generally mild adverse effects, their use in IBD patients is authorized with caution. In addition, the TBE vaccine has been suspected of causing an exacerbation of autoimmune diseases, but a cause-and-effect relationship has not been confirmed. Taking all these factors together, risk-benefit should be weighed and vaccine administered to those patients traveling to high-risk rural or forested areas, especially in the spring or summer.

PREVENTIVE MEASURES DURING THE TRIP

Though vaccination or chemoprophylaxis remains the most effective means of traveler infection prevention, some additional measures upon exposure to environmental factors during the trip should be taken. These include the avoidance of insect bites and the ingestion of safe foods and beverages. Insects are vectors of infections such as malaria, dengue, filariasis, Chagas, leishmaniasis, onchocerciasis, and trypanosomiasis, among others. At the WHO and CDC websites there is updated information about diseases that should be prevented per country, the type of insect vector, and the best means of prevention. Some of these diseases may be partly prevented by the application of insect repellents. However, the ideal mosquito repellent remains to be identified. It should repel multiple species of arthropods, have long-lasting effect, cause no local or systemic toxicity, be resistant to abrasion and rub-off, and be greaseless and odorless. DEET (N,N-diethyl-m-toluamide) remains the gold standard of currently available insect repellents[19]. Used at variable concentrations ranging from 10% to 75%, it is considered that a concentration higher than 50% offers no additional benefit. Other measures, such as avoidance of outdoor exposure during the period of maximum insect activity (for example crepuscular periods for malaria mosquitoes), and wearing long-sleeved shirts, long pants, and a hat outdoors, are also advisable.

It is important to prevent animal bites and scratches to avoid rabies. If suspected exposure to rabies occurs, prompt and thorough cleansing of the wound, together with administration of immunoglobulin added to immunization with the above-mentioned vaccine starting immediately after exposure, virtually guarantees complete protection[16].

IBD patients should pay particular attention to preventing traveler's diarrhea (TD), as there is evidence showing that intestinal infections can trigger the disease[20] or induce relapses[21]. However, infection by enteropathogenic bacteria does not appear to be associated with a poorer clinical outcome of the IBD flare[21]. There is no evidence supporting the need for chemoprophylaxis for IBD travelers to prevent diarrhea, and the majority of specialists prefer not to recommend it to travelers[22]. However, a recent expert review included IBD patients as potential candidates for chemoprophylaxis[22]. An intermediate and very reasonable position might be early self-treatment when a gastrointestinal infection is suspected[4,23]. Fluoroquinolones are the drugs of choice in IBD, followed by azithromycin in patients who take fluoroquinolone as a part of their treatment[4]. The efficacy of Rifaximin against enteroinvasive bacteria (*Campilobacter*,

Table 3 Preventive measures for inflammatory bowel disease patients coming from developing countries (mainly while taking immunosuppressants or before starting)

	South America	Maghreb and Western Orient	Sub-Saharan Africa	Southeast Asia and India	Other
Thick drop	Consider	No	Always	Consider	No
Stool parasite	Always	Consider	Always	Always	No
Urine parasite	No	No	Always		No
Strongyloides (culture, serology)	Always	Consider	Always	Always	No[1]
Trypanosoma (serology)	Always	No	No	No	No
Histoplasma (serology)	Always	No	Always	No	No
HBV and HCV (serology)	Always	Always	Always	Always	Always
Tuberculin skin test or IGRA	Always	Always	Always	Always	Always

[1]Consider in case of Chinese individuals with eosinophilia. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Shigella, and *Salmonella*) has never been proved and consequently it is not an appropriate drug for IBD patients with bloody diarrhea and fever[4,22].

The evidence for the use of vaccines against enterotoxigenic *Escherichia coli* (ETEC) is scarce and it cannot be recommended at the present time for TD prevention. In addition, studies should be performed to demonstrate which foods and beverages have the lowest and the highest risk for TD. Though firm evidence is lacking demonstrating the value of dietary and beverage selection in the prevention of TD, uncooked fruits and vegetables and untreated drinking water are considered to hold the highest risk[22].

PREVENTIVE MEASURES AND CHECK-UPS TO DETECT INFECTIONS HARBORED DURING THE TRIP

Patients with IBD are likely to be treated with steroids, immunomodulators, or biologicals. It has been reported that these therapies can increase the risk of severe infections[24] or OI[25], especially when administered in combination, and even more so in the elderly or when narcotics are also prescribed. Thus, those patients on maintenance therapy with these drugs who travel to developing countries are, at least hypothetically, at increased risk for OI. Of course, the time spent in those countries may be one of the most decisive factors in increasing the risk of such infections, and it should be always taken into account when evaluating these patients. Most OI will present as acute manifestations soon after (or even while) the patient returns home. However, some parasitic or protozoal infections can remain latent for years (e.g. strongyloidosis, Chagas' disease, or histoplasmosis); we must remember this especially in patients who stay for a long period of time in developing countries or, even more importantly, for individuals coming from developing countries who are diagnosed with IBD once in a developed country.

Another important factor is familiarizing oneself with the endemic infections affecting countries from which the patients are returning (Table 3). Depending on the geographic area, our investigations should be directed towards some infective agents or others. However, no specific recommendations are yet available for returning travelers; of course, there is no definition for "long-term" travel or stay in developing countries. Therefore, in this section the authors will try to draw a modest picture of which infections should be investigated, and how.

The most common clinical setting should be that of a patient returning from a short stay in a developing country who presents with diarrhea[26]. In this case, stool samples for microbiological evaluation using conventional cultures (mainly to detect enteric pathogens) should be taken and examination for fecal parasite detection should be carried out. It is important to collect at least three samples from different bowel movements (preferably from different days). If infestation by *Giardia lamblia* or *Criptosporidium parvum* is suspected, immunofluorescence is necessary. The presence of fecal *Entamoeba* cysts must be corroborated by serology; if positive, combined therapy with metronidazole and paromomycin should be started. However, in seronegative patients, paromomycin alone might be sufficient[27].

Among chronic infections, strongyloidosis might reactivate in case of immunosuppression[28]. Infection by *Strongyloides stercolaris* is endemic in tropical and subtropical regions, and it can be also seen anecdotally in Europe, the United States, Japan, and Australia. Evaluation of stool samples remains of paramount importance, although with limited diagnostic yield because of the intermittent presence of ova and larva in feces. Collection of repeated stool samples is strongly warranted, as is the use of concentration techniques or specific cultures that can achieve 80% sensitivity. Although serologic tests are highly sensitive, they do not differentiate past from current infection. Strongyloidosis might present not only by diarrhea, but also by abdominal pain or rectal bleeding. It should be especially suspected in patients coming from endemic areas, with a compatible clinical picture and peripheral eosinophilia[29]. Similarly, schistosomiasis is a prevalent chronic infestation in tropical and subtropical regions. Diagnosis is based in the visualization of ova in urine samples. Chagas' disease, or American trypanosomiasis, is a zoonosis found all over South America. The infection can remain latent for 10 to 30 years, and 30%-40% of individuals will then develop a chronic disease[30]. Interestingly, clinical symptoms will be those

related to the development of visceral involvement; in the case of the GI tract, dysphagia due to megaesophagus and constipation, abdominal pain and bloating due to megacolon make up the most common clinical picture[31]. Parasitological studies are of low diagnostic yield, and serological tests (indirect hemaglutination, indirect immunofluorescence, and ELISA) are preferred; in fact, two positive serological tests are necessary to establish the diagnosis[32]. Although digestive involvement is not usually life threatening, cardiac involvement can result in a fatal outcome.

Malaria and tuberculosis are the most common infectious diseases. *Plasmodium falciparum* can persist as a latent infection for 1 to 5 years, *P. ovale* and *P. vivax* for 3 to 5 years, and *P. malariae* for up to 40 years. Thick drop test in peripheral blood remains the diagnostic test of choice, although PCR tests can be useful in cases of low parasitemia[33,34].

Tuberculosis is nowadays systematically ruled out in patients who might be treated with anti-TNF agents. However, this diagnosis should always be taken into account in IBD patients returning from or coming from developing countries. Tuberculin skin tests and interferon gamma assays can be used for the diagnosis of latent or active TB, as recently stated in the European Crohn's and Colitis consensus[4,35]. Finally, histoplasmosis is widely distributed, although predominantly in America and Africa. Primoinfection is often subclinical, but reactivation can be life threatening. Diagnosis is based on direct observation on microscopy, and it is corroborated by a positive culture of biological samples (sputum, skin lesions, liver biopsy, or bone marrow aspiration). Antigenuria is useful to monitor therapeutic response[36,37].

CONCLUSION

In summary, patients with IBD on immunosuppressanttherapy (including steroids) must be carefully evaluated in case of fever, diarrhea, abdominal pain, or rectal bleeding when returning from or coming from developing countries. Learning about the geographical area and time spent in the area are of paramount importance in identifying one or another OI and appropriately targeting the diagnostic tests. In addition, an accurate medical history, as well as a complete physical examination, will be of great value.

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S- Editor Wang YR **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2715

World J Gastroenterol 2011 June 14; 17(22): 2715-2722
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Familial aggregation in inflammatory bowel disease: Is it genes or environment?

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Supported by Grants from Ministerio de Ciencia e Innovación (SAF2008/03676) and Fundació Miarnau to Sans M
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Received: June 28, 2010 Revised: September 18, 2010
Accepted: September 25, 2010
Published online: June 14, 2010

Abstract

Inflammatory bowel disease (IBD) develops in genetically susceptible individuals due to the influence of environmental factors, leading to an abnormal recognition of microbiota antigens by the innate immune system which triggers an exaggerated immune response and subsequent bowel tissue damage. IBD has been more frequently found in families, an observation that could be due to either genetic, environmental or both types of factors present in these families. In addition to expanding our knowledge on IBD pathogenesis, defining the specific contribution to familial IBD of each one of these factors might have also clinical usefulness. We review the available evidence on familial IBD pathogenesis.

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Key words: Inflammatory bowel disease; Familial aggregation; Familial clustering; Environmental factors; Genetics; Genome wide association studies

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Nunes T, Fiorino G, Danese S, Sans M. Familial aggregation in IBD: Is it genes or environment? *World J Gastroenterol* 2011; 17(22): 2715-2722 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2715.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2715

INTRODUCTION

Our present understanding of inflammatory bowel disease (IBD) pathogenesis considers ulcerative colitis (UC) and Crohn's disease (CD) as complex conditions that develop in genetically susceptible individuals due to the influence of various environmental factors[1-6]. An abnormal recognition of certain antigens of the bowel microbiota by elements of the innate immunity is thought to play a key role, leading to an exaggerated immune response, release of pro-inflammatory molecules and, ultimately, bowel tissue damage.

In past decades a greater incidence of IBD cases among UC and CD relatives, referred to as family aggregation or familial IBD, has been clearly demonstrated[7-11]. The reason for such an increased risk is not straightforward, but genes, the environment or a combination of both could, in theory, account for family aggregation, considering their contribution to the development of IBD. A better understanding of the factors leading to familial IBD might result in clinical applications.

FAMILLIAL IBD

Familial aggregation among IBD patients or "familial IBD" is defined by the occurrence of a trait in more fam-

ily members than expected by chance. Multiple population studies have demonstrated that relatives of an IBD patient have a much higher risk of developing the same condition, compared to the general population[7-11]. The magnitude of familial aggregation depends on several factors, including: (1) type of IBD; (2) population studied; and (3) family relationship.

In respect to type of IBD, familial aggregation has been more frequently reported in CD than UC[7,9]. In first degree relatives, the age-adjusted relative risk of developing the same type of IBD ranges from 2-8 for UC and from 5-10 in the case of CD[7-9]. As elegantly demonstrated by Yang *et al*[9], affected relatives can develop both forms of IBD, although the greatest risk is associated with the appearance of the same disease type occurring in the index case. Additionally, CD patients tend to have a much higher frequency of relatives affected with UC when compared to UC patients having relatives affected with CD[7].

In respect to different populations, it has been shown that Jewish families present more than twice the number of multiple affected families when compared to the non-Jewish population[9]. However, we cannot rule out the possibility that differences amongst other geographical populations are due to their study design.

Finally, frequency of familial IBD also varies according to the degree and type of kinship. The prevalence of IBD in second-degree relatives appears to be lower than in first-degree relatives, especially in those with discordant disease[7]. In addition, although only a few studies have estimated the age-adjusted risk of IBD in relatives, it seems that the risk of IBD in offspring is higher than in parents and similar, or even slightly higher, than in siblings[8,9,11]. A potential source of bias could result from underreported cases in second-degree relatives and older generations, which might have influenced these differences.

In addition to an increased risk of developing the disease, first-degree relatives of IBD patients also have an increased likelihood of sharing the same phenotype[8,12]. This seems to be partly true in CD owing to the striking clinical concordance in families, in respect to disease location and behavior. On the contrary, literature is scarce and presents mixed results for CD severity and complications[8,9,13-16]. In UC families, the phenotype concordance data is less consistent, but a high concordance rate related to colonic extent and extra-intestinal manifestations has been reported[12,14].

The possibility that IBD develops at an earlier age in offspring than in their parents, a phenomenon known as anticipation, has been controversial[17-21]. Although different studies have reported such differences in age of onset, it seems that multiple biases could account for these findings. Whether familial IBD is a different clinical entity was the subject of debate. However, in the largest population-based study including 654 sporadic and familial IBD patients, a positive familial IBD history did not significantly influence clinical course or risk of developing IBD-related complications[22].

ROLE OF GENES IN FAMILIAL IBD

Evidences supporting the role of genetic factors in IBD pathogenesis

The fact that IBD is a genetically mediated disease was initially derived from the physician's perception of a higher prevalence of UC and CD cases among the relatives of IBD patients. This hypothesis was initially supported by case report studies showing clustering in IBD families and was subsequently confirmed by several population-based studies[3-7]. In one of these studies, Yang *et al*[9] described a risk of developing UC and CD among first degree relatives of IBD patients of 1.6% and 5.2%, respectively. The risk of developing IBD also varies according to the ethnic origin of individuals, a fact that is likely to be linked to their genetic background. In that regard, the prevalence of IBD among the Jewish population is 2 to 4 times higher than in any other ethnic group, being greater in Ashkenazi than in Sephardic or Oriental Jewish, with no influence from their geographical location[9,11].

Another source of evidence underlying the key role of genetic factors in IBD steams from twin studies. In these studies the greatest IBD concordance rate was found in monozygotic twins, ranging from 20% to 50% in CD and from 14% to 19% in UC twins, whereas in dizygotic twins concordance rates dropped to 0%-7% in both CD and UC twins[23-26]. The degree of monozygotic-dizygotic twin concordance found in CD point towards a genetically mediated condition with a non-Mendelian inheritance pattern. Of note, the concordance rates observed in CD are greater than these found in type 1 diabetes, asthma or schizophrenia, all of them diseases with a well-established genetic background.

Genes involved in CD pathogenesis

Several genome linkage studies identified a number of CD susceptibility regions in chromosomes 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19 and X[27-34]. After subsequent confirmation, regions on chromosomes 16, 12, 6, 14, 5, 19 were named IBD1 to IBD7, respectively[35]. Of these 7 original loci, only IBD1 (chromosome 16q12) was replicated in all studies, whereas another three loci, IBD2 (chromosome 12), IBD3 (chromosome 6) and IBD4 (chromosome 14) were replicated in some of the studies[36]. Later on, several CD susceptibility genes, such as NOD2, NOD1, toll-like receptors (TRLs) genes, and novel organic cation transporter (OCTN) genes were identified using either a candidate gene approach or positional cloning techniques[37].

The first and most relevant CD susceptibility gene described to date is NOD2. The carriage of one or more of the three main NOD2 variants (Arg702Trp, Gly908Arg and Leu1007incC) is found in 25%-45% of CD Caucasian patients and in only 15%-20% of healthy subjects[38]. A clear gene-dose effect has been described for NOD2 in CD patients. While the risk of developing CD is increased by 2-3-fold in subjects carrying one NOD2 variant allele, it reaches a 20-40-fold increase in subjects with two or more NOD2 variant alleles. In addition to increasing CD

susceptibility, the *NOD2* gene variants can also influence CD behaviour, phenotypes and need of surgery[39]. Similarly, the carriage of NOD2 variants has also been linked to a slight increase in familial CD risk[40]. In spite of being the most powerful CD susceptibility gene found to date, it must be underlined that the *NOD2* gene only accounts for a small proportion of the genetic inheritance of CD in Caucasians. Moreover, *NOD2* gene variants are infrequent in some geographic areas, such as Scotland, Ireland and Scandinavia, or even completely absent in subjects with an Asian and African-American genetic background[37]. In these cases other genes must account for the genetic predisposition to develop CD. This is in keeping with the highly polygenic nature of this disease.

In the last 3 years, the field of IBD genetics has experienced a dramatic transformation. Completion of the human genome project and development of tools capable of simultaneously studying a great number of genes has resulted in a much higher number of genes influencing CD and UC susceptibility than expected. Several genome-wide association studies (GWAS) have been undertaken in CD patients and healthy controls[41-45] and a meta-analysis has been recently published[46]. In this meta-analysis more than 30 independent loci are found to be convincingly associated with CD, providing an extraordinary insight into CD pathogenesis. Interestingly, most CD susceptibility genes are involved in either recognition of bowel microbiota antigens by the innate immunity, the IL-17/IL-23 pathway or autophagy, suggesting that these molecular mechanisms play a key role in CD pathogenesis.

Several studies have evaluated the frequency of CD-related mutations in affected families[47-49]. Jess *et al*[47] studied NOD2 mutation frequency in a population of Danish twins with IBD. In this study, a high prevalence of NOD2 mutation was observed in both CD twins and their healthy siblings. A Swedish study on monozygotic twin pairs reported a NOD2 frequency in both concordant CD siblings of only 22%, although the prevalence of NOD2 was indeed higher in concordant than in discordant twin pairs[48]. Joossens *et al*[49] investigated the prevalence of genetic markers (NOD2, NOD1, TLR4, CARD8) in multiplex and single-case families, healthy relatives and controls. The authors found a significant correlation between the number of genetic mutations per family and an increasing number of first-degree relatives with CD. However, these results could not discriminate between single-case and multiplex families.

Genes involved in UC pathogenesis

Two studies aimed at describing the influence in UC of the well-established CD susceptibility genes reported very interesting findings[50,51]. It became clear there is a genetic overlap between the two forms of IBD, with some genes involved in the development of both CD and UC (3p21.31, NKX2-3, CCNY). On the contrary, other genes have been only associated with UC, but not CD (ECM1 HERC2, STAT3 and PTPN2). In that regard, a very recently published UC GWAS meta-analysis has demonstrated that roughly half of the known CD susceptibility gene loci are shared by UC[52]. In addition, more than 20 exclusive UC loci have been recognized to date including, among others, IL10, ARPC2 and ECM1[52-55]. To summarize our present understanding, we believe that some genetic factors influence the global predisposition to develop IBD, whereas other genes are related to the risk of developing either UC or CD, specifically. Such genetic overlap between UC and CD probably contributes to the existence of a 5% of non-classifiable or indeterminate colitis among the IBD population. Similarly, it also contributes to the fact that first degree relatives of CD or UC patients not only have an increased risk of developing the same type of IBD, but also the other form of IBD, although with a lower frequency in the latter case.

EVIDENCE SUPPORTING AN ENVIRONMENTAL AETIOLOGY IN FAMILIAL IBD

Evidences supporting the role of environmental factors in IBD pathogenesis

The remarkable increase in the IBD incidence in the last few decades cannot be explained by changes in the genetic background of a certain population. Instead, it clearly points towards the existence of potent environmental factors playing a key role in IBD pathogenesis. Several studies performed in Europe that have evaluated IBD prevalence in immigrant populations from low IBD risk areas found that immigrants present a similar or higher risk of developing IBD when compared to the indigenous population[56-58]. These results suggest that differences in prevalence are probably associated with lifestyle and environmental factors, and not with a specific genetic background. It is remarkable though that only very few studies have addressed environmental etiologic factors in respect to familial IBD.

Eating habits, pets and previous infections

One of the largest controlled studies addressing the impact of environmental factors on familial IBD was conducted in Belgium by Van Kruiningen *et al*[59] who investigated 21 families with 3 or more first-degree relatives affected with CD. Subjects were interviewed using an extensive questionnaire on potential environmental factors. In this study affected families and controls presented some remarkable differences in eating habits, domestic factors and medical history. IBD patients ate fewer oats, rye and bran, consumed more unpasteurized cheese and drank more well water, compared to controls. Additionally, an increased frequency of smoking habit, appendectomy and fecal-oral transmitted infections was found in a subject who later developed CD. In respect to domestic habits, affected families also presented a lower daily contact with pets during childhood. Taken together, these results point towards a role of certain gastro-intestinal infections as triggering events contributing to the development of IBD, whereas

the contact with pets during childhood seem to have a protective role favoring the immune system modulation.

Another familial aggregation study by the same Belgian group reported on a large Moroccan family with multiple CD cases[60]. Potential environmental, genetic and serological markers were studied in all family members. No differences in CD susceptibility genes or serological antibodies described in Caucasian populations were found between CD patients and healthy subjects. The study of environmental factors revealed the consumption of a large amount of unpasteurized milk in all family members, which was an environmental factor previously associated with occurrence of familial CD[59].

Another questionnaire-based study on environmental factors in a large monozygotic and dizygotic twin population included more than 300 twin pairs who were discordant for IBD diagnosis[61]. Twins with UC and CD reported recurrent gastrointestinal infections more frequently than their healthy siblings. These findings are in keeping with an increased frequency of fecal-oral transmitted infections reported in multiplex CD families[59] and suggest that past gastrointestinal infections might influence the risk of IBD.

Appendectomy

Appendectomy is associated with a lower risk of developing UC, although the exact mechanisms of this protective role are still not elucidated[62]. While controversial, it seems that the effect of appendectomy requires a certain degree of inflammation (appendicitis or lymphadenitis) and also applies to subjects undergoing appendectomy before the age of 20 years[63]. However, in a recently published study evaluating the usefulness of appendectomy as a therapeutic strategy for distal UC, 40% of patients experienced a complete symptoms resolution after elective appendectomy[64]. Conversely to UC, appendectomy seems to be associated with an increased risk of developing CD, although the studies addressing this issue yield conflicting results[65-70]. These discrepancies might be due to the inclusion of appendectomies performed at CD diagnosis or to methodological differences. More recently, data from large Swedish and Danish cohorts and a meta-analysis have demonstrated that the risk of developing CD is markedly increased only during a short period following appendectomy, disappearing after 5 years[71,72]. This behavior suggests that the association of appendectomy with CD might be a diagnostic bias, instead of a true risk factor.

Tobacco

Smoking habit, particularly cigarette smoking, is the most indisputable example of the influence of the environment on IBD[73,74]. Smoking has striking opposite effects on CD and UC, supporting the notion that distinct mechanisms underlie the pathogenesis of each form of IBD[74]. Subjects who have never smoked and former smokers are at a higher risk of developing UC, whereas present smokers have an increased risk of CD. In addition to the impact on disease susceptibility, smoking habit also modifies the clinical course of disease, increasing the risk of experiencing a relapse and the need for surgery[75-78]. Moreover, it has been demonstrated that tobacco discontinuation improves CD course[79]. Tuvlin *et al*[80] conducted a survey on tobacco use in UC and CD patients in familial IBD. In this study, smokers had double the risk of CD and former smokers had double the risk of UC, in younger age groups. A Danish case-report study on 2 monozygotic female twins with ileo-colonic CD and their non-affected brother and parents showed that though the healthy father. brother and twins all presented a NOD2 variant related to CD, only the affected twins were smokers, had undergone appendectomy and were on oral contraceptive use[81]. To further evaluate the influence of smoking habit in familial IBD, Bridger *et al*[82] analyzed 658 IBD patients, including 339 affected sibling pairs of whom 89 were discordant for smoking when diagnosed. Siblings who were discordant for smoking and IBD type almost always show CD in the smoker and UC in the non-smoker patient. The authors also suggest that the protective effect of tobacco on UC is due to a shift towards development of CD, in subjects prone to undergo bowel inflammation, rather than true protection of from the development of UC.

Oral contraceptive and non-steroidal anti-inflammatory drugs

Several studies have addressed the potential contribution of contraceptive pills to the development of IBD. It has been demonstrated that the risk of IBD in women taking oral contraceptives is greater that in controls, although there is no direct evidence for a causal relationship[83-85]. Data from two meta-analysis suggest a modest association between the use of oral contraceptives and development of IBD, with a pooled relative risk adjusted for smoking habit of 1.46 for CD and 1.26 for UC[86,87]. The most recent meta-analysis also suggests that the risk disappears once the medication is discontinued[87]. Other frequently used types of drug that have also been associated with IBD are non-steroidal anti-inflammatory drugs (NSAIDs)[88-95]. Due to their inhibitory action on protective prostaglandins, these drugs could enhance intestinal permeability facilitating disease activity. A growing body of evidence suggests a true association between NSAIDs and IBD activity, although the existence of multiple confounding factors makes it difficult to establish a formal relationship[96]. These confounding factors include selection of inadequate control groups, publication bias and intestinal tissue damage due not only to IBD activity but also to NSAIDs. Unfortunately, there are no studies evaluating the role of oral contraceptives and NSAIDs in familial IBD.

IBD as an infectious disease

Van Kruiningen *et al*[97] analyzed the pedigrees and time course of IBD development in a group of patients with familial IBD. They found that first-borns and subsequently born siblings were more frequently affected[87,97]. In addition, they described a statistically significant CD clustering that would indicate an infectious etiology supported by

which family members were affected and time to develop symptomatic disease. Another study by Van Kruiningen *et al*[98] assessed 2 French families with multiple CD cases, in an attempt to identify the suspected infectious cause. However, *Campylobacter*, *Yersinia*, *mycobacteria*, *mycoplasma*, *torovirus*, *coronavirus, Brucella*, *Influenza* and animal enteropathogenic infections were all ruled out and no pathogen could be identified. Even though an abnormal recognition of antigens of the intestinal microbiota by innate immunity is thought to play a key role in IBD pathogenesis, there are no studies addressing specifically the interaction between bowel microbiota and familial IBD.

Intestinal permeability

An abnormal gut barrier function, with an increased intestinal permeability, could contribute to CD pathogenesis[99]. It that regard, it has been demonstrated that small intestinal permeability is increased not only in patients with CD but also in their healthy relatives[100,101]. Peeters *et al*[100] reported that 25% of healthy first-degree relatives of CD patients had an increased small intestinal permeability. The mechanisms responsible for these disturbances in bowel permeability are not fully elucidated and its pathogenesis is a much debated topic. In their study in familial and sporadic CD, Peeters *et al*[100] reported no specific genetic pattern accounting for the abnormal permeability found in patients and relatives. In addition, there were no significant differences between families with multiple members affected and families with only one individual affected. Interestingly, almost half of the spouses presented increased intestinal permeability, which clearly suggests that this abnormality is due to environmental and not to genetic mechanisms. In keeping with this finding, Fries *et al*[102] studied the prevalence of intestinal permeability and some CD genetic markers (including NOD2 main variants) in 23 families of CD patients. Authors found no association between increased intestinal permeability and genetic markers in their population. In contrast, other studies have found an association between an abnormal bowel permeability and the presence of NOD2 variations[101,103].

CONCLUSION

IBD is a complex polygenic disorder modulated by a series of environmental factors, some of which are likely yet to be determined. In spite of the significant progress done in the study of both the genetic and environmental factors associated, the exact contribution of each one of the many factors involved is still largely unknown. From a practical point of view, at present no specific recommendations to IBD families, in respect to genetic or environmental counselling, are available. Large, prospective, population-based studies following IBD patients and their healthy relatives will be necessary to untie the knots in this tangled web of genetic and environmental factors.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2723

World J Gastroenterol 2011 June 14; 17(22): 2723-2733
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Comorbidity in inflammatory bowel disease

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Received: August 12, 2010 Revised: September 29, 2010
Accepted: October 6, 2010
Published online: June 14, 2011

Abstract

Patients with inflammatory bowel disease (IBD) can be affected by other unrelated diseases. These are called comorbid conditions, and can include any secondary health problem that affects a person suffering from a primary or main disease, and which is neither linked physiopathologically to the primary condition, nor is it due to the treatments used for the primary condition or to its long-term anatomical or physiological consequences. Different comorbid conditions, as well as their influence on IBD, are discussed.

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Key words: Comorbidity; Comorbid conditions; Crohn's disease; Inflammatory bowel disease; Ulcerative colitis

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San Román AL, Muñoz F. Comorbidity in inflammatory bowel disease. *World J Gastroenterol* 2011; 17(22): 2723-2733 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2723.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2723

COMORBODITY, THE CONTEXT, DEFINITION

It has always been difficult for physicians to attain a balance between specialization and the possession of general medical knowledge that makes it possible to optimize and expand the quality of care delivered to patients. The amount of information that a physician is supposed to manage is huge, and always growing. This is why, even when information tools are better than ever, we have perhaps reached a point in which Medicine achieves such a deep level of knowledge in specific areas, that sometimes the global perspective is lost. Or at least, is lost by physicians.

Health care professionals entrusted to assist patients with inflammatory bowel disease (IBD) must remember that our patients are also unfortunately exposed to other health problems (Table 1). We are not dealing here with the extraintestinal manifestations of IBD, whose importance we recognize and that we have probably learned to manage in a satisfactory way. Neither are we concerned with the adverse effects caused by the diverse therapies applied to our patients, such as osteoporosis or infections in the immunocompromised patients, or with the long-term consequences of the anatomical and physiological alterations induced by the disease. The problem that now demands our attention relates to a series of health problems that do not have a direct relationship to IBD as such, but that could alter the diagnosis, presentation and management of the intestinal disease. These conditions are generically known as "comorbidities". A working definition of comorbid conditions could include in this concept any secondary health problem that affects a person suffering from a primary or main disease, and which is neither linked physiopathologically to the primary condition, nor is it due to the treatments used for the primary condition or to its long-term anatomical or physiological consequences. The delimitation of what is and is not a comorbid condition is not easy, and we are

sure that many readers will think that this review should have perhaps addressed a different scope of diseases. In fact, agreement between the authors has required some debate. Our selection may be incomplete, but we are sure that all the conditions that we have included deserve important consideration when caring for a patient with IBD.

It is of paramount importance to differentiate comorbidity from multimorbidity. The first concept deals with the association of a group of diseases with a given condition, whereas multimorbidity refers to the association of multiple diseases in a single person[1]. Another term bordering the concept of comorbidity, is that of patient complexity. In this concept, other extramedical factors are taken into account, such as personal, cultural or social situations that might significantly influence the way in which the health system has to take care of a given person.

Why is comorbidity important?

Firstly, it is obvious that comorbid conditions cannot be overlooked in a patient with IBD. The existence of comorbidity can significantly change several scenarios of medical practice[2]. (1) Clinical manifestations of IBD and its activity can be altered or confused by associated diseases; (2) Prognosis of IBD will also be influenced; (3) Whenever a patient with significant comorbidity is seen by us, we step outside the realms of medical evidence. As a rule, randomized controlled trials exclude patients with comorbidity, and so their conclusions are not necessarily applicable to such situations; (4) The use of drugs for the treatment of IBD is limited by the increased importance their pharmacologic or collateral effects can have on a person with comorbid conditions; and (5) Frequently, it is more important than ever to set up multidisciplinary teams to empower patient care, or, at least, to assure that channels of collaboration and mutual consultation are as fluid and reliable as possible.

Is it possible to quantify comorbidity? What are its generic consequences?

Different sets of clinical indices have been developed and proposed for the specific study of comorbidity. Not all of them have been developed in the same way, nor have they been applied to similar populations. This is why they are not always comparable, and to pick one or another must be done very carefully. Charlson's index is widely used[3], probably due to its unique combination of simplicity and performance. However, it has been validated in multiple populations, but not in IBD patients.

In the general population, multimorbidity is associated with a significantly worse quality of life[4-6]. The specific case of IBD has not been addressed yet, but it is known that in other chronic conditions there is an increased cost of care and a higher complexity of medical activities[7], which are accompanied by poorer outcomes and unfavorably influence indices such as emotional impact, ability to cope, mortality, days of admission or postoperative complications[8-11].

What is the quantitative importance of comorbidity in IBD?

Data on the importance of comorbidity in IBD patients are scarce. Its prevalence has been poorly studied, and refers to other related diseases[12] such as pulmonary thromboembolism[13], arthritis[14] or immune-based conditions[15]. These immune-mediated inflammatory disorders, which include IBD itself, associate with each other and determine a higher risk for other chronic diseases, with a corresponding increase in resource costs.

Although the main determinant of quality of life in patients with IBD is activity of the disease[16], other comorbidities not directly related also have an impact on the physical scores, especially cardiovascular diseases[17-20].

It is not known whether the presence of several chronic diseases can determine poorer results following the medical treatment of this disease; however, it has been well described that they strongly influence surgical outcomes, because preoperative comorbidities are, alongside with age, the main predictors of the occurrence of postoperative complications[18-20].

Finally, among the identified causes of mortality in IBD are several processes that reflect the patient's comorbidity, such as postsurgical cardiovascular complications, age and infections[19]. It is true that many factors favor infections in IBD patients, such as immunosuppressants and malnutrition, but both age and comorbidity probably have their corresponding effects[20].

HEPATIC COMORBIDITY

The potential association between primary sclerosing cholangitis (PSC) and IBD is well known. However, we shall not deal with this condition here, and instead will focus on other liver diseases which can be observed in our patients, and may well alter the course of their disease.

During the long-term follow-up of a patient with IBD, a transient elevation in liver function tests is frequently observed. The cross-sectional prevalence of high aminotransferase levels varies between 5% and 50%, but more adjusted figures show, that around 20%-40% of patients have elevated aminotransferases at some time during the course of their disease, whereas a chronic alteration in such values can be detected in approximately 10% of these patients[21-23]. These elevations in liver function tests are usually discrete, in a range below twice the upper normal level[21,24]. There is no clear correlation between the degree of alteration of liver function tests and the presence of active IBD. An investigation of the underlying cause is frequently frustrating and unyielding, with a small percentage of definitive diagnoses[22].

The causes of altered hepatic biochemistry are manyfold, but the most frequent causes are steatosis and drug toxicity[21]. The evaluation of such patients has to be sensible and reassuring. The first step is to categorize the type and degree of altered hepatic biochemistry. Four different situations could perhaps be defined: (1) Slight (< 2

Table 1 Putting comorbidity into context: secondary health problems in patients with inflammatory bowel disease

Extraintestinal manifestations	Adverse effects of treatments	Comorbid conditions	Direct consequences of the disease
Peripheral and axial arthritis	Steroids: cataracts, glaucoma, mood changes, osteoporosis…	Cardiovascular	Abdominal and retroperitoneal scarring: hydronephrosis, intestinal obstruction, female infecundity…
Erythema nodosum, pyoderma gangrenosum, oral aphtae	Immunosuppressors: infections, neoplasia, liver toxicity, myelosuppression…	Hepatic, biliary, pancreatic, digestive	Consequences of intestinal resection: malabsorption, short bowel syndrome, oxalate nephrolithiasis
Uveitis, episcleritis, blepharitis	Biologics: infections, neoplasia, demyelinizing disease, infusion	Metabolic: obesity	Persistent inflammation: osteoporosis, amyloidosis
Primary sclerosing cholangitis	Reactions, drug-induced lupus	Neuropsychiatric	

× upper normal level) and transient elevation of aminotransferases (aspartate aminotransferase/alanine aminotransferase), γ-glutamyl transferase, alkaline phosphatase or bilirubin: it is probably appropriate not to alarm the patient and check the altered values after a short period, proceeding to investigate the cause if the alteration persists; (2) Sustained elevation has to be approached as it would in the general population, with special attention to the usual data in the anamnesis (epidemiological sources of exposure, potential liver toxics…) and ordering a battery of tests that could pinpoint the cause of such elevation. No precise indications have been published to determine which tests should be explored, and in what order. A possible selection could include the following: hepatitis B and C serology, and anti-neutrophil-cytoplasma, antitransglutaminase, antinuclear, anti-smooth-muscle and antimitochondrial antibodies; exploring copper and iron metabolism and investigating other less frequent or characteristic causes of altered liver function tests, could be left for a second step. A liver ultrasound should be obtained at an early stage, to explore the presence of a bright pattern, indicative of steatosis[25], detect cholelithiasis and its complications, explore the bile ducts and rule out signs of chronic liver disease or portal hypertension; (3) Some alterations in the liver function tests tend to be more specific, and deserve a different approach, such as predominant cholestasis, suggesting PSC, or elevation of aminotransferases more than 10 times the upper normal level, indicative of acute hepatocellular damage, which should bring to mind the possibilities of acute viral or toxic hepatitis; and (4) The alteration of liver tests in a patient with previously normal values, after starting a new therapy (notably thiopurine immunusuppressors) has to be approached and managed as possible liver toxicity[26].

Some causes of altered liver tests deserve a special comment, due to their prevalence or their importance.

The range of lesions collectively known as steatosis (including both uncomplicated fatty liver and steatohepatitis) are more and more frequent in the general population, but also in IBD patients, in which it accounts for the majority of diagnoses when investigating altered liver tests[21]. The usual causes of liver steatosis in the general population will also be present in IBD patients, such as metabolic syndrome, overweight/obesity and alcohol abuse. However, other possible reasons should be considered in our patients, notably glucocorticosteroid exposure, malnutrition and parenteral nutrition[27]. It is an exclusion diagnosis, and only the presence of a bright liver pattern in the ultrasound examination has been signaled as relatively specific[25]. More specific causes of liver disease have to be ruled out, notoriously those that can be appropriately managed, such as the viral hepatitides, autoimmune liver disease and drug-induced liver injury. A liver biopsy is rarely needed to confirm steatosis, but ultrasound follow-up will detect the potential development of portal hypertension or hepatocarcinoma.

Viral hepatitides B and C are, in older series, somehow more frequent in IBD patients than in the general population[28-31]. However, the current situation is different. In a Spanish national prospective study[32], the presence of markers of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection was prospectively studied in a series of more than 2000 patients. Evidence of current or past infection was present in 9.7% of cases, which is similar to figures observed in the general population: in ulcerative colitis (UC), hepatitis B surface antigen (HBsAg) was present in 0.8%, anti-HBc in 8% and anti-HCV in 1.3%, and in Crohn's disease (CD) HBsAg was present in 0.6%, anti-HBc in 7.1% and anti-HCV in 2.3%. 12% of patients had serological evidence of effective anti-hepatitis B vaccination (anti-HBs without anti-HBc). Multivariate analysis determined that age, family history of hepatitis and moderate to severe IBD were associated with HBV infection or contact, whereas HCV was mainly associated with previous transfusion of blood or blood products, but only if this was performed before 1991, when anti-HCV screening became widespread in local blood banks. Similar data have been communicated by other authors[33]. It is important to remember, that any prevalence of HCV and, especially, HBV markers has to be kept in mind, because the infection can reactivate under immunosuppressive therapy, necessitating prophylactic measures in some cases (antiviral therapy in HbsAg+ patients), and close follow-up in the remaining patients[34].

GASTROINTESTINAL COMORBIDITY

Helicobacter pylori disease

The prevalence of infection by *Helicobacter pylori* (*H. pylori*), as determined by serology, ranges between 15% and 50%. It is always lower than the corresponding prevalence in the general population, and is inversely correlated with sulfasalazine exposure[35-38]. The diminishing use of this drug makes it possible that this situation has

changed lately. The positivity of the urea breath test is also lower then in the general population[39]. However, the prevalence of peptic ulcer in transversal studies can reach 5%[38]. It is important to remember, that about 15% of CD patients will show histologic or even endoscopic lesions in the upper gastrointestinal endoscopy, and that the absence of *H. pylori* in these lesions is an important criterion when identifying them as truly related to IBD.

The indication for gastroprotection in patients exposed to glucocorticoids deserves a mention. The decision to co-prescribing proton pump inhibitors with glucocorticoids is quite usual, at least in our environment, but it is not sustained by medical evidence[40]. Gastroprotection should only be indicated on rare occasions when NSAIDs and glucocorticoids are prescribed jointly. To automatically prescribe proton pump inhibitors in the general patient, will lead to unnecessary higher costs, exposure to yet another set of adverse events and, most importantly, an increased number of tablets taken daily, which generally adversely influences adherence to therapy[41].

Celiac disease /gluten-sensitive enteropathy

Celiac disease and CD share some physiopathological, epidemiological and clinical features[42]. They frequently form part of the differential diagnosis. In older times, it was difficult to rule out celiac disease in a population with IBD, mainly due to the clinical superposition, but also to the possibility of finding very similar histologic changes (duodenal mucosal infiltrate, villous atrophy in Crohn's) and some parallel serologic alterations (positivity of antigliadin antibodies). The higher specificity of both antiendomysium and antitransglutaminase antibodies in the diagnosis of celiac disease, has finally allowed the performance of studies on the prevalence of this condition in patients with an unequivocal diagnosis of IBD.

Following the communication of isolated cases of associated Crohn's and celiac disease[43], an intriguing study was published[44], in which a very high prevalence (15%-20%) of antigliadin or anti endomysium antibodies was detected in a short series of CD patients. The high specificity of these determinations could make it reasonable to deduce that this association is extraordinarily frequent. However, later investigations of better quality have been unable to confirm these results[45-47]. The systematic search for celiac disease in CD patients cannot be recommended, according to the available evidence.

On the other hand, and although data are scarce, it seems that both UC and CD are more frequent in persons with celiac disease than in the general population. This has been described in two clinical series, with an IBD prevalence about 10 times higher than expected[45,48].

Biliary and pancreatic diseases

Cholelithiasis is a very prevalent condition in the general population[49]. Some circumstances explain its increased incidence in IBD patients, mainly the distortion of bile metabolism induced by the functional or anatomical alterations of the gut. Thus, it cannot strictly be considered a comorbid condition. The interest in cholelithiasis as a comorbid condition is mainly due to the possible overlap between its manifestations and those of the IBD or of extraintestinal manifestations, such as PSC. Management does not differ from the approach in the general population.

Pancreatic disease can ensue following the use of medications, the extraintestinal activation of inflammatory phenomena or can appear as an associated autoimmune disease[50]. In any case, it cannot be listed as a comorbid condition.

OBESITY

Presently, obesity is considered an emerging epidemic in Western societies, where it is more prevalent in disadvantaged social classes[51]. It also affects affluent classes in countries with emerging economies. The prevalence of this condition is growing, and some of its causes are sedentary behaviour, changing socioeconomic status and variations in traditional diets[52].

An increasing prevalence of obesity in patients with IBD has been described recently. In the past, this was considered an exception, but today, figures range from 15% to 20% in some series, with a further 40% of patients being overweight[53]. Although there is no evidence that obesity alters the course of the disease, at least in CD[54], obesity is related to unfavorable outcomes such as colonic adenomas, surgical morbimortality, cardiovascular risk and thrombotic disease.

CARDIOVASCULAR COMORBIDITY

Cardiovascular disease (CVD) is the main cause of death in developed countries[55] and its prevalence increases with age. Therefore, IBD patients are very likely to experience these entities throughout their life or, at least, be affected by some of the associated risk factors. The impact of CVD on IBD is the same as for the general population, increasing complications and remaining a common cause of mortality[56], especially when the disease is more severe or surgery is needed[18]. Anecdotally, in the 1950s when Truelove *et al*[57] published the first clinical trial with corticosteroids in UC, which demonstrated a decrease in mortality, the cause of death in 2 of the 5 patients who died was CVD, namely pulmonary thromboembolism. We will discuss IBD as a predisposing factor for CVD in more detail and the possible effects of IBD treatments on cardiovascular morbidity together with the undesired consequences of drugs used in the management of CVD for EII.

Venous thrombosis

Venous thromboembolism (VTE) has been considered a manifestation which is directly related to intestinal inflammatory activity, but in other chronic inflammatory diseases such as rheumatoid arthritis the incidence of VTE is not greater than in the general population[58].

Table 2 Acquired thrombotic risk factors in inflammatory bowel disease

Inflammation
Immobility (surgery or hospitalization)
Surgery
Fluid depletion
Central venous catheters
Drug therapy: Corticosteroids, anti-TNF drugs?
Smoking
Hyperhomocysteinemia (vitamin deficiencies)
Oral contraceptives
Increased levels of lipoprotein A

TNF: Tumor necrosis factor.

Furthermore, IBD patients in remission also have an increased risk of VTE[59], therefore additional factors other than inflammation are involved. This is why we have considered it a manifestation not directly linked with IBD.

In a recent population study[59], the incidence of VTE in IBD was 26 cases/10 000 person-years (PY) with a Hazard ratio of 3.4 (95% CI: 2.7-4.3). These data are similar to those observed in a previous Canadian population study, where the incidence of deep vein thrombosis (DVT) was 31.4/10 000 PY and 10.3/10 000 PY for pulmonary embolism (PE) for CD patients and 30/10 000 PY and 19.8/10 000 PY in patients with UC, respectively[60]. Again, the risk was 3 times higher in IBD compared with controls. There was also a time trend which increased with an annual average of 17% in odds of VTE[61]. There are no major differences between CD and UC, although the incidence in hospitalized patients may be somewhat higher in UC (OR = 1.32), probably because its frequency increases when the colon is involved[61]. IBD activity increases the likelihood of VTE with rates up to 8 times higher than controls[60]. Although VTE episodes are less frequent in outpatients, in this subgroup the differences with controls were even more marked (HR = 15.4) than during hospitalization periods (HR = 3.2)[59]. Similarly, although the majority of thromboembolic events occur in patients over 60 years, differences with controls are greatest in young patients (< 40)[60]. Other aspects that may increase the risk are involvement of the colon in the case of UC (pancolitis in 76% of episodes of VTE *vs* 2% of proctitis) and fistulizing pattern in CD[61].

Causes of thrombosis in IBD are many-fold; in most patients, recognized acquired prothrombotic factors can be identified, such as inflammation, immobilization, surgery, central catheters, corticosteroids, and smoking (Table 2)[62,63]. Nevertheless, in a significant percentage of patients (20%-50%) no obvious cause can be identified[64-66]. This supports the notion, that IBD itself acts as a predisposing factor for thrombosis. Inherited thrombophilias have no role in VTE associated with IBD because VTE is significantly less frequent than in thrombotic non-IBD subjects[67].

The appearance of VTE in a patient with IBD carries a poor prognosis reaching a mortality of 22%-25%[65,66], significantly higher than the control group (OR = 2.1). Hospital stay also increased by 48% and associated health costs doubled[61].

The most common location of VTE is lower extremity DVT with or without PE[58], which would include three-quarters of all episodes, but many locations have been described such as cerebral sinus[68-71], retinal vein[72-75], portal venous system[76-81] and hepatic or cava veins[82-87]. Portal system thrombosis is particularly important both for its potential complications and for its non-negligible frequency, being reported in up to 6% of patients after restorative proctocolectomy and ileal pouch-anal anastomosis[78,88]. A probable long-term complication is portal hypertension, which can be avoided with early anticoagulant therapy, for which clinical suspicion is fundamental, ordering an abdominal Doppler ultrasound and/or abdominal CT in the case of sudden onset of abdominal pain, fever or prolonged ileus after abdominal surgery[89].

Treatment of thromboembolic episodes of IBD is the same as in the general population and is based on the use of anticoagulation, first with heparin (usually low-molecular-weight heparins, LMWH) and then oral anticoagulants which should be sufficient for most patients[90]. Occasionally other measures may be required, such as thrombolysis or placement of an inferior cava filter that can be used as an effective means of preventing pulmonary embolus when anticoagulant therapy is contraindicated or thromboembolism recurs in spite of anticoagulant therapy. These drugs have proven safe in clinical trials where their effectiveness was evaluated as primary treatment. The optimal duration of anticoagulant therapy is unknown, but in general will vary depending on the severity of thrombosis and bleeding risk. In the first episode, 6 mo provided adequate coverage, but will be extended if the risk factor has not disappeared (surgery, immobilization) opting for lifelong anticoagulation in the setting of an inherited hypercoagulable state[89,91]. Despite treatment, up to 13%-26% of patients have a recurrent thromboembolic event[65,92]. Similar rates were found in patients with prior colectomy, thus surgery does not seem to prevent recurrence[65].

Treatment of thromboembolic episodes is important, but so is prevention. Possible measures include the control of disease activity, correcting vitamin and nutritional deficiencies, cessation of smoking, early mobilization after surgery or the use of intermittent pneumatic compression for patients at high risk of bleeding. Drug prophylaxis with LMWH is recommended in patients hospitalized with severe UC[93] or when planning surgery[94]. Outpatients with moderate flares and restricted mobility (old age, motor deficiencies…) are probably also candidates for pharmacologic prevention.

Atherosclerosis and arterial thromboembolic disease

Early atherosclerosis: Early atherosclerosis is a common phenomenon in several immune-based inflammatory diseases, particularly rheumatoid arthritis and systemic

lupus erythematosus[95], and is one of the most important causes of morbidity and mortality in these diseases, justifying the publication of specific recommendations[96]. The inflammatory process is behind the emergence of this phenomenon with a prominent role for the cytokines IL-6 and TNF that are significantly associated with the severity of subclinical atherosclerosis, independent of Framingham risk score[97]. Both cytokines are implicated in the pathogenesis of IBD, making possible the development of early atherosclerosis in these patients. Few data exist on this entity in IBD, although case reports of arterial occlusions in young patients with CD support this possibility[98]. Furthermore, to assess the presence of subclinical atherosclerosis at an early stage, several methods have been proposed, such as assessment of the intima-media thickness of the common carotid artery wall or the measurement of carotid artery stiffness; these have been shown to predict the occurrence of cardiovascular events[99,100]. Both parameters have been found to be altered in IBD patients compared to controls[101,102], although not uniformly in all studies[103].

Cardiovascular risk factors: There are few data on the prevalence of cardiovascular risk factors in IBD, apart from the known relationship with tobacco use[104]. Only in the cohort study by Ha *et al*[105], where the controls were randomly selected, IBD patients had a higher frequency of hypertension and hyperlipidemia, with similar rates of diabetes mellitus. Plasma lipid levels were inversely correlated with inflammatory activity[106], thus do not appear to have a particularly decisive role in predisposition to atherosclerosis in patients with IBD. As mentioned earlier, inflammatory activity *per se* could promote the development of atherothrombotic complications and in relation to this, C-reactive protein has been identified as a risk factor both in chronic inflammatory diseases[107] and in the general population[108,109]; unfortunately there are no specific data for IBD.

Arterial thrombotic events: Only two studies have evaluated the incidence of arterial events in IBD independently of venous thrombosis. The aforementioned study by Ha *et al*[105] found an overall increased incidence compared to the control group, mainly due to a marked increased in the risk of acute mesenteric ischemia (HR = 11.2, $P < 0.001$). The risk of myocardial infarction, conversely, was only discretely increased (HR = 1.6) in women over 40 years; in contrast, stroke was elevated in women below that age. However, Bernstein *et al*[110], found an overall increase in the prevalence of ischemic heart disease (IRR = 1.26) and cerebrovascular disease (IRR = 1.32) regardless of age group, although the latter only applied to patients with CD. In any case, except for acute mesenteric ischemia, in which local factors are probably involved, the rates found were quite similar to those of the control population, and not necessarily clinically relevant.

IBD therapy and cardiovascular diseases

The deleterious effects of glucocorticoids on the cardiovascular system are well known[111,112]: among their adverse effects are hypertension, hyperglycemia, hyperinsulinemia and hyperlipidemia, determining in some contexts increased cardiovascular morbidity, as in renal transplantation[113]. In other diseases, such as rheumatoid arthritis[114], inflammatory activity control can reduce cardiovascular complications. In spite of this, there is no direct evidence that IBD treatments alter the thrombotic tendency of the disease; however, some drugs show a favorable profile on some of the factors involved. For example, aminosalicylates reduce platelet activation and azathioprine inhibits formation of platelet-leukocyte aggregates[62]. There are no specific data for IBD, but methotrexate therapy in rheumatoid arthritis has been shown to decrease the prevalence of metabolic syndrome[115] in this disease and, more particularly, cardiovascular morbidity and mortality[114]. Regarding anti-TNF drugs, the results are somewhat contradictory: initially, endothelial function and insulin resistance transiently improve. On the other hand, infliximab in particular, shows a potential adverse effect on the lipid profile[116]. Likewise, thrombotic events associated with these drugs in patients without other risk factors have been described, although this needs to be confirmed[117].

Finally, it has to be noted, that some of the drugs used in the management of cardiovascular risk factors may be beneficial for IBD. Thus, the anti-diabetes drug rosiglitazone has been proven effective in the treatment of UC in two clinical trials[118,119], atorvastatin could have an antiinflammatory effect in CD[120] and ACE inhibitors have an antifibrogenic effect[121,122], not yet explored in CD.

PSYCHIATRIC COMORBIDITY

Anxiety and mood disorders have been extensively studied in IBD patients, whereas data on other conditions, such as psychoses, are scarce. However, there may be some connection, because some of the drugs used in the management of IBD, such as steroids[123], can precipitate psychotic manifestations and, on the other hand, the treatment of IBD may be followed by improvement of psychiatric illness[124].

As in other chronic diseases, the prevalence of anxiety and depression is higher in patients with IBD than in controls, both in hospital[125-128] and population cohorts[129-132]; their frequency is variable but approaches 24%-27%[129,133], which is two to three times higher than in a control population[129,131]. There are no great differences between UC and CD[130], or with other chronic diseases such as rheumatoid arthritis or diabetes[134].

Psychiatric comorbidity in IBD has been considered a risk factor for the onset of IBD itself, because it can precede its diagnosis[129,132]. However, the relative risks are low, and are limited to the year prior to diagnosis, which could represent the presence of symptoms of an undiagnosed somatic illness[135]. Moreover, the frequency of anxiety and depression increases after diagnosis of IBD,

suggesting that it is a consequence rather than a cause of the disease[132].

The role of daily stress and major life events in the precipitation of flares has also investigated, mainly because patients with higher perceived stress have a greater chance of disease reactivation[136,137]. However, there are several potential factors which may have contributed to this finding. In many studies, the sample size is not enough, and on the other hand, these results may be influenced by the fact that the influence of stress on disease activity is what patients, and probably many physicians, expect to be true[138]. Furthermore, anxiety and depressive symptoms predicted an increased likelihood of functional symptoms[139], and have a decisive influence on variables such as abdominal pain and overall well-being, used in the calculation of indices for most common activity indices. In addition, episodes of recurrence are limited to a relatively restricted period following the initial outbreak[137], which could be attributed to an incomplete improvement of IBD. Finally, several studies did not find such an association[130,140,141] and the results of trials of psychological interventions in IBD have been negative[140]. Thus, although anxiety and depression are more frequent around the periods of IBD activity, they did not seem to be risk factors for flares; also, more importantly, to wrongly assume that stress is responsible for disease exacerbation, may contribute in some way to induce unjustified and harmful feelings of guilt in some patients.

Psychiatric comorbidity has a marked impact on the management of IBD because it is, together with disease activity, the main determinant of quality of life in these patients[142]. Additionally, it is one of the factors associated with poorer adherence to treatment[41] and determines a greater use of health resources[139]. Thus, given the particular importance of these observations, and the frequent association of psychiatric symptoms with IBD, it is essential to identify and properly manage these conditions. In this sense, the use of screening tests with a good balance between sensitivity and specificity, such as the Anxiety and Depression Detector[143,144] or the Goldberg Anxiety and Depression Scale, could be recommended as an initial screening tool[134]. Treatment of psychiatric comorbidity in IBD does not differ from the general approach to these disorders, and the options include different types of psychotherapy[145] or second-generation antidepressants such as selective serotonin reuptake inhibitors or serotonin norepinephrine reuptake inhibitor, with fewer side effects than traditional drugs (tricyclics and MAO inhibitors) and that may be initiated by the gastroenterologist pending evaluation by the appropriate specialist.

CONCLUSION

Throughout the evolution of a patient with IBD, these diseases are frequently associated with other non-related diseases which may change management and prognosis. On the one hand, decision making is complicated because the available evidence does not always apply, as in most clinical trials such patients are excluded. On the other hand, their existence entails taking into account the possible consequences of treatment on other comorbidities, both by the possibility of interactions and by the facilitation of potential adverse effects. Prognosis also changes, especially in the presence of cardiovascular comorbidity, which is associated with a greater overall morbidity and mortality, especially in the surgical context. Finally, the concomitant presence of several diseases requires collaboration and coordination among professionals for joint decision making, and implementation of the proper clinical circuits to facilitate medical attention.

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S- Editor Cheng JX **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2734

World J Gastroenterol 2011 June 14; 17(22): 2734-2739
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Belén Beltrán, MD, PhD, *Series Editor*

Old-age inflammatory bowel disease onset: A different problem?

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Received: August 12, 2010 Revised: May 4, 2011
Accepted: May 11, 2011
Published online: June 14, 2011

Abstract

Inflammatory bowel disease (IBD) in patients aged > 60 accounts for 10%-15% of cases of the disease. Diganostic methods are the same as for other age groups. Care has to be taken to distinguish an IBD colitis from other forms of colitis that can mimick clinically, endoscopically and even histologically the IBD entity. The clinical pattern in ulcerative colitis (UC) is proctitis and left-sided UC, while granulomatous colitis with an inflammatory pattern is more common in Crohn's disease (CD). The treatment options are those used in younger patients, but a series of considerations related to potential pharmacological interactions and side effects of the drugs must be taken into account. The safety profile of conventional immunomodulators and biological therapy is acceptable but more data are required on the safety of use of these drugs in the elderly population. Biological therapy has risen question on the possibility of increased side effects, however this needs to be confirmed. Adherence to performing all the test prior to biologic treatment administration is very important. The overall response to treatment is similar in the different patient age groups but elderly patients have fewer recurrences. The number of hospitalizations in patients > 65 years is greater than in younger group, accounting for 25% of all admissions for IBD. Mortality is similar in UC and slightly higher in CD, but significantly increased in hospitalized patients. Failure of medical treatment continues to be the most common indication for surgery in patients aged > 60 years. Age is not considered a contraindication for performing restorative proctocolectomy with an ileal pouch-anal anastomosis. However, incontinence evaluation should be taken into account an individualized options should be considered

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Key words: Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Eldery population

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Hinojosa del Val J. Old-age inflammatory bowel disease onset: A different problem? *World J Gastroenterol* 2011; 17(22): 2734-2739 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2734.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2734

INTRODUCTION

Old-age colitis refers to patients over 60 years of age who are affected by a group of diseases of the colon, including infections, vasculitis, microscopic colitis, colorectal cancer, ischemic colitis, drug-associated colitis [particularly non-steroid anti-inflammatory drugs (NSAIDs)] and inflammatory bowel disease (IBD)[1]. Diagnosis of IBD in this age group may be difficult because it can be easily confused with other forms of colitis. Of particular relevance is so-called segmental colitis associated with diverticular disease (SCAD), which may simulate IBD both clinically, endoscopically, and histologically[1-3]. SCAD is characterized by the presence of diverticula, most often in the sigmoid colon, and affects middle and old

aged patients. A recent study in a German IBD elderly population showed that 8% of older patients diagnosed as IBD patients were in fact affected by SCAD[4]. Special precautions and attention must be taken in the diagnosis of these patients to avoid errors. New diagnostic tools, such as serological markers or advanced radiological examinations, may be useful in for a correct diagnosis.

FREQUENCY

Approximately 10% of patients at first flare of IBD are > 60 years old, with a similar distribution between ulcerative colitis (UC) and Crohn's disease (CD); 50% of them are diagnosed between 60-70 years of age[4,5]. However, it is difficult to establish the true incidence of IBD in older patients due to problems of case definition, population, and particularly because it may be confused with other clinical conditions, such as NSAID-associated colitis or ischemic colitis[1]. A bimodal age curve for IBD incidence has been suggested in epidemiological studies, with a second peak occurring at 50-70 years[5-7], but this point is currently the subject of debate, and it was not observed in the results of four population studies using strict radiological and endoscopic diagnostic criteria. A clear decrease in incidence occurs from age 40 in patients with CD and then stabilizes from age 60. Although there is a similar trend in patients with UC, the decrease is smaller in this group[8].

Thus, the incidence in patients over 60 years old represents approximately 10% of the cases of IBD; the bimodal distribution is questionable, and care should be taken to avoid including inadequately diagnosed patients.

CLINICAL MANIFESTATIONS

The clinical manifestations of the first flare of disease, both in UC and CD, are similar in the group aged > 60 years and younger age groups. It has been noted that the severity of the symptoms of UC, particularly rectal bleeding and diarrhea, may be lower in patients in this group, but it may also have atypical forms of presentation, such as constipation[9,10]. With regard to disease location, according to the Montreal classification, proctitis and left-sided UC are more common in patients > 60 years old than in younger patients. Severity of recurrence is usually higher in elderly patients (S2-S3 by the Montreal classification), with a longer duration of symptoms, which has influence on the need for steroid treatment[11-14].

In the case of Crohn's disease, the Montreal classification considers three age groups, establishing age > 40 years as the more advanced age group[11]. It has been observed that the form of disease presentation is similar between the different age groups, but that the L2 (colonic) disease location (clinical pattern) is the most common form in the older group (48% colon involvement in > 40 years group versus 28% and 20% in the groups diagnosed between 20-40 years and < 20 years, respectively)[1,15-19]. This frequency rises to 66% in patients aged > 60 years. With regard to the disease behavior, the most common is the inflammatory disease behavior (B1), the stricturing and penetrating pattern being less common than in the 18-60 years age group[1,5,19].

There are few references on the frequency of presentation of extraintestinal manifestations in this subgroup of patients. In the study by Hadithi[5], 17% of patients over 60 years of age had extraintestinal manifestations, which, in order of frequency, were: peripheral arthritis, uveitis, spondylitis, and erythema nodosum.

DIFFERENTIAL DIAGNOSIS

There are various clinical situations that may hinder diagnosis of IBD in elderly patients. IBD may be complicated by infections or by taking certain medications, such as NSAIDs and antibiotics[1,20-22]. Similarly, certain neoplasms, and specifically small bowel lymphoma, may have similar characteristics to those of IBD or be a complication of the disease itself[23-25]. The most common causes of chronic diarrhea and bowel inflammation in patients aged > 60 years are indicated in Table 1[25-31].

Segmental colitis associated with diverticular disease (SCAD) warrants special consideration[1,25-27]. Diverticulosis affects more than half of the people over 60 years of age. SCAD is clinically manifested by abdominal pain, rectal bleeding, and altered bowel habits, but only 3%-8% of patients have mucosal inflammation around the diverticula. It involves only the sigmoid colon, with sparing of the rectum and proximal colon. Endoscopic examination shows a circumferential distribution of erythema, granularity, and friability, and sparing of the diverticular ostium and cavity with a diffuse periosteal distribution of inflammation confined to the area of the diverticula[24,25]. Histopathologically, inflammation analogous to that seen in the UC and nonspecific chronic inflammation that may contain granulomas has even been reported, leading to misdiagnosis with CD. In general, SCAD may be suspected when colitis is confined to the diverticular segment of the left colon (excluding the rectum), the rectum and proximal colon are endoscopically and histologically normal, and if surgical resection of the affected segment is required, when no recurrence of segmental colitis occurs.

There are no randomized studies specifically assessing treatment of SCAD[1,5]. Overall response to oral (2.4-3 g/d) or topical (enemas 1-2 g/d) mesalazine was good, with a clinical response of 80% at six weeks and a maintenance of response in 90% of patients at six months[5,27]. The addition of fiber and antibiotics (rifaximin) during the acute phase may improve the response. If there is no improvement with this combination treatment, steroids applied topically as enemas may improve the clinical response. Exceptionally, oral prednisone has been evaluated in some cases of non-responders. These are patients who will rarely require surgery with segmental resection for SCAD.

MEDICAL TREATMENT

The principles governing medical management of IBD in patients aged > 60 years are the same as in other age groups. The location, extent, severity, and disease behavior,

Table 1 Differential diagnostic of colitis in patients older than 60 years

Disease	Clinical characteristics	Endoscopic findings
Colitis associated to diverticulosis	Rectal bleeding, abdominal pain, diarrhea	Segmentary distribution, peridiverticular, sigma affected, rectum and proximal colon are normal
Ischemic colitis	Abdominal pain rectorragia acute onset	Segmentary colitis (sigma/left colitis), majority are non-obstructive
Microscopic colitis	Watery diarrhea, no rectal bleeding, no fever, frequent cause of diarrhea in the eldery	Normal endoscopy, multiple biopsies from colon, celiac disease association
Infectious colitis	Disenteriforme diarrhea, different agents, *C.difficile* to be ruled out	Difuse effects on the colon Increased morbidity and mortality in older-aged population
NSAIDs colitis	Recurrent abdominal pain; obstruction, perforation, hemorrhage; chronic anemia	Any part of the intestine, isolated lesions, aggravate previous UC and CD

NSAIDs: Non-steroid anti-inflammatory drugs; UC: Ulcerative colitis; CD: Crohn's disease.

both in UC and CD, will determine the medical treatment options to be used[32-37]. Special precautions and the most generally used treatments in IBD old-aged patients are summarized below. In brief, depending on the kind and severity of IBD suffered by patients, we should choose the best treatment, taking special care of side effects caused by advanced age or by drugs associated with another diseases.

Mesalazine

Oral and/or topical mesalazine is the main option for medical treatment in patients with a mild to moderate flare of UC. The availability of new distal release mesalazine preparations with the MMX formulation may facilitate compliance and, therefore, adherence to treatment. It is similar in terms of efficacy to other oral preparations of mesalazine, regardless of its form of release[38]. However, in Crohn's disease, the efficacy of mesalazine is questionable and it has a limited role in the treatment of mild flares of ileal or colonic location[36,37].

Oral or topic steroids

Beclomethasone combined with mesalazine has been shown to be effective for treating moderate flares of left UC when administered orally as a single 5 mg dose for 2-4 wk, obtaining remissions in about 60% of patients[39]. Although there is no specific study by age subgroups, it remains an option to consider that may avoid the need for systemic steroids[40,41]. Nevertheless, we have to bear in mind that local or systemic steroids administered intrarectally are absorbed and therefore not free from their side effects.

Antibiotics

A detailed case history should be made in the group of patients > 60 years old, looking for symptoms suggestive of sensory neuropathy in the use of metronidazole[42].

Immunomodulators

The prevalence of corticosteroid resistance or dependence in this group of patients has not been specifically evaluated, but it is estimated at about 30%[43]. Although there is little objective data on which to base this practice, treatment was started with conventional immunomodulatory agents (azathioprine, mercaptopurine, and methotrexate) in this group of patients without evidence of differences in terms of efficacy, metabolism, and toxicity between the patients aged > 60 years and younger patients[35-37].

Biological agents

Their indications remain similar to those for patients aged < 60 years[44-46]. However, one of the most important aspects regarding the use of these agents is the safety profile, particularly that related to the risk of infections. The trend observed in the subgroup of elderly patients with rheumatoid arthritis > 65 years old to develop infections has not been confirmed in any controlled study. It is not uncommon for patients to be treated with combination therapies (corticosteroids, conventional immunomodulators, and biological agents) because of the disease's complexity. In this regard, the higher rate of infection found in the TREAT registry in patients treated with anti-TNF agents (infliximab (IFX) in this case) may have been due to the greater severity of these patients and the greater use of steroids[47]. In fact, the only independent risk factor for infections was the concomitant use of steroids (RR = 2.33, 95% CI = 1.50-3.62). However, the results of series describing the clinical experience in treatment with these drugs will best reflect what we would encounter in our clinical practice, particularly when they report observations collected over years. In this regard, the results of the Leuven group comparing adverse effects in 734 patients treated with IFX versus 664 control patients, with a follow-up of 58 to 144 mo, showed no significant differences in the risk of severe infections, mortality, and malignant conditions. No specific analysis by age subgroups was done[48].

In the Stockholm cohort study[44], it was observed that the use of infliximab in the subgroup of patients aged > 60 years was associated with an increased risk of serious side effects and higher mortality, which was also suggested in the multivariate analysis of the Lichtenstein registry[41]. However, more safety data are required on the use of biological therapies in patients aged > 60 years. In the meantime, it seems logical to use them according to recommended usage guidelines and with strict adherence to performing all tests prior to administration. It should be kept in mind that in this age group, and particularly in countries where the prevalence of tuberculosis is higher, it is more likely that an increased frequency of cases of latent TB will be observed.

Surgical indications

In older patients surgical indications are the same as in younger patients, with failure of medical treatment being the most common cause of surgery[49]. In general, the need for surgery is lower in CD patients with an age at diagnosis > 40 years and colonic disease location, and more common in the elderly. However, it is similar in ileocecal disease[49-51].

In UC, restorative proctocolectomy with ileal pouch-anal anastomosis continues to be the surgical technique of choice[52,53]. In general, results are not as good in older patients compared to younger patients, but they are both reasonably good in terms of efficacy and morbidity. Only an increased frequency of diurnal incontinence and nocturnal leakage has been reported among patients aged > 65 years. Generally, overall quality of life was good, to the extent that in the study by Delaney[52] 89% of patients over 65 years of age were satisfied with surgery and 96% would recommend it. Therefore, age is currently not considered a contraindication for performing an ileal-anal pouch.

MORTALITY

Mortality from UC is similar to that of the general population, with more controversial results in the case of CD[1,5,41,54,55]. In UC, age at diagnosis is not associated with increased mortality, whereas in CD, there appears to be a slight increase in the risk of mortality in older aged patients (> 55 years) with long-standing disease[56-58]. However, we should be cautious, because some of the results may be biased by the type of study, study population, follow-up period, *etc*.

There are a number of aspects to consider regarding possible factors that may be associated with an increased relative risk of mortality. In this regard, there were more hospitalizations in patients > 65 years than in the younger group, representing 25% of all admissions for IBD occurring in the USA (33% of hospitalizations for UC and 20% for CD)[41]. In general, the disease course of UC is more severe in elderly patients and mortality in hospitalized patients, both with UC and CD, is estimated to be 3-5 times higher than in age subgroups < 65 years. In the study by Ananthakrishnan *et al*[41], hospital mortality was higher (OR: 3.91; 95% CI: 2.50-6.11) and age was an independent risk factor in the group of IBD patients aged > 65 years; other variables significantly associated with hospital mortality were fistulizing disease, malnutrition, and need for intestinal resection for stenosing disease. Interestingly, in this study, a greater difference was observed in mortality between unoperated and operated patients, possibly because medical salvage therapies, including immunomodulatory and/or biological therapies, may increase morbidity and mortality, suggesting that "early" surgery is associated with lower mortality in older patients. However, hospital stays were longer, particularly in the group of operated patients. Although postoperative complications were similar between this age group and those younger than 60 years, the risk of cardiovascular or pulmonary complications was greater, these being aspects to be taken into account in the postoperative period of patients.

Related to the use of biological agents, there is a study[59] that points out that 3 out of 5 deaths attributable to infliximab treatment were older than 70 years. However, these patients had a longer evolution of disease (15-26 years) and they suffered from severe diseases and comorbidities.

The risk of suffering dysplasia and cancer is higher in extended Crohn's colitis patients > 45[60]. However there is an increased incidence of these complications related to disease duration (increased risk among patients with disease duration longer than 20 years). Therefore, older and long-evolution patients should be under colonoscopic surveillance, however, only being of advanced age when an IBD outcome occurs is not enough to maintain different colonoscopic surveillance compared to naïve younger patients.

Little is known about effects of age on colonoscopy risks. In a large retrospective study of safety, feasibility, and tolerability of ileonoscopy in IBD patients[61], few severe complications where detected (0.3%-0.8%) and none of them were related to the advanced age of patients or disease activity.

Table 2 Summary of inflammatory bowel disease onset in the elderly

Home messages
10%-15% patients with inflammatory bowel disease > 60 years
12% UC patients
16% CD patients
Differential diagnosis with colitis associated to diverticulosis, ischemic colitis, infectious colitis (*C.difficile*)
Ulcerative colitis: proctitis/left colitis is more frequent
CD: Granulomatous colitis > frequent than ileocecal
Medical treatment
Same treatment options as younger patients
Increased rate of complications with steroids
Similar response, but recurrence less frequent
Surgery: Equal indications, ileoanal reservoir is secure
More hospitalizations
Similar mortality, increased in hospitalized patients, comorbidity

UC: Ulcerative colitis; CD: Crohn's disease.

CONCLUSION

The main messages regarding IBD in the elderly are summarized in Table 2. The prevalence of IBD in patients aged > 60 years is difficult to determine, but accounts for 10%-15% of cases of the disease. Diagnosis may be difficult because there are a number of clinical conditions that may mimic IBD, particularly ischemic colitis or colitis associated with diverticular disease. Diagnostic methods are the same as for other age groups. The clinical pattern in UC is proctitis and left-sided UC, while granulomatous colitis is more common in CD. Its disease course is similar to that of the younger groups. The treatment options are those used in younger patients, but a series of considerations related to potential pharmacological interactions and side effects of the drugs must be taken into account.

The safety profile of conventional immunomodulators and biological therapy is acceptable, but more data are required on the safety of use of these drugs in the elderly population. The overall response to treatment is similar in the different patient age groups, but elderly patients have fewer recurrences. The number of hospitalizations in patients > 65 years is greater than in younger groups, accounting for 25% of all admissions for IBD. Mortality is similar in UC and slightly higher in CD, but significantly increased in hospitalized patients. Failure of medical treatment continues to be the most common indication for surgery in patients aged > 60 years. Age is not considered a contraindication for performing restorative proctocolectomy with an ileal pouch-anal anastomosis.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2740

World J Gastroenterol 2011 June 14; 17(22): 2740-2747
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Ulcerative colitis in smokers, non-smokers and ex-smokers

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Received: August 13, 2010 Revised: November 16, 2010
Accepted: November 23, 2010
Published online: June 14, 2011

Abstract

Smoking is a major environmental factor that interferes in the establishment and clinical course of ulcerative colitis (UC). Firstly, the risk of smoking status impact in the development of UC is reviewed, showing that current smoking has a protective association with UC. Similarly, being a former smoker is associated with an increased risk of UC. The concept that smoking could have a role in determining the inflammatory bowel disease phenotype is also discussed. Gender may also be considered, as current smoking delays disease onset in men but not in women. No clear conclusions can be driven from the studies trying to clarify whether childhood passive smoking or prenatal smoke exposure have an influence on the development of UC, mainly due to methodology flaws. The influence of smoking on disease course is the second aspect analysed. Some studies show a disease course more benign in smokers that in non-smokers, with lower hospitalizations rates, less flare-ups, lower use of oral steroids and even less risk of proximal extension. This is not verified by some other studies. Similarly, the rate of colectomy does not seem to be determined by the smoking status of the patient. The third issue reviewed is the use of nicotine as a therapeutic agent. The place of nicotine in the treatment of UC is unclear, although it could be useful in selected cases, particularly in recent ex-smokers with moderate but refractory attacks of UC. Finally, the effect of smoking cessation in UC patients is summarised. Given that smoking represents a major worldwide cause of death, for inpatients with UC the risks of smoking far outweigh any possible benefit. Thus, physicians should advise, encourage and assist UC patients who smoke to quit.

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Key words: Smoking; Ulcerative colitis; Nicotine; Inflammatory bowel disease; Colectomy; Pouchitis

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Bastida G, Beltrán B. Ulcerative colitis in smokers, non-smokers and ex-smokers. *World J Gastroenterol* 2011; 17(22): 2740-2747 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2740.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2740

INTRODUCTION

The development of inflammatory bowel disease (IBD) is the result of an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host. Some environmental factors, such as cigarette smoking and appendectomy, have been shown to play a significant role in the pathogenesis of IBD. Cigarette smoking may cause lung cancer, atherosclerotic vascular disease, other kinds of cancers and chronic obstructive pulmonary disease.

Nowadays, smoking represents a major cause of preventable morbidity and is probably the most important and preventable cause of premature mortality in developed and developing countries.

As smoking is a major environmental factor that interferes in the clinical course of ulcerative colitis (UC), in this article we will review its influence on patients with UC.

RISK OF DEVELOPING UC

It is now fully accepted that UC predominantly affects non-smokers and former smokers, and that smoking exerts a universal protective effect against developing UC[1].

This relationship between smoking and UC has been described for more than 30 years. The first to report this association was Samuelsson[1], who noted a lack of smokers among UC patients compared with matched control subjects. The author attributed this observation to interaction with medication rather than to pathogenesis. In 1982, Harries *et al*[2] confirmed this observation; in a study on 23 UC patients, they found only 8% were current smokers compared with 44% of matched control subjects [patients with Crohn's disease (CD) or a cohort evaluated for fractures]. After the first reports, numerous other studies also confirmed this association. The first meta-analysis, including nine case-control studies, revealed a pooled odds ratio (OR) for non-smoking as the risk factor for acquiring UC of 2.9 (95% CI: 2.6-3.2)[3]. The authors also demonstrated a higher risk for former smokers compared to non-smokers with a pooled OR of 1.64 (95% CI: 1.36-1.98). Additionally, they found a dose-response association with an overall pattern of decreased risk of disease with increased level of smoking. A subsequent meta-analysis[4] that included new information from 11 741 patients with UC in 13 studies confirmed the previous data. When they compared current smoking with never smoking, all studies, with the exception of one, found an OR of less than 1.0, indicating a protective association of current smoking and UC; current smoking decreased the risk for UC (OR: 0.58, 95% CI: 0.45-0.75), suggesting that current smoking is associated with an approximately 42% reduced risk of an UC diagnosis. In contrast to current smoking, when former smokers were compared with never smokers among patients with UC *vs* controls, being a former smoker was associated with an increased risk of UC, with an overall OR of 1.79 (95% CI: 1.37-2.34).

The influence of smoking in genetically predisposed IBD patients has been analyzed in family studies. It is well known that there is high concordance within a family between smoking habits and the IBD phenotype, with UC developing in non-smokers and CD in smokers[5]. Thus, some of the apparent protection that smoking exerts on sporadic UC may be due not only to a therapeutic effect of tobacco usage, but rather that in some instances, it is CD rather than UC which develops as a result of the influence of smoking on the pathogenic processes. Bridger *et al*[6] examined 89 sibling pairs with CD or UC discordant for both smoking and IBD phenotype to investigate whether smoking determines the type of IBD that develops in individuals with very similar genetic susceptibility. Of 89 sibling pairs discordant for smoking at diagnosis, 23 were also discordant for disease type. In 21 of these, CD occurred in the smoker and UC in the non-smoker, suggesting that tobacco consumption may act on the IBD genetic predisposition to shift the phenotype from UC towards CD. This role of smoking habits on the IBD phenotype has been confirmed in twins[7]. Among 103 pairs (at least one twin who suffered from IBD), the frequency of smokers was lower among twins with UC. Furthermore, smoking habits were found to be of significance for discordance of disease.

Another issue that should be noted is whether gender may influence the effect of tobacco smoking on UC. Motley *et al*[8] noticed that current smoking delayed disease onset in men but not in women. The effect of gender was also described in the aforementioned article by Bridger *et al*[6], who noted different effects of smoking on the incidence of CD or UC in females and males. This paper showed more pronounced protection from UC (OR 0.18, 95% CI: 0.11-0.3) in females smoking at diagnosis compared with non-smokers (OR 0.47, 95% CI: 0.28-0.79). Later, Cosnes *et al*[9] studied a cohort of 1784 consecutive adult patients (978 with UC). In this study, the beneficial effect of smoking in UC was modulated importantly by gender: they described a more marked increase in disease presentation in men during the few years after smoking cessation.

One important question that should be resolved is whether childhood passive smoking or prenatal smoke exposure has the same influence on the development of UC. This issue was analyzed in a meta-analysis of 13 studies[10]; the results revealed that, in contrast to the inverse association between active smoking and UC development, there was no significant association between childhood passive smoke exposure or prenatal smoke exposure and the development of UC. A dose-response relationship is a possible explanation for the failure of this meta-analysis to show a protective effect of childhood passive smoke exposure on UC; thus it is possible that the level of exposure does not reach a threshold level which is required for the protective effect that has been well documented in active smokers. Moreover, the size of the studies regarding prenatal smoke exposure and the development of IBD were too small to reach a definitive conclusion. After the publication of the meta-analysis, van der Heide *et al*[11] published a new study that addressed this issue; the authors did not find that passive smoking had a beneficial effect. Furthermore, passive smoking UC patients had a higher prevalence of ileal disease (pouchitis and backwash ileitis) than non-passive smoking UC patients.

All the presented data support the view that current smoking is associated with a low risk of UC, but before drawing conclusions we should be aware of the possible

bias that may be present in the studies analyzed, such as differences in study methods, measurement error, or a lack of verification of self-reported smoking status. The lack of uniformity in smoking definitions is probably a serious weakness in many of the published studies. Another important issue that should be noted is that most of the studies are based strictly on the effects of smoking on the non-Jewish white population. Certain races have not demonstrated associations between smoking behaviors and IBD[12,13], suggesting that different races may have varying degrees of susceptibility to IBD, although these differences are probably more important in CD than in UC. In fact, in Israeli Jewish patients, there was no association between smoking and CD patients, although the opposite association exists in UC[14].

INFLUENCE OF SMOKING ON DISEASE COURSE

Active tobacco smoking has a protective effect on the severity of UC; the disease course is more benign in smokers than in non-smokers[15,16]. Flare-up, hospitalization rates, the need for oral steroids and, more importantly, colectomy rate are reported to be lower in smokers compared with non-smokers, though this has not been observed in all studies[17-19].

The link between smoking and colectomy in UC patients is controversial. In a retrospective analysis of a large series of UC patients, current smoking was found to decrease the 10-year cumulative colectomy risk from 0.42 to 0.32[18]. A subsequent meta-analysis with a total of 1489 UC patients found the risk for colectomy to be lower (OR: 0.57, 95% CI: 0.38-0.85) in current smokers compared with non-smokers[20].

In agreement with these results, a population-based cohort study performed in Europe[16] with 771 UC patients prospectively included and followed for 10 years revealed a lower relapse rate (Hazard ratio: 0.8, 95% CI: 0.6-0.9) in smokers compared with non-smokers. Another similar study carried out in the Netherlands by van der Heide *et al*[11] with 295 UC patients identified smoking after diagnosis as a protective factor for colectomy (OR 0.27, 95% CI: 0.11-0.67), whereas pancolitis at diagnosis (OR 3.18, 95% CI: 1.85-5.48) was a risk factor.

On the other hand, a study by Beaugerie *et al*[21], designed to determine the impact of cessation of smoking on the course of UC, analyzed the disease severity in 32 patients with UC who stopped smoking after the diagnosis compared with 32 non-smokers and 32 continuing smokers matched for sex, age, and age at onset. There was no significant difference in colectomy rate among quitters during the 5-year period after smoking cessation and either non-smokers or continuing smokers during the matched periods. Nevertheless, smokers who quit experienced an increase in disease activity, hospital admissions, and the need for major medical therapy (oral steroids, immunosuppressants) within the first years following the cessation of smoking.

As well as the study by Beaugerie *et al*[21], Boyko *et al*[17] reported a lower hospitalization rate in patients who were smoking at the onset of UC, but could not identify a difference in the colectomy rate between smokers and non-smokers.

In agreement with the presented data, improvements in symptoms and a milder course of disease have been reported in ex-smokers who returned to smoking[22,23]. In fact, many patients noted symptom exacerbation when they stopped smoking, followed by symptom relief when they smoked again[8].

Interestingly, some studies reported a gender association; when compared to non-smokers, male UC who smoked ran a more benign disease course as assessed by the decreased need for immunomodulators, whereas this difference was not observed in females[3,9,20]. Additionally, smoking delayed the onset of the disease, although only in males.

Smoking seems to be a protective factor against proximal extension. In patients with distal UC at diagnosis, retrograde extension of the disease process was less frequent in smokers than in non-smokers[24]. A retrospective analysis in France showed that in a subgroup of patients with limited disease at onset of symptoms, the percentage of smokers developing pancolitis was lower than among non-smokers[18]. More recently, Meucci *et al*[25], in a cohort of 273 patients, described proximal extension of the disease in 27.1%. The cumulative rate of proximal extension was higher in non-smokers, in patients with more than three relapses per year and in patients requiring systemic steroid or immunosuppressive treatment.

Not all the studies could identify a protective effect of smoking on the retrograde extension of UC. Pica *et al*[26] retrospectively reviewed 138 patients with ulcerative proctitis; in this series, proximal extension of the disease was seen in 30% of patients. The prevalence of smoking habit was not higher in patients with extended disease.

Finally, it is important to note that many studies failed to identify a beneficial effect of smoking on the course of UC[27-30]. For instance, Roth *et al*[31] included 102 consecutive patients in a survey to assess the natural history of the disease and to determine predictors of future disease severity at the time of diagnosis. Delay from symptoms to diagnosis of UC, gender, family history of IBD, smoking status and disease severity at the time of diagnosis did not significantly predict the disease severity. Similar results were described by Romberg-Camps *et al*[32] in a population-based survey designed to predict the disease course in 630 UC patients. In this study, disease severity, cumulative medication use, and "surgical" and "nonsurgical" recurrence rates were calculated as outcome parameters. A protective effect of smoking on disease recurrence in UC could not be confirmed in this study.

Tobacco smoking also influences other clinical scenarios in patients with UC. Smoking may also prevent the development of primary sclerosing cholangitis (PSC), or pouchitis after colectomy and ileal pouch anal anastomo-

Table 1 Studies of the use of nicotine in ulcerative colitis

Ref.	Formulation	Dose (mg)	*n*	Type of disease	Study	Comparator	Results
[44]	Nicotine gum	4	1	Active UC	Uncontrolled	--	100%
Perera *et al*[45]	Nicotine gum	4	11	Active UC	Uncontrolled	--	27%
Watson *et al*[46]	Nicotine gum	--	1	Active UC	Uncontrolled	--	100%
Srivastava *et al*[47]	Transdermal nicotine	22	18	Active UC	Uncontrolled	--	78%
Guslandi *et al*[48]	Transdermal nicotine	15	3	Active UC	Uncontrolled	--	66%
Guslandi *et al*[49]	Transdermal nicotine	15	10	Active UC	Uncontrolled	--	70%
Lashner *et al*[50]	Nicotine gum	20	7	Active UC	Cross-over	Placebo	NI 43% *vs* PL 0%
Pullan *et al*[51]	Transdermal nicotine	15-25	72	Active UC	Controlled	Placebo	NI 49% *vs* PL 24%
Sandborn *et al*[52]	Transdermal nicotine	11-24	64	Active UC	Controlled	Placebo	NI 39% *vs* PL 9%
Thomas *et al*[53]	Transdermal nicotine	15-25	61	Active UC	Controlled	Prednisone	NI 20% *vs* PR 45%
Guslandi *et al*[54]	Transdermal nicotine	15	38	UC in remission	Controlled	Prednisone	NI 71% *vs* PR 88%
Thomas *et al*[55]	Transdermal nicotine	15	80	UC in remission	Controlled	Placebo	NI 45% *vs* PL 50%
Green *et al*[57]	Nicotine enemas	4	22	Active UC	Uncontrolled	--	73%
Sandborn *et al*[58]	Nicotine enemas	6	7	Active UC	Uncontrolled	--	71%

NI: Nicotine; PL: Placebo; UC: Ulcerative colitis.

sis[33-36]. PSC is a chronic cholestatic liver disease of unknown etiology that is associated with UC. Loftus *et al*[33] published a case-control study to determine whether the relationship between smoking and PSC is similar to that found between smoking and UC. Like UC, PSC was found to be a disease of non-smokers, as the odds of having PSC in current smokers compared with never-smokers was 0.13. The protective effect was independent of whether or not the PSC patient had underlying IBD; the odds of having disease among former and current users of any tobacco relative to never-users were 0.41 regardless of the presence or absence of IBD. Another study published recently by van der Heide *et al*[11] found that never smoking was a risk factor for the development of PSC in UC patients (OR 4.32, 95% CI: 1.52-12.25). In this article, proctitis (OR 0.09, 95% CI: 0.02-0.39) and left-sided colitis at diagnosis (OR 0.35, 95% CI: 0.13-0.93) were associated with a lower risk for PSC.

NICOTINE AS A THERAPEUTIC AGENT IN UC

Four different formulations of nicotine have been used as a therapeutic agent in patients with UC: nicotine gum, transdermal nicotine, delayed release oral capsule and enema (Table 1).

Chewing polacrilex gum containing up to 4 mg of nicotine results in peak blood nicotine concentrations that are usually observed with cigarette smoking. However, the extraction of nicotine from gum is incomplete and variable (53%-72%) for a variety of reasons, including: variable nicotine extraction because of differences in chewing intensity and duration, variable saliva production (the basic pH of saliva enhances absorption), variability in the amount of nicotine retained in the mouth for buccal absorption *vs* the amount swallowed (which undergoes first pass hepatic metabolism) and inconsistent administration schedules[37,38].

Transdermal nicotine administration provides a steady plasma nicotine concentration that is directly proportional to the nicotine dose and leads to peak nicotine concentrations of approximately two thirds of those measured during smoking[39]. Nevertheless, compared with smoking cigarettes, no sharp increases in blood nicotine concentrations have been observed using transdermal application.

Topical administration of nicotine directly to the colon *via* enema or delayed release oral capsule formulations decrease the systemic absorption and side effects, and may be clinically beneficial. Nicotine is rapidly and extensively metabolized, primarily in the liver *via* the cytochrome P450 enzyme pathway[40,41]. When nicotine is ingested orally, bioavailability is low (20%-44%) due to the first pass hepatic metabolism[42,43].

A pharmacokinetic study in healthy volunteers demonstrated that nicotine tartrate administered as a liquid enema had low systemic absorption (mean systemic bioavailability for various formulations ranged from 15% to 25%) and was well tolerated after a single dose[42].

Uncontrolled studies reported that nicotine gum 4 mg, 5-7 sticks/d was beneficial in some ex-smokers with active UC[44-46] but, in contrast, nicotine gum had no benefit and was poorly tolerated in those with active UC who had never smoked[45]. Similarly, uncontrolled studies reported that transdermal nicotine 15 mg/24 h and 30 mg/24 h (22 mg delivered) was beneficial in patients with active and steroid-dependent UC[47-49]. As happened with the nicotine gum, patients who had never smoked were reported to tolerate nicotine poorly[47]. Following the uncontrolled studies, six controlled trials have assessed the utility of nicotine therapy in UC patients[50-56].

A study in seven patients with active UC confirmed that nicotine gum 2 mg administered 5-7 times/d was beneficial compared with placebo in three of four ex-smokers and in none of three who had never smoked[50].

Two randomized, placebo-controlled trials of transdermal nicotine for active UC showed that nicotine was useful when compared with placebo[51,52]. Both studies began with lower nicotine doses (15 mg and 11 mg) to improve patient tolerance, followed by dose escalation after 1-2 wk. The first study, conducted by Pullan *et al*[51]

treated 72 patients with active left-sided UC with either transdermal nicotine patches (up to 25 mg/24 h) or placebo over a period of 6 wk. The standard medication, which included mesalamine (all patients) and glucocorticoids (12 patients), continued to be administered during the study. Most patients tolerated the nicotine doses. Patients in both the nicotine and placebo groups improved, although the improvement in clinical and histological grades was greater in the group treated with nicotine. Seventeen of 35 (48.6%) patients in the nicotine group had complete remission, compared with only nine of 37 (24.3%) patients in the placebo group.

Similar results were found by Sandborn *et al*[52], who compared transdermal nicotine (22 mg/24 h) for 4 wk with placebo. Nicotine treatment led to a clinical benefit in 39% of patients compared with 9% receiving placebo. No improvement in histology was observed.

A fourth randomized controlled trial compared transdermal nicotine at the highest tolerated dose (up to 25 mg/16 h) with prednisolone 15 mg/d in active UC[53]. No significant differences were found between the two groups, although the corticosteroid therapy tended to be more efficacious.

A fifth randomized controlled trial compared transdermal nicotine 15 mg/24 h for 5 wk to prednisone 30 mg/d tapered over 5 wk and demonstrated equivalence, although again there was a tendency towards prednisone being more efficacious[54]. After following up 30 patients with remission of distal UC after therapy with either mesalazine plus transdermal nicotine or mesalazine plus oral prednisolone for 12 mo, Guslandi *et al*[56] suggested that nicotine-induced remission of UC lasts longer than that obtained by therapy with oral corticosteroids. With respect to the maintenance of remission in patients with UC, no beneficial effect was noted with a lower dose of transdermal nicotine (up to 15 mg/16 h) compared with placebo over a period of 6 mo in the sixth randomized, placebo-controlled trial[55].

In two studies in which liquid nicotine enemas were used to treat patients with active distal UC, the results suggested a clinical benefit in the absence of detectable serum nicotine concentrations[57,58]. Adverse events were of minimal consequence. Nevertheless, the liquid enemas were difficult to retain. Further controlled trials are needed to determine the therapeutic value of topical nicotine administration.

Finally, in patients with PSC, a pilot study of oral nicotine tartrate capsules delivered to the gastroduodenum did not report a beneficial effect[59].

Adverse events when nicotine is therapeutically administered are common (> 50%), but generally mild. They include contact dermatitis, nausea, lightheadedness, headache, sleep disturbance, central nervous system stimulation, sweating, tremor, and tachycardia. The side effects are more frequent in non-smokers than in ex-smokers. Non-smokers have fewer nicotine-associated adverse events if they are initially administered a low dose patch, subsequently escalating the nicotine dose, presumably because they have not yet developed a tolerance to nicotine side effects[60].

Table 2 Possible mechanisms in the pathogenic interactions of smoking and inflammatory bowel disease[64]

Modulation of cellular and humoral immunity
Changes in cytokine levels
Modification of eicosanoid-mediated inflammation
Reduction of antioxidant capacity, production of oxygen free radicals
Release of endogenous glucocorticoids
Colonic mucus effects
Alteration of mucosal blood flow
Pro-thrombotic effects and promotion of microvascular thrombosis
Alteration of gut permeability
Modification of gut motility

PATHOGENIC MECHANISMS OF SMOKING ON UC PATIENTS

The exact mechanisms of action of nicotine and smoking in UC patients is not well known (Table 2)[61]. Tobacco smoke contains hundreds of different substances including nicotine, free radicals and carbon monoxide. It is suspected that the main metabolite responsible for the impact on the course of UC is nicotine, however there is no absolute proof of nicotine being the sole active moiety. In consequence, probably the mechanisms are diverse and considering that the pathogenesis of UC is only partially understood, any dissertation on their possible mechanisms can only be hypothetical.

Nicotine increases mucin synthesis in UC patients[62]. Patients with UC are shown to have a significantly thinner mucus layer in the left colon and rectum compared with healthy (smoking and nonsmoking) control subjects[63]. Nevertheless, no effect of transdermal nicotine on mucin gene expression in UC patients could be demonstrated by Louvet *et al*[64].

Nicotine also affects the immune system. Heavy smokers may have reduced levels of immunoglobulin A in both saliva and intestinal secretions[65,66]. Heavy smoking also influences cellular immune-defense mechanisms, leading to a state resembling immune suppression with an increased level of suppressor CD8+ T cells and a diminished ratio of CD4/CD8 T cells, which is reversible after the cessation of smoking[67]. Nicotine has been shown to decrease the synthesis of proinflammatory molecules, for instance, interleukin (IL)-1β and tumor necrosis factor (TNF)-α by mouse colonic mucosa as well as the production of mucosal eicosanoids[68] and some proinflammatory cytokines by human mononuclear cells (e.g. IL-2[69], IL-8, and TNF-α[70]).

Smokers with UC have a significant reduction in mucosal cytokine levels, specifically, IL-1b and IL-8[71]. Beneficial effects of nicotine in active UC may be associated with a decrease in IL-8 expression. In rats, DNBS-induced colonic damage was improved in passive-smoking rats involving changes in colonic cytokine levels[72]. The increase

of leukotriene B4, TNF-α, IL-1β, and IL-6 levels was alleviated. In contrast, the deprivation of IL-10 during UC was preserved. The exact meaning of these changes in the cytokine balance still remains poorly understood.

Smoking has a deleterious effect on phagocyte function, decreasing their bactericidal and bacteriostatic activity[73]. Chronic exposure of rats to nicotine inhibits the antibody-forming cell response, impairs the antigen mediated signaling in T-cells and induces T-cell anergy[74].

Smokers have a greater capacity for the generation of free oxygen radicals, with reduced antioxidant capacity[75]. This fact correlates with the harmful effect of smoking on patients with CD but does not offer an explanation for the beneficial effects on UC patients.

Other effects of nicotine or smoking on the intestine include hypoperfusion of the rectum and of acutely damaged colonic tissue[76], the alteration of gut motility, the reduction of smooth muscle tone and contractility (modulated by nitric oxide)[77], altered permeability[78], and alterations in the microcirculation[79]. Patients with IBD have an increased intestinal permeability[80]. Smoking was found to decrease the intestinal permeability in healthy control subjects[81]. However, no such observation could be made in smokers with UC compared with nonsmokers with UC[82], refuting the notion that the protective effect of smoking on UC is due to the moderation of an increased intestinal permeability.

SMOKING CESSATION

Based on all the available data, it is clear that smoking cessation in a patient with UC could exacerbate its symptoms as well the disease activity. For this reason, in order to decide whether we should recommend that UC patients quit smoking, we need to balance the decision with the patient.

First and foremost, the patient has to be aware that active cigarette smoking causes a broad spectrum of diseases; it is a major cause of vascular disease, cancer and chronic obstructive pulmonary disease. In addition to these, cigarette smoking also causes other respiratory symptoms, adversely affects reproductive outcomes and is a cause of diminished health status. Furthermore, exposure to second-hand smoke is an established cause of coronary heart disease and lung cancer, as well as a host of other adverse health effects.

Secondly, patients should be given truthful information; it is important to explain to them that there is the possibility of disease exacerbation (flares, hospitalization or the need for oral steroids) following cessation of smoking. We should also explain that the risk of colectomy in the short term appears not to be increased in the case of quitting smoking.

Additionally, patients should be given other important information about the effects of smoking on the course of UC. Patients with UC had a significantly decreased risk of pulmonary cancer, which may primarily be explained by the smoking habits of the patients with UC[83]. Patients should also be informed about the influence of smoking on the risk of colon cancer (CRC). It is well accepted that UC is associated with an increased risk of colon cancer. A recent meta-analysis of 36 studies showed that current smokers had a significantly increased risk of CRC incidence[84].

Finally, the patient should be aware that nowadays there is an increasing number of treatments that can be used to control inflammation in the case of disease exacerbation after smoking cessation, such as immunosuppressants, leukocyte apheresis or anti-TNF-α products[85].

Based on all the deleterious health effects combined with the substantial prevalence, cigarette smoking represents a major worldwide cause of death. In patients with UC, the risks of smoking far outweigh any possible benefit, so physicians should advise, encourage and assist UC patients who smoke to quit.

CONCLUSION

(1) Smoking is protective in UC, with a lower incidence of disease in smokers; (2) Current smoking protects against UC and, after onset of the disease, improves its course, reducing the need for colectomy; (3) Ex-smokers have an increased risk of developing an unfavorable clinical course; (4) PSC is observed almost exclusively in nonsmokers; (5) Nicotine is efficacious for active UC, but in most studies, side-effects (nausea, headache, dermatitis) were frequent and tended to overcome the clinical benefit; (6) The place of nicotine in the algorithm of treatment of UC patients is unclear, although it may be useful in selected cases, particularly in recent ex-smokers with moderate but refractory attacks of UC; and (7) Since smoking is associated with several additional deleterious effects (e.g. cardiovascular, lung cancer risk), gastroenterologists should encourage both UC and CD patients to quit smoking. Before stopping, UC patients should be informed about the potential risk of an increase in disease activity, without a higher risk for surgery.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2748

World J Gastroenterol 2011 June 14; 17(22): 2748-2773
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

miRNA studies in *in vitro* and *in vivo* activated hepatic stellate cells

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Received: May 19, 2010 Revised: September 14, 2010
Accepted: September 21, 2010
Published online: June 14, 2011

Abstract

AIM: To understand which and how different miRNAs are implicated in the process of hepatic stellate cell (HSC) activation.

METHODS: We used microarrays to examine the differential expression of miRNAs during *in vitro* activation of primary HSCs (pHSCs). The transcriptome changes upon stable transfection of rno-miR-146a into an HSC cell line were studied using cDNA microarrays. Selected differentially regulated miRNAs were investigated by quantitative real-time polymerase chain reaction during *in vivo* HSC activation. The effect of miRNA mimics and inhibitor on the *in vitro* activation of pHSCs was also evaluated.

RESULTS: We found that 16 miRNAs were upregulated and 26 were downregulated significantly in 10-d *in vitro* activated pHSCs in comparison to quiescent pHSCs. Overexpression of rno-miR-146a was characterized by marked upregulation of tissue inhibitor of metalloproteinase-3, which is implicated in the regulation of tumor necrosis factor-α activity. Differences in the regulation of selected miRNAs were observed comparing *in vitro* and *in vivo* HSC activation. Treatment with miR-26a and 29a mimics, and miR-214 inhibitor during *in vitro* activation of pHSCs induced significant downregulation of collagen type I transcription.

CONCLUSION: Our results emphasize the different regulation of miRNAs in *in vitro* and *in vivo* activated pHSCs. We also showed that miR-26a, 29a and 214 are involved in the regulation of collagen type I mRNA.

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Key words: Hepatic stellate cells; miRNA; miR-146a; Nuclear factor-κB

Peer reviewers: Dr. Katja Breitkopf, Department of Medicine II, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany; Richard A Rippe, Professor of Medicine, Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina, Chapel Hill, NC 27599-7032, United States

Maubach G, Lim MCC, Chen J, Yang H, Zhuo L. miRNA studies in *in vitro* and *in vivo* activated hepatic stellate cells. *World J Gastroenterol* 2011; 17(22): 2748-2773 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2748.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2748

INTRODUCTION

Liver fibrosis, characterized by an overproduction of extracellular matrix (ECM), is a common outcome of

different chronic liver diseases[1]. Hepatic stellate cells (HSCs) are one of the major cell types responsible for the production of ECM molecules like collagens, laminin, proteoglycans and fibronectin[2]. The production of different ECM molecules is increased upon transdifferentiation (activation) of HSCs from a quiescent to an activated myofibroblast-like state[3,4]. Consequently, the regulation of the complex process of HSC activation is of great interest to the research community. Understanding this process should lead to the discovery of therapeutic strategies for liver fibrosis. Due to the complexity of the activation of HSCs, the number of regulatory steps is expected to be overwhelming[5], and requires addressing many different targets at the same time, either with different compounds or with one compound that is able to work on many different targets.

miRNAs are small approximately 23-nt non-coding RNAs, which are able to regulate hundreds of different proteins. The versatility of miRNAs is attributed to the imperfect binding (seed region) to the 3'-UTR of mRNAs, which results in, contrary to siRNA, many binding partners. The regulation by miRNAs is also different to siRNAs because it leads to a translational repression and/or mRNA destabilization[6,7]. That miRNAs fulfill regulatory functions has been established by their involvement in many different processes and diseases[8,9]. Therefore, it is tempting to use these molecules in order to treat liver fibrosis; a condition that is caused by a deregulation of biological processes. To succeed in this attempt, we need to identify the miRNAs, which are differentially regulated in the normal and diseased liver, and more specifically in the HSCs; one cell type that is responsible for the fibrotic process.

The purpose of this study was to identify differentially regulated miRNAs in *in vitro* activated HSCs, in order to study them in an *in vivo* animal model, and finally, to determine their role in the activation process.

MATERIALS AND METHODS

Isolation of rat primary HSCs and cell culture conditions

Wistar rats were used to isolate primary HSCs (pHSCs) according to a published pronase/collagenase *in situ* perfusion protocol[10]. The isolation protocol was approved by the Institutional Animal Care and Use Committee under #080389. For *in vitro* activation, the cells were seeded into 75-cm^2 culture flasks and harvested after 3, 5, 7 or 10 d. Primary cells and the HSC-2 cell line were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in a 5% CO_2 humidified incubator.

HSC-2 is a spontaneous immortalized cell line derived from the pHSCs of a male Wistar rat. The primary cells were passaged several times before clonal selection by limiting dilution[11].

The purity of pHSCs from rats on normal and choline-deficient ethionine supplemented (CDE) diet was assessed using vitamin A autofluorescence or real-time polymerase chain reaction (PCR), respectively (Figure 1A). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

In vivo activation of rat HSCs

Six- to eight-week-old male Wistar rats were fed the CDE diet (CDE model) (MP Biomedicals, Solon, OH, USA, #0296021410) for 4 wk (Figure 2). Livers were isolated, perfused with PBS and fixed in neutralized formalin (paraffin embedding) or *in vivo* activated pHSCs were isolated.

Isolation of miRNA for microarray and analysis

miRNA was extracted from quiescent (freshly isolated) and 10-d *in vitro*-activated pHSCs using the PureLink purification kit (K1570-01; Invitrogen). The miRNA microarray (NCode Multi-Species miRNA microarray V2) was performed according to the manufacturer's manual (MIRLS-20; Invitrogen). For each experiment, a dye swap was performed. The arrays were scanned using a GenePix 4200AL array scanner. The raw datasets were deposited under #GSE19463 at the Gene Expression Omnibus (GEO) repository[12]. For two-color miRNA arrays, averaging of dye-swapped arrays was performed to minimize the dye effects prior to normalization using the Cross-Correlation method[13]. The targets of differentially regulated miRNAs (Table 1) were predicted by three different methods, TargetScan 5.1[14], mirBASE target[15], and miRNA Viewer[16] using default parameters. Targets predicted by at least two tools were selected and grouped into upregulated and downregulated miRNAs, respectively. These two groups of targets were subjected to pathway analysis using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA). A ratio was calculated whereby the number of predicted targets in a given pathway was divided by the total number of molecules in that pathway. The Fisher's exact test was used by the software to calculate a *P* value. This *P* value represented the probability that the association between the predicted targets and the pathway could not be explained by chance alone. The *P* value cutoff was set at $P \leqslant 0.001$. The x axis was the negative logarithm of *P* value with a base of 10 ($-\log_{10} P$ value).

Real-time PCR

The verification of the microarray data and subsequent miRNA assessments were performed for let-7b, let-7c, miR-16, 26a, 29a, 31, 125b, 143, 146a, 150 and 214 by using the respective Taqman MicroRNA assays (P/N 4427975, Applied Biosystems, Foster City, CA, USA). The U6 snRNA assay (ID 001973) served as a normalization control. Total RNA was isolated using the NucleoSpin RNAII kit (Macherey-Nagel, Germany). Total RNA and miRNA were isolated using the same kit but with a small modification. Briefly, the cell lysate was adjusted to contain 35% ethanol and passed through the RNAII column to bind the total RNA. The ethanol concentration of the flow through was then adjusted to > 70% and passed through the same column in order to bind the miRNA.

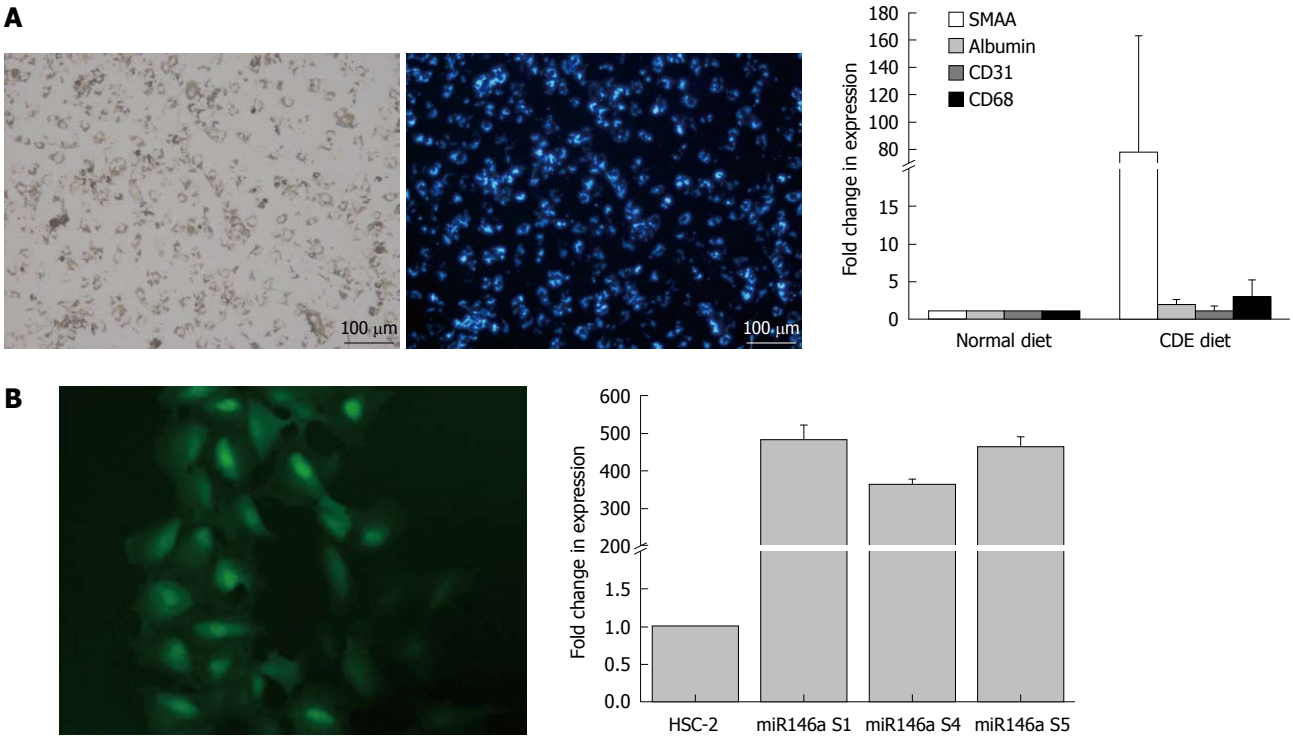

Figure 1 Primary hepatic stellate cells and over-expression of miR-146a in hepatic stellate cell-2 cell line. A: Bright-field image of 1 d cultivated primary hepatic stellate cells and the corresponding vitamin A autofluorescence image are shown. Scale bar represents 100 μm. Real-time polymerase chain reaction (PCR) for *in vivo* activated hepatic stellate cells from rats on normal (n = 2) and choline-deficient ethionine supplemented (CDE) diet (n = 4). The mean ± SE for each diet model is shown; B: A representative image for the over-expression of miR-146a as visualized by the reporter GFP is shown. Real-time PCR for three independent clones confirmed the expression of miR-146a. The data represent the mean ± SE of triplicate reactions. SMAA: Smooth muscle α-actin.

The Cells-to-Ct kit (Invitrogen, P/N 4391848) was used for some experiments to quantify the miRNA expression with the respective miRNA assays. The reverse transcription and real-time PCR were performed according to the assays protocol using the ABI 7500 Fast Real Time PCR System (Applied Biosystems). Taqman assays used were smooth muscle α-actin (SMAA) (Rn01759928_g1), Col1a1 (Rn01463849_g1), interleukin (IL)-6 (Rn00561420_m1), cyclooxygenase-2 (Cox-2) (Rn00568225_m1), RelA (Rn01502266_m1), CD31 (Rn01467259_m1), Albumin (Rn01413833_m1), CD68 (Rn01495643_g1) and tissue inhibitor of metalloproteinase (TIMP)-3 (Rn00441826_m1).

Nuclear factor-κB siRNA transfection

HSC-2 cells were seeded at a density of 10^6 per 100 mm cell culture dish and incubated at 37℃. The siRNA was mixed at a final concentration of 10 nmol/L with 1 mL DMEM without serum and 120 μL HiPerfect transfection reagent (Qiagen, Germany) and incubated for 10 min. The mixture was added drop-wise to the cells and incubated for 48 h. For the mock control, only the HiPerfect reagent was used. The ON-Targetplus nuclear factor (NF)-κB siRNAs used were J-080033-11 and J-080033-12 (Dharmacon, Lafayette, CO, USA). These conditions were tested for transfection efficiency using FITC-labeled siRNA and FACS analysis.

Overexpression of miR-146a in an HSC cell line

The vector was constructed by amplification of a 487-bp fragment containing the rno-miR-146a from rat genomic DNA using the following primer pair: sense 5'-*AAGCTT*GCCACCAGTCCCATCCTTCACC-3' (*Hin*dIII), anti-sense 5'-*GGATCC*TTCCTCTGTGCTGGGATTACAGGGTG-3' (*Bam*HI). After sub-cloning, the rno-miR-146a was excised using *Bam*HI/*Eco*RV and cloned into pcDNA6.2/GW EmGFP-miR (Invitrogen). The HSC-2 cells were stably transfected with the construct using Lipofectamine 2000 (Invitrogen) and selected in cell culture medium supplemented with 10 μg/mL Blasticidin. The clonal selection was achieved using FACS.

Gene expression array and analysis

Total RNA from HSC-2 cells overexpressing miR-146a and control cells (two different passages) were used to study the transcriptome changes using the GeneChip Rat Genome 230 2.0 (Affymetrix, USA). The preparation of the samples was performed according to the technical manual P/N 702232 Rev. 3 (Affymetrix) using one-cycle cDNA and target labeling. The chips were scanned using a Genechip Scanner 3000 (Affymetrix). The raw datasets were deposited under #GSE19463 at the GEO repository[12].

The microarray probe set data was summarized using the Robust Multi-Array Average expression measure method, and pre-processed to correct unreliable (small) intensities for each array. The pre-processed data were then normalized using the Cross-Correlation method[13]. For

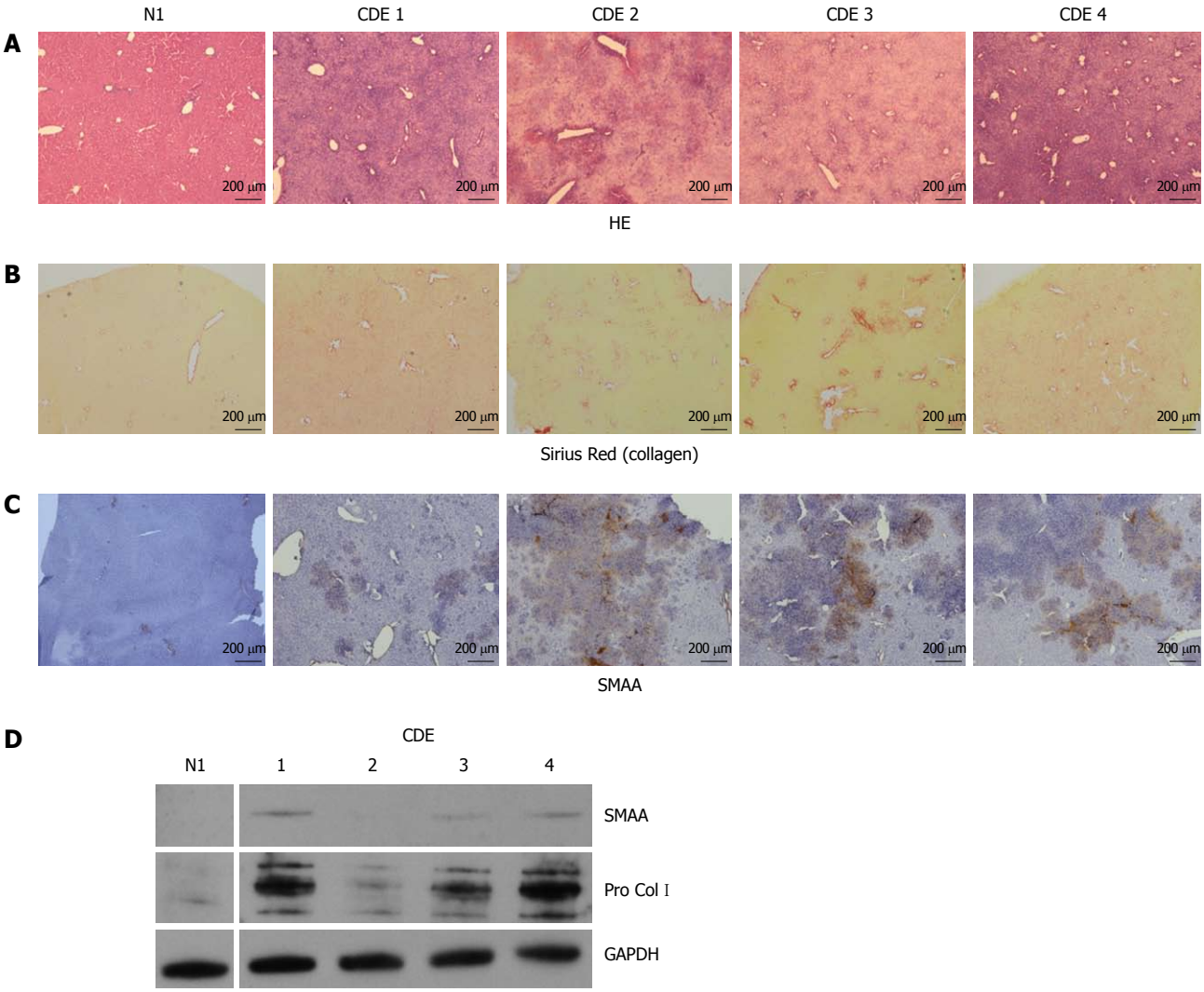

Figure 2 Histological and immunohistochemical analysis of livers from rats receiving choline-deficient ethionine supplemented diet for 4 wk. A: HE staining shows the structural changes between control and choline-deficient ethionine supplemented (CDE) diet livers. No severe steatosis is observed; B: The Sirius Red staining depicts the deposition of collagen around the portal area and the whole liver; C: The increase in smooth muscle α-actin (SMAA) staining reflects the increasing number of myofibroblasts seen in patches throughout the liver. Scale bar represents 200 μm; D: The Western blotting data confirm the increase in SMAA and Col I.

each gene, a fold change value was calculated for samples *vs* control. Differentially expressed genes (DEGs) were selected based on the criterion of fold change > 2. The *P* values of DEGs were obtained using one-tailed Student's *t* test. Pathway analysis was carried out on the DEGs using Ingenuity Pathway Analysis (Ingenuity Systems).

Transfection of miRNA mimics and hairpin-inhibitor

Cells were seeded at 20 000 per well in 48-well plates 24 h prior to transfection. The miRNA mimics or hairpin-inhibitor were added at the required final concentration (miR-26a, 146a, controls and quadruple transfection: 50 nmol/L each; miR-29a and 214: 200 nmol/L each) to 750 μL DMEM without serum, followed by 10 μL Hi-Perfect transfection reagent. The mixture was incubated for 10 min. The medium from each well was aspirated and replaced by 250 μL of the mixture. The transfection was performed in triplicate. Controls were either Hi-Perfect reagent only (mock) or control miRNAs for the mimic and/or inhibitor.

SDS-PAGE and Western blotting

Cells were lysed in ProteoJet lysis buffer (#K0301; Fermentas, Glen Burnie, MD, USA) and the protein concentration was estimated using the BCA method (Thermo Scientific, USA). The samples were separated in 4%-12% Bis-Tris NuPage gels (Invitrogen) and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature using 5% non-fat milk in TBS-Tween (TBS-T). The primary antibodies were applied in the following dilutions: interleukin receptor associated kinase 1 (IRAK1) (sc-7883; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) 1:400; tumor necrosis factor receptor associated factor 6 (TRAF6) (sc-7221; Santa Cruz Biotechnology) 1:400; IκBα (#4814; Cell Signaling, Danvers, MA, USA) 1:1000; pIκBα (#2859; Cell Signaling) 1:750; Cox-2 (sc-1747; Santa Cruz Biotechnology) 1:5000; and β-actin (ab-8227; Abcam, Cambridge,

Table 1 Differentially regulated miRNAs as identified by miRNA microarray

miRNA name	Fold change	*P* value
Upregulated compared to day 0		
rno-let-7b	4.70	0.0242
rno-let-7c	3.75	0.0236
rno-let-7e	2.77	0.0340
rno-miR-125b	11.98	0.0113
rno-miR-132	1.97	0.0184
rno-miR-143	17.05	0.0014
rno-miR-145	2.29	0.0483
rno-miR-152	3.01	0.0255
rno-miR-199a	3.46	0.0415
rno-miR-21	5.73	0.0142
rno-miR-210	2.34	0.0186
rno-miR-214	18.44	0.0011
rno-miR-22	3.11	0.0392
rno-miR-221	10.09	0.0007
rno-miR-222	2.67	0.0317
rno-miR-31	8.91	0.0013
Downregulated compared to day 0		
rno-let-7f	-2.17	0.0327
rno-miR-10a	-3.53	0.0417
rno-miR-122a	-349.63	0.00002
rno-miR-125a	-2.36	0.0474
rno-miR-126	-170.32	0.0003
rno-miR-146a	-16.83	0.0352
rno-miR-150	-9.81	0.0325
rno-miR-151*	-3.72	0.0345
rno-miR-16[1]	-4.38	0.0366
rno-miR-181a[1]	-4.50	0.0346
rno-miR-192	-6.08	0.0206
rno-miR-194	-6.08	0.0168
rno-miR-195	-14.50	0.0130
rno-miR-207	-1.93	0.0449
rno-miR-26a	-5.17	0.0163
rno-miR-26b	-4.81	0.0300
rno-miR-296	-1.93	0.0292
rno-miR-29a[1]	-2.38	0.0644
rno-miR-30a-5p	-5.35	0.0327
rno-miR-30b	-10.51	0.0075
rno-miR-30c	-9.69	0.0138
rno-miR-30d	-8.68	0.0093
rno-miR-335	-3.74	0.0500
rno-miR-422b[1]	-8.49	0.0455
rno-miR-483	-2.49	0.0451
rno-miR-99a	-2.97	0.0383

[1]Data from two experiments.

UK) 1:5000. After three washes in TBS-T, the appropriate HRP-conjugated secondary antibody was given at 1:2000 dilution in blocking solution. After three washes in TBS-T, the membrane was developed using the chemiluminiscence substrate (Millipore, Billerica, MA, USA). Primary and secondary antibodies were incubated at 4℃ overnight and 1 h at room temperature, respectively.

Electrophoretic mobility shift assay

Nuclear protein extract from rno-miR-146a-overexpressing clones was obtained using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). The electrophoretic mobility shift assay (EMSA) was performed using the NF-κB(I) EMSA kit according to its protocol (AY1030; Panomics, USA), as described previously[17]. The samples were separated in a 6% non-denaturing polyacrylamide gel (Invitrogen) and transferred to a nylon membrane.

Immunohistochemistry and staining of liver sections

Slides were de-paraffinized and the antigen retrieved by heat exposure in the Target Retrieval Solution pH 9 (S2367; Dako, Glostrup, DK) using a 2100-Retriever retrieval steamer for 45 min. The endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 min. Protein was blocked in 10% normal goat serum in PBS for 20 min. The slides were incubated with mouse anti-human SMAA (M0851; Dako) at 1:100 dilution for 1 h, washed and incubated with an anti-mouse HRP-conjugated antibody (K4001; Dako) for 30 min, and developed with DAB (K3468; Dako). All incubations were carried out at room temperature. Nuclei were counter stained with hematoxylin. Hematoxylin and eosin and Sirius Red staining was performed according to standard protocols on paraffin sections. Bright-field images were taken with the LEICA RMB-DM epifluorescence microscope (LEICA, Germany).

Statistics

All quantitative data were presented as mean ± SE. Experimental data were analyzed using the two-tailed Student's *t* test assuming equal variances. $P \leqslant 0.05$ was considered significant. The time-dependent changes during *in vitro* HSC activation were tested for significance at the 0.05 level using one-way ANOVA and Bonferroni's *post-hoc* test. The array data were normalized and analyzed as described in the respective sections above.

RESULTS

Identification of differentially regulated miRNAs in in vitro activated pHSCs and comparison to in vivo activated pHSCs

In 10-d *in vitro* activated pHSCs, 16 miRNAs were upregulated and 26 were downregulated significantly in comparison to quiescent pHSCs (Table 1). We included miR-29a, although the *P* value was above the threshold of 0.05, for further studies because of its predicted targets, which consisted of a number of collagens. The microarray data were confirmed for a number of chosen miRNAs (let-7b, 7c, miR-16, 26a, 29a, 31, 125b, 143, 146a, 150 and 214) using real-time PCR in three additional experiments (Figure 3A). Using isolated *in vivo* activated pHSCs from rats on CDE diet, we found that only miRNAs let-7b, 7c, miR-31, 143 and 214 showed the same regulation as observed for the *in vitro* activated pHSCs (Figure 3B).

Pathway analysis for differentially regulated miRNAs in in vitro activated pHSCs

We performed a pathway analysis using the predicted targets of the differentially regulated miRNAs. The enrich-

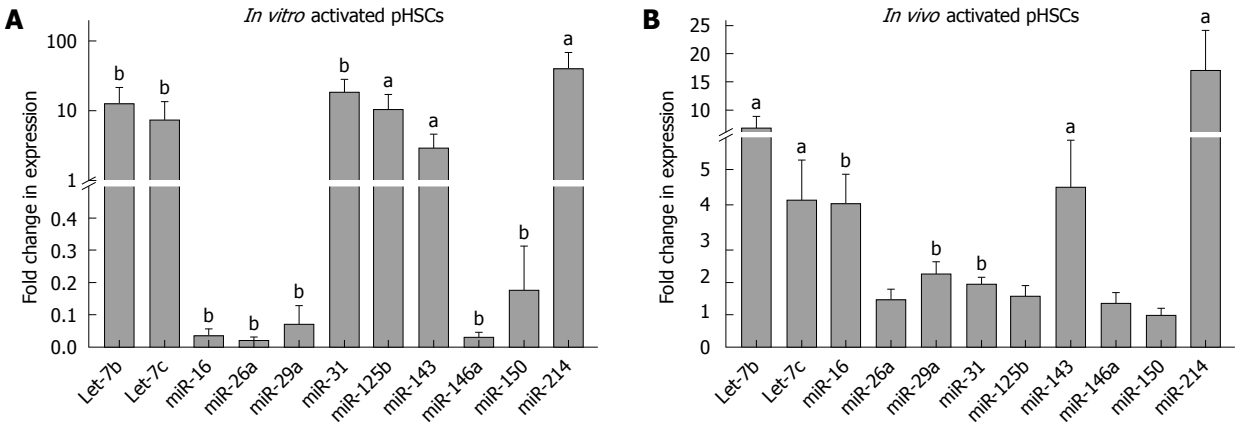

Figure 3 Verification of microarray data by real-time polymerase chain reaction of 11 differentially regulated miRNAs and their regulation upon *in vivo* activation of hepatic stellate cells. A: The graph depicts the changes in the miRNA expression of 11 miRNAs detected by real-time polymerase chain reaction, comparing quiescent with 10-d culture activated primary hepatic stellate cells (pHSCs). The data represent the mean ± SE of three independent experiments ($^{a}P \leqslant 0.05$, $^{b}P \leqslant 0.005$); B: The graph illustrates the relative expression levels of miRNAs in isolated *in vivo* activated pHSCs ($n = 4$, choline-deficient ethionine supplemented diet) compared to normal diet ($n = 2$) ($^{a}P \leqslant 0.05$, $^{b}P \leqslant 0.005$).

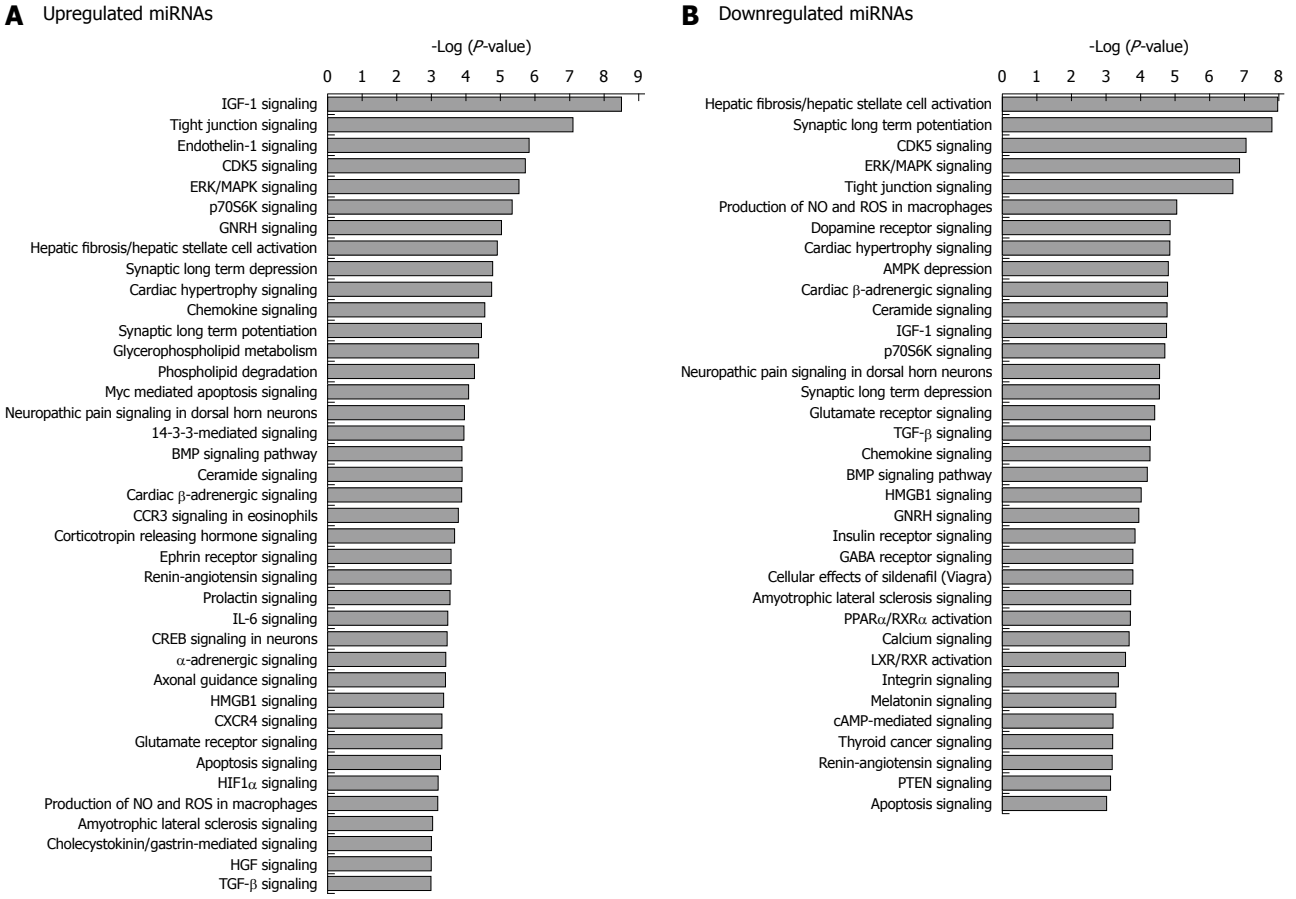

Figure 4 Predicted targets of all differentially regulated miRNAs during *in vitro* activation of primary hepatic stellate cells (Table 1) were analyzed. The two charts represent the enrichment of molecules in affected pathways for the upregulated (A) and downregulated (B) miRNAs. Only pathways with $P \leqslant 0.001$ are shown. IGF-1: Insulin-like growth factor-1; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; BMP: Bone morphogenetic protein; IL: Interleukin; ROS: Reactive oxygen species; AMPK: AMP activated protein kinase; TGF: Transforming growth factor; PTEN: Phosphatase and tensin homolog; LXR: Liver X receptor; RXR: Retinoid X receptor; PPAR: Peroxisome proliferator-activated receptor; HGF: Hepatocyte growth factor.

ment of genes in single pathways is shown as the -log of the P value ($P \leqslant 0.001$). Signaling pathways which were affected include endothelin-1, cyclin-dependent kinase 5, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), $p70^{S6K}$, chemokine, bone morphogenetic protein (BMP) and IL-6 for the upregulated miRNAs, as well as ERK/MAPK, production of NO and reactive oxygen species (ROS), AMP

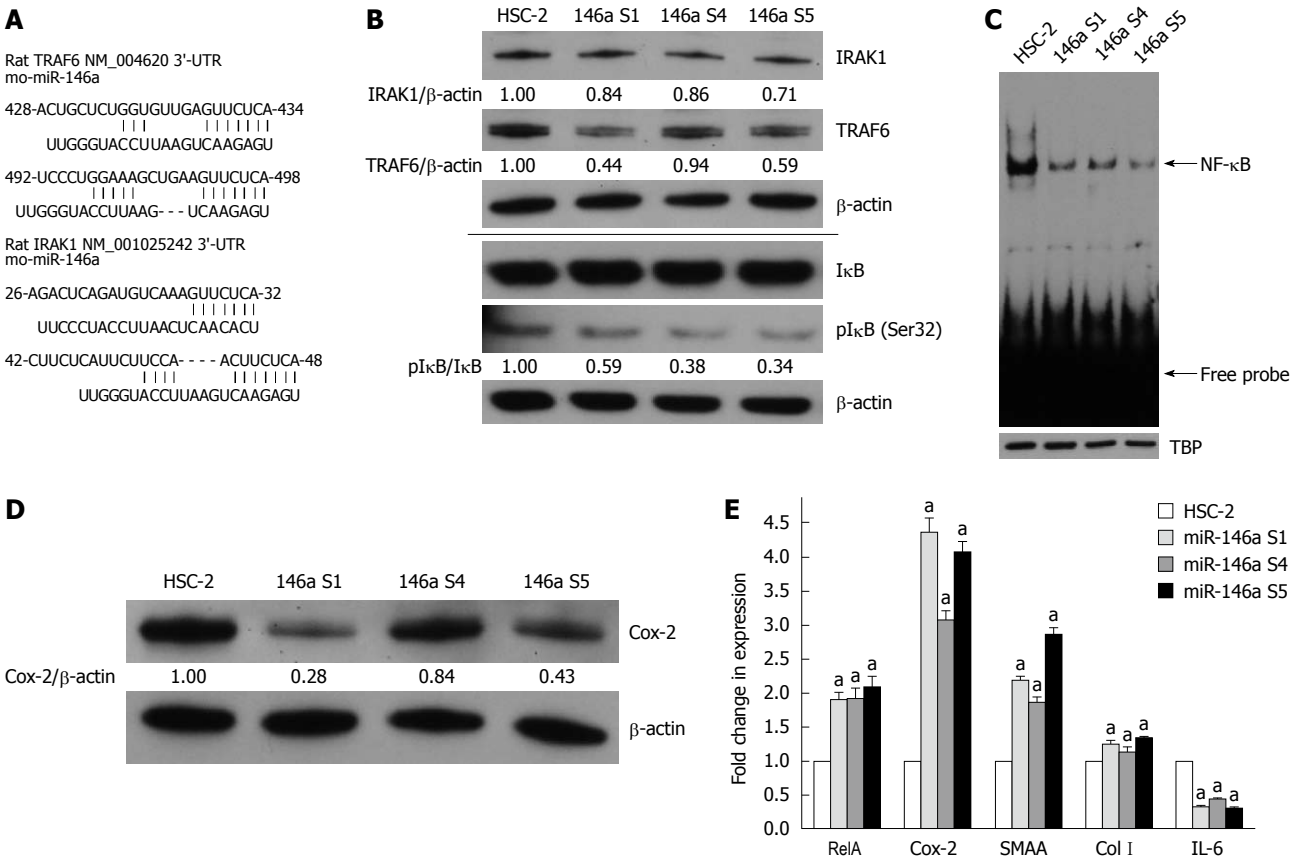

Figure 5 Changes during overexpression of rno-miR-146a in the hepatic stellate cell-2 cell line. A: Depicted are two putative binding sites of miR-146a to the 3'-UTR of rat tumor necrosis factor receptor associated factor 6 (TRAF6) and rat interleukin receptor associated kinase 1 (IRAK1), respectively; B: The Western blotting data show the suppression of TRAF6 and IRAK1, resulting in the decreased phosphorylation of IκB, although the expression of IκB remained unchanged. A representative Western blotting for two independent experiments is shown; C: Electrophoretic mobility shift assay (EMSA) results demonstrated a decrease in nuclear factor (NF)-κB DNA binding activity due to the overexpression of miR-146a. TATA binding protein (TBP) showed equal loading of samples. A representative EMSA experiment is shown out of three independent samples for each clone; D: miR-146a-overexpressing clones showed a reduced level of cyclooxygenase-2 (Cox-2) protein. The Western blotting shown is representative of two independent experiments; E: The relative fold change in mRNA expression between hepatic stellate cell (HSC)-2 and miR-146a-overexpressing HSC-2 cells for five different targets [NF-κB (RelA), Cox-2, smooth muscle α-actin, Col Ⅰ, interleukin-6] is shown. The data represent the mean ± SE of two independent experiments ($^aP \leq 0.005$).

activated protein kinase (AMPK), transforming growth factor (TGF)-β, integrin, cAMP-mediated signaling and phosphatase and tensin homolog (PTEN) for the down-regulated miRNAs (Figure 4A and B).

Overexpression of miR-146a in HSC-2 and transcriptome analysis

Studies have shown that miR-146a is linked to inflammation and the NF-κB pathway through the two known targets IRAK1 and TRAF6[18,19]. In order to study the function of miR-146a in activated HSCs *in vitro*, we overexpressed this miRNA in a HSC cell line HSC-2[11]. The level of miR-146a in this cell line is very low, making it suitable for the overexpression. The expression of the reporter green fluorescent protein and the real-time PCR validation of the miR-146a expression (Figure 1B) provided evidence for the successful overexpression of miR-146a in three different clones (S1, S4 and S5).

IRAK1 and TRAF6 are direct targets of miR-146a with two target sites for each mRNA (Figure 5A). We were able to show downregulation of these proteins in all three clones (Figure 5B). The functional consequence of this downregulation can be seen by suppression of the phosphorylation of IκB at Ser32 (Figure 5B). The reduced phosphorylation of IκB in turn should lead to the retention of NF-κB in the cytoplasm. Indeed, our EMSA illustrated that there was reduced nuclear binding activity of NF-κB to an NF-κB probe in all clones (Figure 5C). One of the genes regulated by NF-κB is Cox-2, which is functionally related to HSCs due to its pro-apoptotic effect on HSCs[20,21]. Therefore, we investigated the protein level of Cox-2 in the miR-146a-overexpressing clones, and found the expected downregulation (Figure 5D). Surprisingly, further investigation revealed that the mRNAs of NF-κB and Cox-2 were upregulated (Figure 5E). In contrast, we observed a significant downregulation of IL-6 mRNA, another target of NF-κB, in the clones S1, S4 and S5 (Figure 5E). We also found a significant upregulation of SMAA and collagen Ⅰ (Col Ⅰ) mRNAs, a HSC activation and a fibrotic marker, respectively (Figure 5E).

In order to establish a link between the regulation of

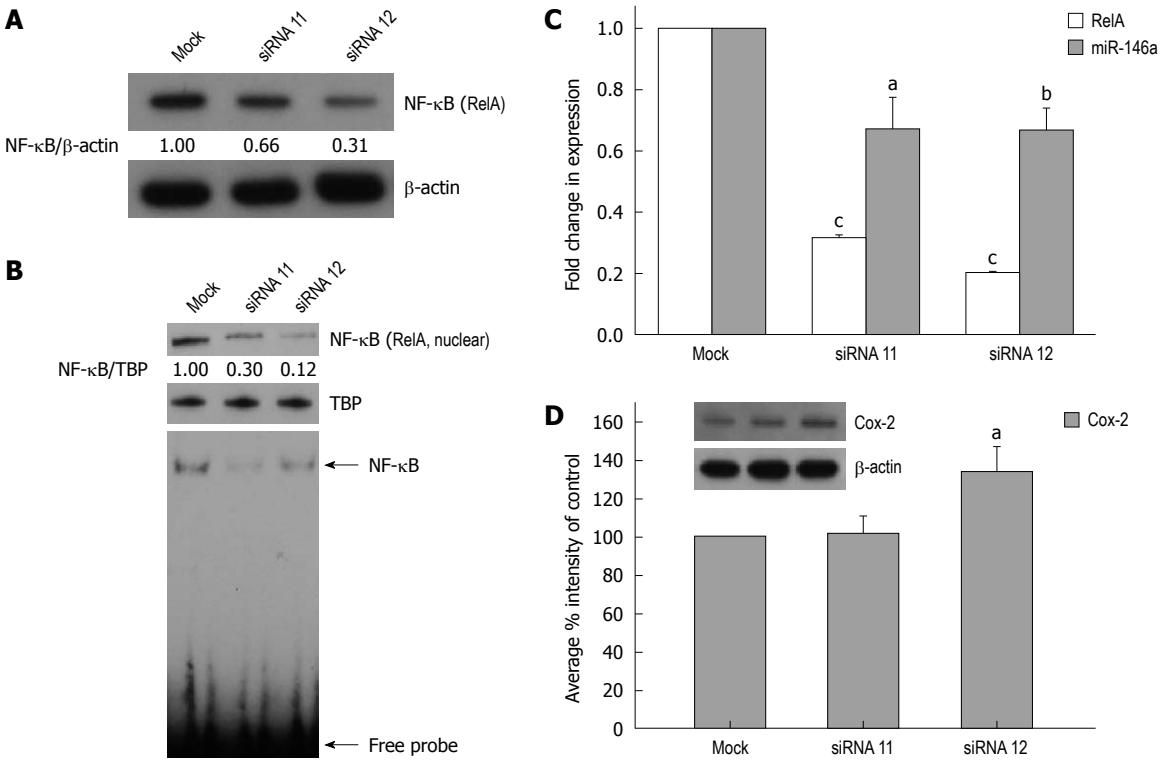

Figure 6 Regulation of miR-146a by nuclear factor-κB. A: Knock-down experiments using nuclear factor (NF)-κB siRNAs showed a reduced level of cellular NF-κB (RelA) protein; B: The nuclear level of NF-κB (RelA) was decreased and showed a diminished DNA binding activity. Depicted is a representative Western blotting and electrophoretic mobility shift assay from three independent experiments; C: Downregulation of NF-κB (RelA) mRNA due to NF-κB siRNA transfection was accompanied by a decrease in miR-146a after 24 h. The data represent the mean ± SE of two independent experiments ($^{a}P \leq 0.05$, $^{b}P \leq 0.01$, $^{c}P \leq 0.001$); D: Cyclooxygenase-2 (Cox-2) protein was upregulated after NF-κB siRNA transfection. Shown are a representative Western blotting and the densitometric analysis of six independent experiments ($^{a}P \leq 0.05$).

miR-146a and NF-κB activity, as proposed by Taganov *et al*[18], we transfected NF-κB siRNAs into HSC-2 cells. The efficiency of the transfection was shown by the downregulation of NF-κB in total cell lysates and nuclear extracts, which resulted in a decrease in NF-κB DNA binding activity (Figure 6A and B, respectively). We also found downregulation of miR-146a in NF-κB siRNA-transfected cells, thereby confirming a regulation of miR-146a by NF-κB in HSCs (Figure 6C). Surprisingly, we noticed an increase in the Cox-2 protein expression (Figure 6D), which implied a yet unclear involvement of miR-146a in the regulation of this enzyme.

The differences in the NF-κB-dependent regulation of Cox-2 and IL-6 have already hinted at the intricacy of the influence of the miR-146a overexpression has on the gene expression in activated HSCs. In order to get an overview of the transcriptome changes, we performed a gene expression analysis of the three miR-146a-overexpressing clones, and compared them with control cells using a cDNA microarray. The analysis yielded 485 up- and 309 downregulated transcripts (Supplementary Tables 1 and 2), which satisfied a P value ≤ 0.05 and at least twofold change. Among the upregulated genes were Lmcd1, CD81, FGF13, Col4a1, Cadherin 11 and BMP-4. The highly downregulated genes included Col15a1, MMP-2, Thy-1, IL-1RL1 and Cadherin 13.

We further analyzed the pathways which were significantly enriched, using a P value ≤ 0.05 as a threshold. Here, we observed enrichment for signaling pathways like integrin-linked kinase, hepatic fibrosis/HSC activation and caveolar-mediated endocytosis, calcium, cAMP-mediated signaling, integrin, endothelin-1 for the upregulated genes (Figure 7 A), and hepatic fibrosis/HSC activation, lipopolysaccharide (LPS)/IL-1-mediated inhibition of retinoid X receptor (RXR) function and nitrogen metabolism, and liver X receptor/RXR activation for the downregulated genes (Figure 7 B).

The most interesting finding was the robust upregulation of TIMP-3 mRNA (Supplementary Tables 1), verified by real-time PCR (Figure 8 A), which is an inhibitor of the tumor necrosis factor-α converting enzyme[22], and has been proposed as a tumor suppressor. Similarly, pHSCs treated with miR-146a mimic also showed induction of TIMP-3 mRNA (Figure 8 B).

Time-dependent expression of different miRNAs during in vitro activation of pHSCs

The fact that miR-146a was not downregulated in *in vivo* activated pHSCs (CDE diet) prompted us to study the time-dependent expression of this miRNA during the *in vitro* activation of pHSCs, together with miR-26a, 29a and 214. The expression of miR-146a was indeed downregulated at day 3 already, and recovered subsequently until day 10. Although the miR-146a level at day 10 was

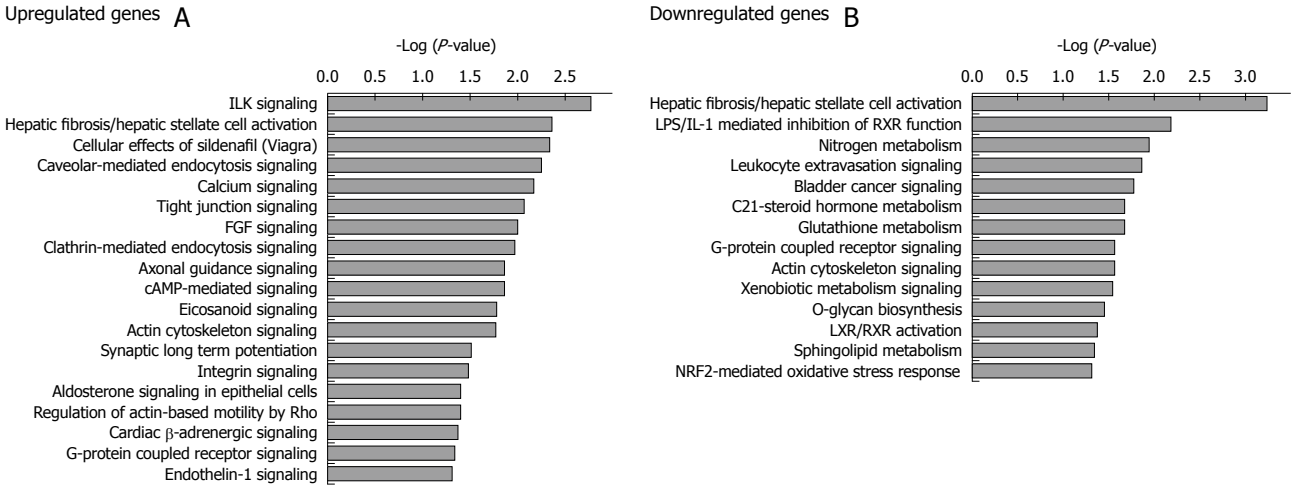

Figure 7 Pathway analysis for the differentially expressed genes of miR-146a over-expressing clones. The charts depict the pathways affected by the (A) or (B) of genes upon stable transfection of miR-146a into hepatic stellate cell-2 cells. Only pathways with $P \leqslant 0.05$ are shown. ILK: Integrin-linked kinase; FGF: Fibroblast growth factor; IL: Interleukin; LPS: Lipopolysaccharide; LXR: Liver X receptor; RXR: Retinoid X receptor.

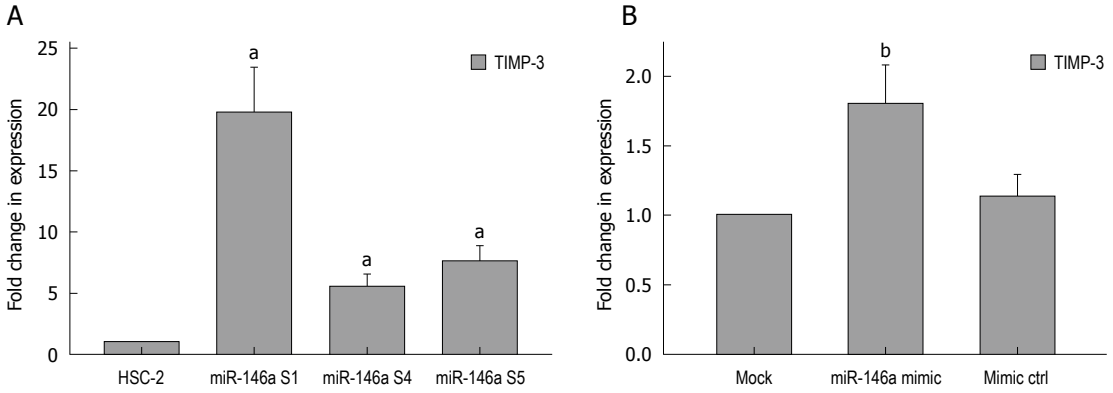

Figure 8 Relative expression of tissue inhibitor of metalloproteinase-3 mRNA in rno-miR-146a-overexpressing hepatic stellate cell-2 cells. A: The graph depicts the relative changes in tissue inhibitor of metalloproteinase (TIMP)-3 mRNA of three rno-miR-146a-overexpressing clones detected by real-time polymerase chain reaction (PCR). The data represent the mean ± SE of two different passages for each clone ($^aP \leqslant 0.005$); B: Primary hepatic stellate cells were treated with 50 nmol/L miR-146a mimic, and the expression of TIMP-3 mRNA was analyzed by real-time PCR and expressed as fold change relative to mock controls. The data represent the mean ± SE of three independent experiments ($^bP \leqslant 0.01$). HSC: Hepatic stellate cell.

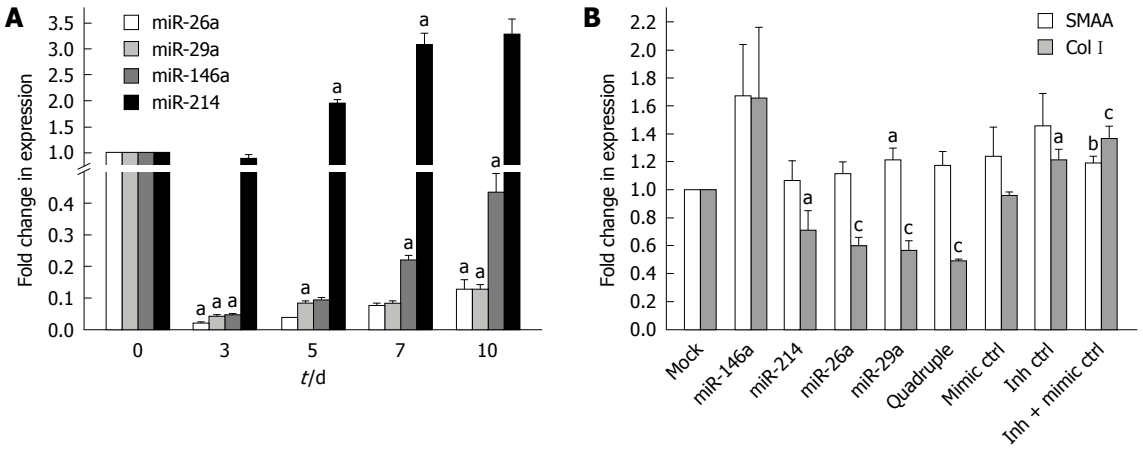

Figure 9 Time-dependent changes in the expression of different miRNAs during *in vitro* activation and miRNA mimic and inhibitor transfection of primary hepatic stellate cells. A: The relative changes in the expression level after 3, 5, 7 and 10 d of *in vitro* primary hepatic stellate cells (pHSCs) activation is shown for miR-146a, 26a, 29a and 214 ($^aP \leqslant 0.05$, one-way ANOVA). The data represent one of two independent experiments performed in triplicate; B: pHSCs were transfected with miR-146a, 26a, 29a mimics or miR-214 inhibitor or in combination. The control transfection consisted of control miRNAs for the mimic and/or inhibitor. The smooth muscle α-actin and Col I mRNA expression was analyzed as fold change relative to mock controls. The data represent the mean ± SE of two independent experiments, each performed in triplicate ($^aP \leqslant 0.05$, $^bP \leqslant 0.01$, $^cP \leqslant 0.001$). SMAA: Smooth muscle α-actin.

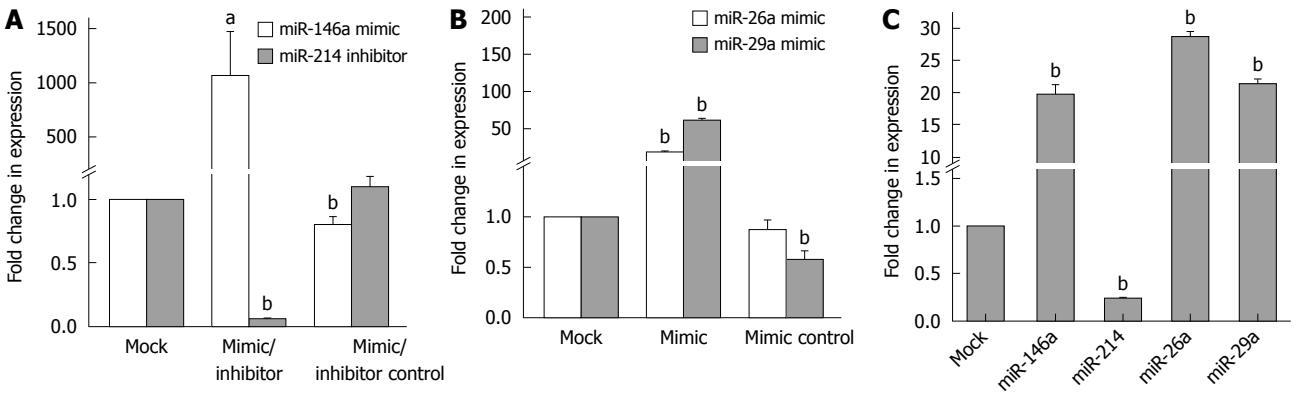

Figure 10 Transfection of primary hepatic stellate cells with different miRNA mimics and inhibitor. The miRNA expression was analyzed as fold change relative to mock transfected primary hepatic stellate cells (pHSCs). Shown are data for the transfection of miR-146a mimic and miR-214 inhibitor with respective control (A), for the transfection of miR-26a and miR-29a mimic with respective control (B) and for the quadruple transfection of miR-146a, miR-26a, miR-29a mimic and miR-214 inhibitor (C) ($^{a}P \leq 0.05$, $^{b}P \leq 0.005$).

still lower than that in quiescent pHSCs at day 0, there was still a 10-fold increase between day 3 and 10 (Figure 9A). In contrast, the expression of miR-26a and 29a did not change as dramatically from day 3 to day 10. We also noticed that miR-214 started to increase only from day 5 onwards (Figure 9A).

Regulation of SMAA and Col I transcripts in pHSCs by different miRNA mimics and inhibitor

In order to study the effect of different miRNA mimics or inhibitor on the *in vitro* activation process, we transfected 3-d *in vitro* activated pHSCs for 3 d with miR-146a, 26a, 29a mimics, miR-214 hairpin inhibitor or all combined. The impact on the HSC activation was followed using real-time PCR to study the changes on the mRNA levels of SMAA and Col I . The high efficiency of transfection was demonstrated by real-time PCR (Figure 10). We found moderate upregulation of the activation marker SMAA by miR-146a (Figure 9B); an observation seen also for the miR-146a-overexpressing clones (Figure 5E and cDNA microarray data). In fact, all cells transfected with the mimics, the inhibitor or combined showed an upwards trend for SMAA mRNA compared to the mock control, although the level did not always change significantly (Figure 9B). For the Col I expression, we noted again an increase caused by miR-146a mimic (not significant) and a decrease by miR-26a, 29a mimic and miR-214 inhibitor. The quadruple transfection led to a suppression of Col I mRNA (Figure 9B).

DISCUSSION

The aim of this study was to gain a deeper insight into the regulation of miRNAs during the activation process of pHSCs, as well as the influence of up- or downregulation of miRNAs on the gene expression and activation of HSCs.

The expression analysis of miRNAs between quiescent and *in vitro* activated pHSCs yielded a number of induced and suppressed miRNAs (Table 1), some of which (miR-143, 16, 122, 146a, 92b, 126) confirmed the findings of Guo *et al*[23]. On the other hand, there were some differences in the regulation of certain miRNAs (miR-328, 207), which could be attributed to the dynamic nature of miRNA regulation and the different use of quiescent pHSCs (day 0 *vs* day 2).

When evaluating the miRNAs expression profile of the *in vitro* and *in vivo* activated pHSCs, a clear distinction was seen in the expression of miR-16, 26a, 29a, 125b, 146a and 150 (compare Figure 3A and B); a phenomenon which could be explained by the distinct HSC activation process. This has been shown at the gene expression level by De Minicis *et al*[24]. The *in vivo* activation was performed over a period of 4 wk, whereas the *in vitro* activation was monitored over 10 d, which could also account for some differences in the miRNA expression, assuming a dynamic regulation.

On the other hand, we found that certain miRNAs (let-7b, 7c and miR-214) were regulated in the same way during *in vitro* and *in vivo* activation of pHSCs. It also became clear to us that miR-214 could be a potential candidate for a diagnostic approach, because this miRNA always shows robust upregulation.

Pathway analysis of the miRNA microarray data was performed to obtain information on signaling cascades involving predicted targets of the differentially regulated miRNAs in *in vitro* activated pHSCs (Figure 4A and B). NO and ROS are known to play a role in the activation process and apoptosis of HSCs[25,26]. The pathways for AMPK, ERK/MAPK, PTEN and TGF-β are also implicated in HSC activation[27-30]. We noticed that a number of pathways were present in the charts for both up- and downregulated miRNAs, which could denote the complexity of regulated targets by each single miRNA, and possibly a cooperative effect between up- and downregulated miRNAs.

A number of publications have shown that miR-146a is involved in inflammatory diseases, regulation of the immune response and NF-κB[19,31-33]. In the early events of liver fibrosis, the activation of HSCs is in part driven by

the hepatic inflammatory process, during which different cytokines are secreted by various liver cells, like Kupffer cells, endothelial cells and hepatocytes[34,35]. Involvement of NF-κB in HSC activation has also been shown in several research papers[36,37]. Therefore, we overexpressed miR-146a in an HSC cell line and observed changes consistent with the findings from Bhaumik *et al*[19]. The detected increase in the NF-κB transcript (Figure 5E) could be explained by a feedback mechanism to the reduced nuclear activity, which leads to the upregulation of the mRNA.

Cox-2 is inducible in activated HSCs by various stimuli and is thought to regulate proliferation[21]. Others have shown that the inhibition of this enzyme has a beneficial antifibrotic effect[20,38,39]. The seemingly discrepant findings of the protein (lower) and transcript (elevated) level for Cox-2 in the miR-146a-overexpressing HSCs (Figure 5D and E) hint at independent pathways for the regulation of Cox-2. These pathways have been shown for intestinal myofibroblasts[40] and during ischemic injury of ileal mucosa[41]. Lasa *et al*[42] and others have shown that the p38 MAPK signaling cascade is able to stabilize the Cox-2 mRNA[43], which could also explain an elevated transcript level. We also cannot exclude that other mechanisms could be involved in stabilizing the Cox-2 mRNA and/or a regulation of Cox-2 by other miRNAs like miR-26a or 143, which are also present in the cell line HSC-2 and for which Cox-2 is a predicted target.

In contrast, the IL-6 mRNA, another molecule regulated by NF-κB, was downregulated (Figure 5E). This observation implies that IL-6 regulation in HSCs is more tightly associated with NF-κB than that of Cox-2.

We were also interested to know whether downregulation of the NF-κB DNA binding activity triggered by miR-146a overexpression could facilitate a feedback loop in HSCs; a notion supported by the fact that the promoter region of miR-146a contains a number of NF-κB binding sites[18]. As expected, a reduction in the NF-κB DNA binding activity (Figure 6B) leads to a decrease in miR-146a (Figure 6C). The observed upregulation of Cox-2 protein (Figure 6D) was somewhat surprising and again substantiated the speculation that other pathways such as p38 MAPK, C-Jun N-terminal kinase and ERK could participate in the regulation of Cox-2 in HSCs[40,44,45].

The microarray analysis revealed that the transcriptome changes caused by miR-146a overexpression are complex and numerous pathways are affected (Figure 7, Supplementary Tables 1 and 2). We found that several DEGs coincided with data from earlier publications on HSC activation[24,46], suggesting that a number of genes affected by miR-146a overexpression are also involved in the activation process. Pathway analysis of the DEGs (Figure 7) confirms a link between miR-146a and inflammation (LPS/IL-1 mediated inhibition of RXR function, eicosanoid signaling, nitrogen metabolism and NRF2-mediated oxidative stress response pathways). That the miR-146a overexpression in HSC-2 cells leads to changes in the pathway called hepatic fibrosis/HSC activation emphasizes that these changes are specific for the HSCs. The upregulation of TIMP-3 (Supplementary Table 1 and Figure 8) again emphasizes the involvement of miR-146a in inflammatory processes and immunity, by linking it to the TNFα activity[47].

We noticed a robust downregulation of miR-146a during *in vitro*, but a missing regulation of miR-146a during *in vivo* activation of pHSCs (CDE diet). We hypothesized that there is a dynamic component in the regulation of miR-146a. We effectively found that there is a time-dependent regulation of miR-146a over 10 d of *in vitro* activation of pHSCs. From an *in vivo* perspective, it could be a possibility that miR-146a is decreased following the first insult to the liver, but reaches almost a normal level during the developing fibrosis, as seen for the *in vivo* activated pHSCs (CDE diet). The mechanism behind this miR-146a regulation is not clear, but the involvement of different transcription factors [NF-IL6, interferon regulatory factor (IRF 3/7)] binding to its promoter region is conceivable[18].

The dynamic nature of miRNA regulation during the *in vitro* activation of pHSCs could also partially explain the differences in the expression pattern of the miRNAs *in vitro* and *in vivo*. The dynamic nature of miRNA expression has been shown for the T-cell development[48], and it makes sense if we consider the multitude of effects a single miRNA can have due to the imperfect complementarity to its target sequence.

The *in vivo* targets of a miRNA treatment are pHSCs, therefore, we assessed the effects of several miRNAs mimics (miR-26a, 29a, 146a) and inhibitor (miR-214) on the activation state of pHSCs. The transfection with a combination of all mimics and inhibitor was performed so as to examine possible cooperative effects between different miRNAs, as a first step to understand the cooperativity of miRNA expression changes during HSC activation. The miR-26a, 29a mimics and miR-214 inhibitor showed a significant suppression of the Col I mRNA (Figure 9B). This is somewhat surprising because even though a number of collagens are predicted targets for miR-26a and 29a, none has a perfect binding site, which would explain regulation by mRNA degradation. Therefore, we conclude that the mechanism by which miR-26a, 29a and 214 downregulate the Col I mRNA is indirect, as also suggested by van Rooij *et al*[49] for miR-29a. The downregulation of Col I by the quadruple transfection shows some synergistic effect between the miRNAs.

Our findings showed the differential regulation of miRNAs in *in vitro* and *in vivo* activation of pHSCs, and particularly, the involvement of miR-26a, 29a and 214 in the regulation of Col I mRNA. Moreover, miR-146a overexpression or treatment with miR-146a mimic upregulates TIMP-3 mRNA, which suggests an association between miR-146a, TNFα activity and inflammation. In conclusion, our observations help build a global picture of the miRNA regulation during HSC activation *in vitro* and *in vivo*, and may have important implications when considering a therapeutic approach for treating liver fibrosis using miRNAs.

Supplementary Table 1 Upregulated genes in miR-146a-transfected hepatic stellate cell-2

Probe ID	Representative public ID	Gene symbol	Gene title	Log2	Fold change	*P*-value
1383164_at	AW524366			5.065854	33.494550	3.49E-05
1379902_at	BE108170			4.568453	23.726920	0.000154
1382211_at	AI602542			4.401705	21.137100	0.008197
1394456_at	AW525722			3.905818	14.988850	0.000414
1373740_at	AA851385			3.777603	13.714240	0.002694
1393437_at	AW142608			3.417765	10.686850	3.36E-05
1389579_at	BI284372			3.412511	10.648010	0.010102
1374065_at	BG378920			3.327195	10.036580	0.000137
1393018_at	AI071984			3.323743	10.012590	0.000201
1392105_at	AW527533			3.229460	9.379166	0.005625
1373062_at	BM388650			3.174909	9.031143	0.000330
1373776_at	AI406341			3.127483	8.739090	3.23E-06
1378457_at	AI179450			2.993031	7.961450	0.001547
1391428_at	AI639162			2.959079	7.776274	0.003728
1393314_at	BI289840			2.826721	7.094600	8.14E-05
1379382_at	AI144865			2.810339	7.014492	0.017550
1391481_at	BE104424			2.742723	6.693324	0.000235
1376435_at	BI303340			2.688229	6.445217	0.000280
1395327_at	AW522341			2.651476	6.283098	0.017329
1371506_at	AA891207			2.611623	6.111911	0.013546
1394833_at	BE120930			2.604706	6.082676	3.53E-06
1375230_at	AA800192			2.512936	5.707806	0.000375
1374811_at	AA858705			2.505232	5.677406	0.035302
1377934_at	BF387289			2.461691	5.508619	0.005853
1383240_at	BE110753			2.374072	5.184023	0.004602
1372921_at	AI073219			2.355729	5.118529	0.036703
1376800_at	AA892496			2.321059	4.996990	0.000124
1384137_at	AI030318			2.265411	4.807915	0.000711
1398597_at	AI044699			2.200111	4.595148	0.000220
1393782_at	BF396790			2.190070	4.563275	0.003901
1382330_at	BE116838			2.188725	4.559023	0.000179
1398657_at	AI045896			2.164482	4.483053	5.91E-05
1376617_at	BE107482			2.109346	4.314957	0.001578
1378111_at	AI576002			2.104523	4.300555	0.023000
1379936_at	AA875132			2.096645	4.277136	0.005892
1377946_at	BF420043			2.071766	4.204010	3.19E-05
1377675_at	AI177743			2.063136	4.178936	0.005112
1380940_at	BF402603			2.046820	4.131942	0.007683
1381335_at	BE349658			2.046725	4.131669	7.30E-05
1374971_at	AA818954			2.036171	4.101556	0.000603
1397781_at	BF414751			1.968385	3.913299	0.004705
1391841_at	BE103537			1.948096	3.858649	0.003749
1388546_at	AI013328			1.918544	3.780415	0.005524
1379444_at	BF283694			1.836166	3.570599	0.000940
1392627_x_at	BI282114			1.828818	3.552459	9.63E-05
1374432_at	BE118251			1.820423	3.531847	0.023594
1389239_at	BM384377			1.820056	3.530949	3.85E-06
1394578_at	BI299761			1.783117	3.441689	0.019487
1376734_at	BI279030			1.776063	3.424903	0.021384
1392820_at	BI285064			1.763119	3.394312	0.000366
1382212_at	AI385201			1.761883	3.391405	0.008303
1374273_at	BG665433			1.740316	3.341083	0.001848
1390471_at	BM383411			1.732112	3.322138	0.003308
1396009_at	BE108258			1.726446	3.309117	2.68E-05
1382294_at	AI576111			1.698452	3.245526	0.004164
1390459_at	BG670247			1.683832	3.212802	0.000503
1381577_at	AI170131			1.674706	3.192544	9.98E-05
1388720_at	BM390713			1.669188	3.180356	0.000841
1371394_x_at	BG664827			1.660846	3.162018	0.000385
1379719_at	AI408386			1.629839	3.094784	8.76E-06
1392876_at	BG375098			1.597297	3.025758	0.000425
1377881_at	AA997027			1.583295	2.996534	0.000249
1392924_at	BG371591			1.573984	2.977258	2.15E-05
1378152_at	AI170349			1.572157	2.973490	0.000818
1390987_at	AI406858			1.539611	2.907161	0.005124
1390300_at	BM383635			1.539317	2.906568	0.001988

1381996_at	BG666712	1.524793	2.877454	0.007289
1392893_a_at	AA926239	1.492644	2.814042	1.82E-05
1378462_at	BE107396	1.484439	2.798083	0.027693
1391028_at	AI511126	1.480597	2.790641	0.002716
1385978_at	AI072788	1.480070	2.789622	0.015570
1392813_at	AI548994	1.472777	2.775556	0.028907
1379903_at	AI059853	1.456459	2.744339	0.005294
1382291_at	AI454332	1.447251	2.726879	0.021889
1396539_at	BE119221	1.444627	2.721924	0.003250
1395381_at	BF542239	1.428428	2.691532	0.018331
1394709_at	AI406967	1.426754	2.688412	0.013879
1378780_at	BF410325	1.420940	2.677598	0.009832
1374172_at	AI010883	1.407256	2.652321	0.005074
1377551_at	BE118580	1.406921	2.651706	0.000613
1378172_at	AI008119	1.390207	2.621164	9.29E-05
1389744_at	AW527194	1.371756	2.587854	0.029149
1393728_at	AA964541	1.361978	2.570373	0.006640
1389284_at	BI275747	1.352525	2.553586	0.009308
1377309_at	AA963085	1.344539	2.539490	0.000217
1394012_at	BI303933	1.325072	2.505453	9.03E-06
1396886_at	BF387869	1.319644	2.496045	0.000671
1389172_at	AI179391	1.318725	2.494456	5.03E-06
1376011_at	AI411359	1.315430	2.488765	0.013276
1374171_at	AI170507	1.313956	2.486224	0.003317
1382802_x_at	AW920828	1.313169	2.484867	0.003200
1395211_s_at	BE118557	1.294366	2.452692	0.014857
1391727_at	BG662710	1.294339	2.452646	0.001351
1373079_at	BI296427	1.291969	2.448621	0.040034
1375005_at	BF403824	1.291727	2.448210	0.001959
1382174_at	AI227996	1.290673	2.446421	0.006458
1380088_at	AW533021	1.284168	2.435415	5.37E-05
1383910_at	BF398220	1.280890	2.429889	0.003117
1381498_at	AA956116	1.226183	2.339473	0.001176
1391936_a_at	BI289110	1.212511	2.317406	0.041362
1379089_at	BM382838	1.208370	2.310764	0.000681
1372993_at	BI299621	1.204492	2.304561	0.000924
1390671_at	AI044666	1.203424	2.302856	0.001722
1394916_at	AW526714	1.202724	2.301739	0.001978
1389397_at	AI234012	1.202426	2.301263	0.000195
1376637_at	AI102401	1.198002	2.294217	0.027098
1386552_at	BF284027	1.197145	2.292856	0.003348
1377792_at	AW524891	1.195075	2.289568	0.013872
1384952_at	AI028968	1.184453	2.272772	0.001744
1376768_at	BM386807	1.182682	2.269984	0.008644
1389127_at	BF552908	1.178770	2.263837	0.001451
1392140_at	BF419584	1.174126	2.256561	0.010974
1375707_at	AA817993	1.171668	2.252720	0.017511
1377994_at	AI501237	1.165986	2.243866	2.56E-05
1372027_at	AI009713	1.165036	2.242388	0.004861
1374290_at	AI408191	1.161834	2.237416	0.000514
1372820_at	BE109102	1.157167	2.230190	6.76E-05
1395629_at	BE105336	1.152754	2.223379	0.001122
1373628_at	AA818342	1.148702	2.217143	0.003268
1383697_at	AW530905	1.147530	2.215342	0.000199
1384269_at	BF386887	1.144996	2.211455	0.039498
1379733_at	BF396474	1.144326	2.210428	0.000479
1379682_at	BI281668	1.138058	2.200846	0.001290
1393334_at	AW528448	1.134957	2.196120	0.000111
1372583_at	AI009094	1.134834	2.195932	0.000776
1390193_at	BF389884	1.132371	2.192188	0.007448
1373114_at	AI408442	1.132006	2.191632	0.026188
1395586_at	BF545930	1.130756	2.189735	0.009671
1389085_at	BI296359	1.122011	2.176502	0.033565
1378898_at	BE109293	1.117104	2.169111	0.018502
1384433_at	AI072153	1.116005	2.167459	0.008374
1392778_at	AA891634	1.112387	2.162030	0.000734
1372515_at	BI281177	1.109794	2.158148	0.000373
1394727_at	AI407942	1.104267	2.149896	0.000884
1391617_at	AI171103	1.103278	2.148422	0.001730
1384680_at	AA924336	1.103174	2.148268	1.20E-05
1374246_at	BF402392	1.100807	2.144746	0.001207

1395350_at	AW919190			1.100062	2.143640	0.026720
1397343_at	BE113258			1.098815	2.141787	0.000702
1384051_at	BF390066			1.096884	2.138923	0.000370
1381654_at	BF398637			1.086982	2.124292	0.008757
1392619_at	BE118107			1.081379	2.116058	0.010153
1376747_at	BE107075			1.079554	2.113382	0.014697
1380763_at	AI101194			1.078476	2.111804	0.008553
1393653_at	BM384831			1.077241	2.109997	0.029779
1379844_at	AW531072			1.073984	2.105239	0.004020
1394948_at	BI303527			1.073384	2.104364	0.028955
1393911_at	AI502300			1.069942	2.099349	0.001054
1378006_at	AI233832			1.069145	2.098190	0.000230
1379451_at	AI549081			1.069122	2.098155	0.025226
1391643_at	BI290758			1.065858	2.093415	0.009922
1390136_at	BE109274			1.062274	2.088220	0.003245
1389256_at	BG381256			1.061409	2.086969	0.027360
1376465_at	BI295240			1.055842	2.078931	0.025628
1390515_at	AA998383			1.052460	2.074063	0.003395
1390205_at	BE108876			1.052183	2.073665	0.000145
1374728_at	BG671786			1.050605	2.071399	0.043671
1394883_at	AI179616			1.050405	2.071111	0.038398
1372104_at	BF289002			1.043745	2.061573	0.000585
1379252_at	AW522833			1.041529	2.058408	0.000451
1377151_at	AI102833			1.040228	2.056553	0.003640
1377880_at	AI170633			1.038506	2.054099	0.000230
1389908_at	BE107167			1.035223	2.049431	0.015670
1373082_at	AA893743			1.033904	2.047558	0.017691
1373537_at	BE113175			1.028307	2.039629	0.005797
1392578_at	AI070875			1.021081	2.029439	0.010470
1377705_at	BF549971			1.019922	2.027809	0.001107
1371854_at	BG374451			1.019759	2.027580	0.012677
1375647_at	BG671943			1.006159	2.008556	0.017266
1385903_at	AA859627			1.004338	2.006023	0.000426
1398265_at	NM_013040	Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1.437830	2.709130	0.001785
1387287_a_at	D83598	Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1.223092	2.334465	0.004574
1397375_at	BM384537	Acsl5	Acyl-CoA synthetase long-chain family member 5	1.153318	2.224249	0.015680
1386926_at	NM_053607	Acsl5	Acyl-CoA synthetase long-chain family member 5	1.053592	2.075692	0.015907
1370857_at	BI282702	Acta2	Smooth muscle α-actin	2.229514	4.689760	0.000129
1398294_at	NM_031005	Actn1	Actinin, α 1	1.131925	2.191509	0.001329
1368223_at	NM_024400	Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.048565	4.136942	0.028144
1376481_at	BF416285	Adamts9	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9	2.392634	5.251154	0.000223
1374535_at	BI283881	Afap1l2	Actin filament associated protein 1-like 2	2.203032	4.604460	0.000634
1368869_at	BG663107	Akap12	A kinase (PRKA) anchor protein 12	1.529713	2.887284	0.019479
1368868_at	NM_057103	Akap12	A kinase (PRKA) anchor protein 12	1.010475	2.014575	0.013205
1387493_at	NM_133515	Akap5	A kinase (PRKA) anchor protein 5	1.569515	2.968049	0.003304
1370043_at	NM_031753	Alcam	Activated leukocyte cell adhesion molecule	1.603863	3.039561	0.010096
1383469_at	BG377269	Aldh1a3	Aldehyde dehydrogenase 1 family, member A3	1.116847	2.168725	0.002910
1370638_at	AF069525	Ank3	Ankyrin 3, epithelial	1.818441	3.526998	0.008330
1367664_at	NM_013220	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	1.668973	3.179881	0.020907
1367665_at	L81174	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	1.503501	2.835300	0.035713
1372069_at	BF284716	Ankrd15	Ankyrin repeat domain 15	1.333618	2.520339	0.003331
1367974_at	NM_012823	Anxa3	Annexin A3	3.372176	10.35443	7.22E-05
1367975_at	BF283732	Anxa3	Annexin A3	1.914777	3.770554	0.000113
1395313_s_at	AI179982	Anxa3	Annexin A3	1.717191	3.287957	0.000365
1373654_at	BM389254	Anxa8	Annexin A8	1.142590	2.207770	0.000179
1392815_at	BE114489	Arap2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	1.501088	2.830562	0.000231
1387018_at	NM_053770	Argbp2	Arg/Abl-interacting protein ArgBP2	1.513378	2.854778	0.039786
1373315_at	AI176425	Arnt2	Aryl hydrocarbon receptor nuclear translocator 2	2.041125	4.115662	0.023949
1378134_at	BI291629	Atp8b1	ATPase, Class Ⅰ, type 8B, member 1	1.061725	2.087426	7.68E-05
1368485_at	NM_024401	Avil	Advillin	2.121568	4.351667	0.012237
1370823_at	AF387513	Bambi	BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis)	1.526089	2.880041	0.003285
1372613_at	AI232784	Bdh2	3-hydroxybutyrate dehydrogenase, type 2	1.177434	2.261742	0.013257
1387232_at	NM_012827	Bmp4	Bone morphogenetic protein 4	3.074538	8.424189	0.000456
1380459_at	AI555023	Btbd14a	BTB (POZ) domain containing 14A	1.357670	2.562709	3.53E-06
1386995_at	BI288701	Btg2	B-cell translocation gene 2, anti-proliferative	1.228055	2.342509	0.004008
1377086_at	AI233530	C1qtnf3	C1q and tumor necrosis factor related protein 3	2.513168	5.708723	0.002654
1376657_at	BE117767	Cadm1	Cell adhesion molecule 1	1.040910	2.057525	0.001137
1393452_at	BM391835	Car9	Carbonic anhydrase 9	2.223630	4.670671	0.000110

1390101_at	AI170609	Ccdc107	Coiled-coil domain containing 107	1.152965	2.223704	0.000166
1398827_at	NM_013087	Cd81	Cd81 molecule	3.901095	14.93986	0.000330
1388936_at	BI296340	Cdh11	Cadherin 11	3.493679	11.26425	0.016743
1370371_a_at	U23056	Ceacam1 /// Ceacam10	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) /// carcinoembryonic antigen-related cell adhesion molecule 10	2.350728	5.100814	0.001261
1393142_at	BF562621	Cep70	Centrosomal protein 70 kDa	1.414308	2.665318	5.96E-05
1368675_at	NM_032084	Chn2	Chimerin (chimaerin) 2	1.111248	2.160324	0.00013
1389368_at	AW253242	Cnksr3	Cnksr family member 3	1.115693	2.166991	0.002136
1376868_at	BM389293	Cobll1	Cobl-like 1	2.807857	7.002435	0.002758
1372439_at	AI176393	Col4a1	Collagen, type Ⅳ, α 1	3.587058	12.01745	4.28E-05
1373245_at	BE111752	Col4a1	Collagen, type Ⅳ, α 1	3.148631	8.868134	9.98E-05
1388494_at	BI281705	Col4a2	Collagen, type Ⅳ, α 2	2.800595	6.967276	0.000478
1393891_at	BE128699	Col8a1	Collagen, type Ⅷ, α 1	1.151470	2.221401	0.003468
1367782_at	NM_012812	Cox6a2	Cytochrome c oxidase, subunit Ⅵa, polypeptide 2	2.245734	4.742785	0.001312
1386921_at	NM_013128	Cpe	Carboxypeptidase E	2.564823	5.916823	0.021482
1382037_at	AI600057	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	2.041047	4.115440	0.002850
1391448_at	BI289620	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	1.915600	3.772706	0.000387
1398622_at	AI703807	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	1.808390	3.502512	0.000527
1376457_at	AI175861	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	1.377770	2.598664	0.018183
1387922_at	AF109674	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	1.195518	2.290270	0.003604
1368059_at	NM_053955	Crym	Crystallin, mu	1.288402	2.442574	0.000395
1383590_at	AA963863	Csgalnact1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	3.304007	9.876547	0.022018
1370057_at	NM_017148	Csrp1	Cysteine and glycine-rich protein 1	1.030407	2.042601	0.001628
1388583_at	BF283398	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.092111	4.263716	0.042845
1387655_at	AF189724	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.643136	3.123441	0.028532
1369633_at	AI171777	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.567476	2.963858	0.045339
1368290_at	NM_031327	Cyr61	Cysteine-rich, angiogenic inducer, 61	1.261651	2.397699	0.022554
1371436_at	AI176924	Ddah2	Dimethylarginine dimethylaminohydrolase 2	1.710173	3.272001	0.006371
1368013_at	NM_080399	Ddit4l	DNA-damage-inducible transcript 4-like	1.056235	2.079498	0.002531
1389894_at	BF399476	Dlc1	Deleted in liver cancer 1	1.068336	2.097013	0.002194
1377835_at	BM390876	Dock8	Dedicator of cytokinesis 8	2.230319	4.692377	0.005526
1388506_at	AW144509	Dsp	Desmoplakin	1.830721	3.557148	9.48E-05
1368146_at	U02553	Dusp1	Dual specificity phosphatase 1	1.560299	2.949149	0.003438
1368949_at	NM_053820	Ebf1	Early B-cell factor 1	1.316466	2.490553	0.000315
1369519_at	NM_012548	Edn1	Endothelin 1	1.366023	2.577591	0.009786
1368541_at	NM_053719	Emb	Embigin	1.064032	2.090767	0.031817
1377752_at	BE112998	Emp2	Epithelial membrane protein 2	1.392599	2.625512	0.000189
1373617_at	AA818807	Emp2	Epithelial membrane protein 2	1.347238	2.544246	0.000131
1377311_at	AI045616	Emx2	Empty spiracles homeobox 2	1.032095	2.044992	0.001019
1369096_at	NM_134331	Epha7	Eph receptor A7	1.427798	2.690358	0.001357
1385788_at	AW534949	Ephb3	Eph receptor B3	1.321665	2.499544	0.000783
1369182_at	NM_013057	F3	Coagulation factor Ⅲ (thromboplastin, tissue factor)	2.945169	7.701657	8.99E-05
1377940_at	BF398271	Fam101b	Family with sequence similarity 101, member B	1.069187	2.098250	0.047471
1384507_at	AA817708	Fam105a	Family with sequence similarity 105, member A	1.169405	2.249190	0.005950
1389146_at	BF283267	Fam107b	Family with sequence similarity 107, member B	1.016680	2.023257	0.000409
1393910_at	BF563961	Fam13a1	Family with sequence similarity 13, member A1	4.490284	22.475530	0.003831
1379625_at	BG664461	Fam164a	Family with sequence similarity 164, member A	1.550421	2.929026	0.001732
1384648_at	AA963844	Fam164a	Family with sequence similarity 164, member A	1.219281	2.328307	0.000456
1391944_at	BI296237	Fam184a /// RGD1560557	Family with sequence similarity 184, member A /// similar to minichromosome maintenance protein 8 isoform 1	1.268560	2.409209	0.001168
1373286_at	AA875261	Fblim1	Filamin binding LIM protein 1	1.055977	2.079126	0.000141
1376500_at	AI639044	Fbxo23	F-box only protein 23	1.064218	2.091036	0.001619
1386614_at	BG671466	Fbxo23	F-box only protein 23	1.016912	2.023583	0.010871
1368114_at	NM_053428	Fgf13	Fibroblast growth factor 13	3.654623	12.593640	0.004204
1370106_at	NM_019199	Fgf18	Fibroblast growth factor 18	2.489420	5.615522	0.016971
1369313_at	NM_031677	Fhl2	Four and a half LIM domains 2	1.491713	2.812227	0.011052
1371951_at	AA800031	Fhl2	Four and a half LIM domains 2	1.319164	2.495215	0.010936
1372825_at	BI290551	Fnbp1	Formin binding protein 1	1.481808	2.792985	0.003539
1376784_at	BI274481	Fnbp1	Formin binding protein 1	1.407572	2.652903	0.007946
1369471_at	NM_138914	Fnbp1	Formin binding protein 1	1.142482	2.207604	0.012674
1377342_s_at	BE105446	Fnbp1	Formin binding protein 1	1.043905	2.061801	0.023433
1370829_at	M69056	Fntb	Farnesyltransferase, CAAX box, β	2.001250	4.003468	0.001272
1368711_at	NM_012743	Foxa2	Forkhead box A2	3.125513	8.727165	0.004980
1380387_at	BE105492	Foxp2	Forkhead box P2	1.808179	3.501999	0.000372
1383721_at	AI556075	Fzd8	Frizzled homolog 8 (Drosophila)	1.891668	3.710641	8.54E-05
1372016_at	BI287978	Gadd45b	Growth arrest and DNA-damage-inducible, β	1.287102	2.440374	0.007019
1369735_at	NM_057100	Gas6	Growth arrest specific 6	2.030906	4.086614	0.000694

1367627_at	NM_031031	Gatm	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	1.704989	3.260264	0.001931
1390557_at	BF394809	Gca	Grancalcin	1.294802	2.453433	0.015190
1379031_at	BM390697	Gca	Grancalcin	1.132824	2.192876	0.016810
1374903_at	AI234819	Gcnt2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1.780391	3.435192	0.000760
1370375_at	J05499	Gls2	Glutaminase 2 (liver, mitochondrial)	1.381886	2.606088	0.000222
1392888_at	AI071251	Gpc4	Glypican 4	1.149833	2.218883	0.017197
1373773_at	BF394166	Gpm6a	Glycoprotein m6a	1.492990	2.814717	0.004487
1370389_at	AB036421	Gpm6b	Glycoprotein m6b	1.729687	3.316558	0.041126
1382955_at	BI284296	Gpr126	G protein-coupled receptor 126	1.691914	3.230851	0.002139
1373693_at	BF414143	Gprc5c	G protein-coupled receptor, family C, group 5, member C	1.172596	2.254169	0.000245
1368618_at	NM_031623	Grb14	Growth factor receptor bound protein 14	2.263062	4.800093	0.001165
1368401_at	M85035	Gria2	Glutamate receptor, ionotropic, AMPA 2	1.185577	2.274543	0.038866
1383897_at	BE117477	H2afy2	H2A histone family, member Y2	1.467448	2.765322	0.003118
1384541_at	BM391441	Hapln1	Hyaluronan and proteoglycan link protein 1	2.720224	6.589753	0.021358
1370125_at	NM_019189	Hapln1	Hyaluronan and proteoglycan link protein 1	2.532368	5.785206	0.010848
1368983_at	NM_012945	Hbegf	Heparin-binding EGF-like growth factor	1.057962	2.081988	0.018982
1376867_at	BE095833	Hspc159	Galectin-related protein	2.821242	7.067707	0.000688
1373515_at	BI275737	Hspc159	Galectin-related protein	2.640040	6.233487	0.000522
1387028_a_at	M86708	Id1	Inhibitor of DNA binding 1	1.097822	2.140313	0.005605
1390507_at	BI296097	Isg20	Interferon stimulated exonuclease 20	2.272905	4.832953	0.040035
1394824_at	BF398348	Itga11	Integrin, α 11	1.885728	3.695395	0.000279
1393558_at	AI137931	Itga6	Integrin, α 6	1.341112	2.533464	0.001146
1382439_at	AI070686	Itgb6	Integrin, β 6	1.316970	2.491424	0.005869
1387907_at	J05510	Itpr1	Inositol 1,4,5-triphosphate receptor, type 1	1.709204	3.269803	0.001386
1368725_at	NM_019147	Jag1	Jagged 1	1.045878	2.064622	0.010981
1398124_at	AI071356	Jazf1	JAZF zinc finger 1	1.325135	2.505564	1.36E-05
1396701_at	BE110052	Kalrn	Kalirin, RhoGEF kinase	1.050512	2.071265	0.000530
1369144_a_at	NM_031739	Kcnd3	Potassium voltage gated channel, Shal-related family, member 3	1.978834	3.941744	0.019750
1394039_at	BM382886	Klf5	Kruppel-like factor 5	1.168271	2.247422	0.001471
1368363_at	NM_053394	Klf5	Kruppel-like factor 5	1.107290	2.154406	0.000169
1388932_at	BI274917	Lama5	Laminin, α 5	1.499220	2.826898	0.008496
1367880_at	NM_012974	Lamb2	Laminin, β 2	1.055487	2.078420	0.000777
1370993_at	AA997129	Lamc1	Laminin, γ 1	1.144416	2.210566	0.001299
1388422_at	BI275904	Lims2	LIM and senescent cell antigen like domains 2	3.226389	9.359224	0.000228
1376632_at	AI602501	Lmcd1	LIM and cysteine-rich domains 1	3.919424	15.130880	0.000893
1381798_at	BE114958	Lmo7	LIM domain 7	1.648918	3.135983	0.000383
1375726_at	BI284480	Lmo7	LIM domain 7	1.223048	2.334393	0.000945
1381190_at	AI598833	Lmo7	LIM domain 7	1.051248	2.072321	0.001218
1375523_at	BE108178	LOC294446	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81)	1.286331	2.439070	0.000713
1370948_a_at	M59859	LOC294446 /// LOC681252 /// Marcks	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81) /// similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate 80 kDa protein) /// myristoylated alanine rich protein kinase C substrate	1.113014	2.162971	3.48E-05
1370949_at	M59859	LOC294446 /// LOC681252 /// Marcks	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81) /// similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate 80 kDa protein) /// myristoylated alanine rich protein kinase C substrate	1.078213	2.111419	5.02E-05
1381434_s_at	AW253721	LOC302022	Similar to nidogen 2 protein	1.462223	2.755326	0.002301
1373232_at	AI008975	LOC302022	Similar to nidogen 2 protein	1.153904	2.225152	0.007223
1390158_at	BI290752	LOC304903	Similar to Pappalysin-2 precursor (Pregnancy-associated plasma protein-A2) (PAPP-A2) (Pregnancy-associated plasma protein-E1) (PAPP-E)	1.089462	2.127947	0.013140
1384907_at	AI411835	LOC306096	Similar to Dachshund homolog 1 (Dach1)	2.365997	5.155086	0.015526
1383888_at	AA998264	LOC307495	Similar to biliverdin reductase B (flavin reductase (NADPH))	1.345301	2.540832	6.23E-05
1379465_at	AW527596	LOC311134	Hypothetical LOC311134	1.691930	3.230885	0.004884
1392074_at	AA926082	LOC500046	Similar to hypothetical protein FLJ21986	1.886585	3.697589	0.042067
1392592_at	AI137045	LOC679869	Similar to transcription factor 7-like 2, T-cell specific, HMG-box	1.098548	2.141391	0.000254
1394497_at	AI535239	LOC679869 /// LOC683733	Similar to transcription factor 7-like 2, T-cell specific, HMG-box /// similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.055505	2.078445	0.000768
1373088_at	BI295811	LOC682888	Hypothetical protein LOC682888	1.243831	2.368265	1.11E-06
1388447_at	AA800701	LOC683626	Similar to limb-bud and heart	1.193694	2.287377	0.007020
1379815_at	AI713959	LOC683733	Similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.406898	2.651664	1.15E-05

1377156_at	BI273936	LOC683733	Similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.332040	2.517583	0.001391
1383488_at	AA817785	LOC687536	Similar to Forkhead box protein F1 (Forkhead-related protein FKHL5) (Forkhead-related transcription factor 1) (FREAC-1) (Hepatocyte nuclear factor 3 forkhead homolog 8) (HFH-8)	1.534125	2.896127	0.003143
1386120_at	BF393607	LOC689147	Hypothetical protein LOC689147	1.753785	3.372422	0.003982
1393414_at	AW142650	LOC689176	Similar to transmembrane protein 64	1.201660	2.300042	0.047653
1376691_at	AI103213	LOC689176	Similar to transmembrane protein 64	1.150453	2.219836	0.044249
1374016_at	AI502597	Lpar1	Lysophosphatidic acid receptor 1	1.299750	2.461863	0.003803
1370048_at	NM_053936	Lpar1	Lysophosphatidic acid receptor 1	1.268034	2.408331	0.001243
1389913_at	BI276990	Lrrfip1	Leucine rich repeat (in FLII) interacting protein 1	1.260408	2.395635	0.000794
1368448_at	NM_021586	Ltbp2	Latent transforming growth factor β binding protein 2	2.071905	4.204415	0.002220
1367768_at	NM_031655	Lxn	Latexin	1.381643	2.605649	0.000525
1374933_at	BI277043	Mcam	Melanoma cell adhesion molecule	1.008050	2.011191	0.034411
1369218_at	NM_031517	Met	Met proto-oncogene	1.253867	2.384798	0.000472
1384617_at	AI385260	MGC72614	Hypothetical LOC310540	1.318517	2.494096	0.001888
1398387_at	AI009530	MGC72614	Hypothetical LOC310540	1.263746	2.401184	0.001149
1367568_a_at	NM_012862	Mgp	Matrix Gla protein	3.353436	10.220800	0.010514
1384150_at	AA901038	Mid1	Midline 1	1.062584	2.088669	0.000603
1370072_at	NM_012608	Mme	Membrane metallo endopeptidase	3.646308	12.521260	0.003799
1372457_at	BF284182	Mtus1	Mitochondrial tumor suppressor 1	1.629140	3.093285	5.84E-06
1380321_at	BI287786	Mtus1	Mitochondrial tumor suppressor 1	1.402213	2.643066	0.000159
1378970_at	AW252385	Mybphl	Myosin binding protein H-like	1.080283	2.114451	0.043933
1370158_at	AA946388	Myh10	Myosin, heavy chain 10, non-muscle	1.192392	2.285314	0.005978
1388298_at	BI279044	Myl9	Myosin, light chain 9, regulatory	1.368369	2.581786	0.025217
1389507_at	AI072446	Nedd4l	Neural precursor cell expressed, developmentally down-regulated 4-like	1.010244	2.014252	0.002318
1369679_a_at	AB060652	Nfia	Nuclear factor I/A	1.018188	2.025374	0.008943
1388618_at	BM389302	Nid2	Nidogen 2	1.740644	3.341843	0.002285
1368883_at	NM_030868	Nov	Nephroblastoma overexpressed gene	1.914944	3.770993	0.010007
1371412_a_at	BE107450	Nrep	Neuronal regeneration related protein	1.740346	3.341152	0.003311
1369783_a_at	U02319	Nrg1	Neuregulin 1	1.341273	2.533748	0.000850
1370607_a_at	U02323	Nrg1	Neuregulin 1	1.322000	2.500124	0.000298
1371211_a_at	U02315	Nrg1	Neuregulin 1	1.304653	2.470242	0.000467
1382814_at	AW521702	Odz3	Odz, odd Oz/ten-m homolog 3 (Drosophila)	2.654920	6.298115	0.004174
1377702_at	BG380173	P2ry5	Purinergic receptor P2Y, G-protein coupled, 5	1.073159	2.104036	0.000211
1367687_a_at	M25719	Pam	Peptidylglycine α-amidating monooxygenase	1.192716	2.285826	0.021615
1398487_at	BF419639	Pbx1	Pre-B-cell leukemia homeobox 1	1.092533	2.132481	0.006868
1393966_at	AW530825	Pbx1	Pre-B-cell leukemia homeobox 1	1.018972	2.026474	0.000957
1370490_at	L43592	Pcdhb12	Protocadherin β 12	1.403580	2.645572	0.026763
1377042_at	BI288196	Pcgf5	Polycomb group ring finger 5	1.189904	2.281376	0.002754
1392773_at	AA859578	Pcsk5	Proprotein convertase subtilisin/kexin type 5	1.589348	3.009134	0.002877
1393467_at	BF549923	Pcsk5	Proprotein convertase subtilisin/kexin type 5	1.332597	2.518556	0.000461
1387812_at	NM_012999	Pcsk6	Proprotein convertase subtilisin/kexin type 6	3.748654	13.441790	0.001431
1382345_at	AA955299	Pctk2	PCTAIRE protein kinase 2	1.020874	2.029147	5.29E-05
1374157_at	AA858930	Pde4b	Phosphodiesterase 4B, cAMP specific	2.492088	5.625918	0.004862
1369044_a_at	AF202733	Pde4b	Phosphodiesterase 4B, cAMP specific	1.307088	2.474415	0.025797
1374616_at	BM384311	Pdgfrl	Platelet-derived growth factor receptor-like	1.872122	3.660707	1.96E-06
1368703_at	NM_053326	Pdlim5	PDZ and LIM domain 5	1.360465	2.567680	0.000723
1386913_at	NM_019358	Pdpn	Podoplanin	2.706385	6.526842	0.015510
1374969_at	AA799832	Pgm5	Phosphoglucomutase 5	1.912300	3.764087	4.83E-05
1368860_at	NM_017180	Phlda1	Pleckstrin homology-like domain, family A, member 1	1.236279	2.355901	0.031298
1378106_at	AI029402	Phlda2	Pleckstrin homology-like domain, family A, member 2	1.063574	2.090104	0.007195
1388539_at	BE113268	Pkp2	Plakophilin 2	1.216333	2.323553	0.004529
1382659_at	BF289229	Pla2r1	Phospholipase A2 receptor 1	1.819950	3.530690	5.56E-05
1387122_at	NM_012760	Plagl1	Pleiomorphic adenoma gene-like 1	6.377022	83.114140	0.001611
1386962_at	NM_024353	Plcb4	Phospholipase C, β 4	1.727990	3.312660	0.008873
1370489_a_at	U57836	Plcb4	Phospholipase C, β 4	1.479277	2.788090	0.007828
1369029_at	NM_057194	Plscr1	Phospholipid scramblase 1	1.321345	2.498990	0.002555
1370247_a_at	AA943163	Pmp22	Peripheral myelin protein 22	1.312653	2.483979	0.000418
1372531_at	BE106488	Ppfibp2	PTPRF interacting protein, binding protein 2 (liprin β 2)	1.798887	3.479517	0.023491
1393082_at	AI044747	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c	1.263639	2.401006	0.009828
1368716_at	NM_133425	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c	1.085918	2.122725	0.010686
1370012_at	NM_031557	Ptgis	Prostaglandin I2 (prostacyclin) synthase	1.878328	3.676486	0.007631
1368527_at	U03389	Ptgs2	Prostaglandin-endoperoxide synthase 2	1.723796	3.303044	0.037120
1377427_at	BE104739	Ptpn14	Protein tyrosine phosphatase, non-receptor type 14	1.140142	2.204028	1.10E-05
1374774_at	BF552241	Ptpn14	Protein tyrosine phosphatase, non-receptor type 14	1.065112	2.092332	3.73E-05
1368035_a_at	X83505	Ptprf	Protein tyrosine phosphatase, receptor type, F	2.196463	4.583543	0.005199

1384227_at	AI044031	Ptprk	Protein tyrosine phosphatase, receptor type, K, extracellular region	2.172519	4.508097	4.14E-05
1390034_at	BF393945	Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	1.192882	2.286089	0.025134
1367791_at	NM_031645	Ramp1	Receptor (G protein-coupled) activity modifying protein 1	1.907320	3.751116	0.017043
1368660_at	NM_021690	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	1.018953	2.026448	0.003623
1390159_at	AA819332	Rasgrp3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	1.433832	2.701633	0.016442
1383322_at	BG375198	Rasl11b	RAS-like family 11 member B	1.757780	3.381775	0.008884
1387581_at	NM_022959	Rassf9	Ras association (RalGDS/AF-6) domain family (N-terminal) member 9	2.580119	5.979890	2.33E-07
1383247_a_at	BI291029	rCG_35099	Spinster homolog 2	1.270831	2.413004	0.006005
1388791_at	BI275911	RGD1309930	Similar to 2810022L02Rik protein	1.327569	2.509794	0.016780
1395336_at	BE098691	RGD1309930	Similar to 2810022L02Rik protein	1.309761	2.479005	0.004198
1374898_at	AW527473	RGD1311422	Similar to CG8841-PA	1.039847	2.056009	0.002294
1373584_at	BE113205	RGD1559643	Similar to hypothetical protein A430031N04	1.019520	2.027245	0.000710
1372380_at	AI231308	RGD1561067	Similar to RNA binding protein gene with multiple splicing	3.350875	10.202670	0.002486
1375898_at	AW252379	RGD1561067	Similar to RNA binding protein gene with multiple splicing	3.091972	8.526606	0.004328
1376619_at	AI412803	RGD1561090	Similar to protein tyrosine phosphatase, receptor type, D	2.033850	4.094962	1.02E-06
1374591_at	AI409042	RGD1561090	Similar to protein tyrosine phosphatase, receptor type, D	1.973152	3.926251	2.32E-05
1376919_at	BG665267	RGD1562317	Similar to expressed sequence AW212394	1.376378	2.596157	0.000204
1388879_at	BG669292	RGD1562717	Similar to ABI gene family, member 3 (NESH) binding protein	1.796298	3.473277	0.026049
1388906_at	BM389311	RGD1564174	Similar to novel protein similar to Tensin Tns	1.157511	2.230722	4.32E-05
1383398_at	AI059150	RGD1564327	Similar to integrin α 8	1.649388	3.137005	0.000121
1385354_at	BE120766	RGD1564327	Similar to integrin α 8	1.472111	2.774276	9.05E-07
1376546_at	BE120498	RGD1565432	Similar to hypothetical protein	1.921295	3.787629	0.009661
1396347_at	BF395640	RGD1565926	RGD1565926	1.020952	2.029258	0.010695
1371731_at	AI408151	RGD1566215	Similar to Coatomer γ-2 subunit (γ-2 coat protein) (γ-2 COP)	1.928401	3.806332	5.12E-05
1380425_at	AI012859	Rnasel	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	1.671401	3.185237	0.002887
1377116_at	BI301478	Rnasel	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	1.626917	3.088523	0.001458
1381533_at	AI144754	Rnd1	Rho family GTPase 1	1.196579	2.291955	0.000495
1379693_at	AI409154	Robo2	Roundabout, axon guidance receptor, homolog 2 (Drosophila)	1.245877	2.371626	0.001543
1390632_at	BE107414	Rspo3	R-spondin 3 homolog (Xenopus laevis)	1.049128	2.069279	0.005955
1388356_at	AI406499	S100a16	S100 calcium binding protein A16	1.404636	2.647510	0.004150
1368379_at	NM_054001	Scarb2	Scavenger receptor class B, member 2	2.124624	4.360893	0.001346
1393338_at	AW528719	Scx	Scleraxis	1.644999	3.127476	0.007845
1368394_at	AF140346	Sfrp4	Secreted frizzled-related protein 4	1.610004	3.052527	0.003747
1367802_at	NM_019232	Sgk1	Serum/glucocorticoid regulated kinase 1	1.265947	2.404849	0.007546
1389779_at	AA800626	Sh2d4a	SH2 domain containing 4A	1.417920	2.672000	0.032404
1392301_at	AI237897	Sh3tc1	SH3 domain and tetratricopeptide repeats 1	1.214738	2.320987	0.006644
1392556_at	BF410961	Shroom3	Shroom family member 3	1.454496	2.740607	9.57E-05
1376040_at	BI290044	Sipa1l2	Signal-induced proliferation-associated 1 like 2	1.185619	2.274609	0.012637
1368565_at	NM_019225	Slc1a3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.926317	3.800836	0.003174
1376165_at	BE098153	Slc24a3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	1.145511	2.212244	0.000291
1398295_at	NM_031684	Slc29a1	Solute carrier family 29 (nucleoside transporters), member 1	1.222426	2.333387	0.003869
1369074_at	NM_130748	Slc38a4	Solute carrier family 38, member 4	2.257810	4.782650	0.000540
1392349_at	BE116021	Slc5a3	Solute carrier family 5 (inositol transporters), member 3	1.031672	2.044392	0.000967
1387968_at	L22022	Slc6a15	Solute carrier family 6 (neutral amino acid transporter), member 15	2.581534	5.985759	0.018733
1368920_at	NM_031321	Slit3	Slit homolog 3 (Drosophila)	1.652522	3.143828	0.030840
1384437_at	AI576309	Smarca1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	1.832940	3.562623	4.14E-05
1377695_at	BF281135	Smtnl2	Smoothelin-like 2	2.018633	4.051996	0.047065
1375349_at	BI295776	Sorbs1	Sorbin and SH3 domain containing 1	2.508532	5.690409	0.000169
1372728_at	BE103745	Sort1	Sortilin 1	1.319517	2.495825	0.006034
1371004_at	AI070124	Sort1	Sortilin 1	1.117923	2.170343	0.001420
1379611_at	BF416979	Spsb1	splA/ryanodine receptor domain and SOCS box containing 1	1.025012	2.034976	0.009283
1373554_at	BE349698	Spsb1	splA/ryanodine receptor domain and SOCS box containing 1	1.010972	2.015269	0.008275
1389142_at	AI013361	Sqrdl	sulfide quinone reductase-like (yeast)	2.278473	4.851642	0.001121
1368109_at	NM_031337	St3gal5	ST3 β-galactoside α-2,3-sialyltransferase 5	1.683094	3.211158	5.13E-05
1370907_at	M83143	St6gal1	ST6 β-galactosamide α-2,6-sialyltranferase 1	1.294325	2.452622	0.000575
1389420_at	BI279446	Stap2	Signal transducing adaptor family member 2	1.208781	2.311423	0.019358
1370680_at	AF483620	Stau2	Staufen, RNA binding protein, homolog 2 (Drosophila)	1.015615	2.021764	0.000276
1372602_at	BI295979	Stbd1	Starch binding domain 1	2.761499	6.781006	2.53E-05
1373590_at	BI295949	Stom	Stomatin	1.267382	2.407243	0.003323
1379732_at	AW920037	Stx11	Syntaxin 11	1.976724	3.935983	9.77E-05
1368771_at	NM_134378	Sulf1	Sulfatase 1	2.238326	4.718492	0.000659

1376572_a_at	AI045848	Svil	Supervillin	1.320708	2.497887	0.019303
1367570_at	NM_031549	Tagln	Transgelin	1.696733	3.241660	0.002280
1367859_at	NM_013174	Tgfb3	Transforming growth factor, β 3	1.509832	2.847768	0.003273
1375951_at	AA818521	Thbd	Thrombomodulin	1.311404	2.481829	0.020049
1370474_at	J03819	Thrb	Thyroid hormone receptor β	2.219205	4.656368	0.002376
1387983_at	J03933	Thrb	Thyroid hormone receptor β	1.184729	2.273206	0.003624
1383623_at	BM383909	Thyn1	Thymocyte nuclear protein 1	1.043826	2.061688	0.002044
1375138_at	AA893169	Timp3	TIMP metallopeptidase inhibitor 3	6.264691	76.888260	0.015514
1389836_a_at	AI599265	Timp3	TIMP metallopeptidase inhibitor 3	5.105264	34.422120	0.013029
1372926_at	AI009159	Timp3	TIMP metallopeptidase inhibitor 3	4.004944	16.054920	0.025103
1368989_at	NM_012886	Timp3	TIMP metallopeptidase inhibitor 3	2.253301	4.767725	0.015049
1373847_at	AW435343	Tm4sf1	Transmembrane 4 L six family member 1	2.114633	4.330798	0.000242
1378305_at	AI578087	Tm4sf1	Transmembrane 4 L six family member 1	1.938512	3.833100	0.000317
1390832_at	BI294696	Tmcc3	Transmembrane and coiled-coil domain family 3	2.119391	4.345106	0.005139
1376623_at	AI409186	Tmem204	Transmembrane protein 204	1.085094	2.121514	7.30E-05
1383314_at	BE110098	Tmem51	Transmembrane protein 51	1.344562	2.539531	3.99E-05
1371361_at	BI278826	Tns1	Tensin 1	1.158595	2.232399	0.000276
1370288_a_at	AF372216	Tpm1	Tropomyosin 1, α	2.037181	4.104428	0.000937
1395794_at	BF395218	Tpm1	Tropomyosin 1, α	1.997138	3.992073	0.008961
1371241_x_at	AF370889	Tpm1	Tropomyosin 1, α	1.663566	3.167985	0.013541
1370287_a_at	M23764	Tpm1	Tropomyosin 1, α	1.590740	3.012039	5.95E-05
1368724_a_at	NM_019131	Tpm1	Tropomyosin 1, α	1.054891	2.077561	0.018900
1372219_at	AA012755	Tpm2	Tropomyosin 2	1.064563	2.091537	2.16E-05
1398759_at	NM_013043	Tsc22d1	TSC22 domain family, member 1	1.051368	2.072494	0.000844
1377630_at	AI408602	Tspan13	Tetraspanin 13	1.481687	2.792752	0.019678
1375057_at	BG377313	Tspan18	Tetraspanin 18	1.454071	2.739802	0.030870
1398476_at	AW527349	Vcl	Vinculin	1.244474	2.369321	0.000134
1372905_at	AW433888	Vcl	Vinculin	1.136321	2.198197	3.11E-05
1369098_at	NM_013155	Vldlr	Very low density lipoprotein receptor	1.505396	2.839027	0.001107
1387455_a_at	NM_013155	Vldlr	Very low density lipoprotein receptor	1.471028	2.772194	0.001621
1389611_at	AA849857	Vldlr	Very low density lipoprotein receptor	1.435945	2.705593	0.000947
1368854_at	AI227991	Vsnl1	Visinin-like 1	2.472695	5.550796	0.007104
1368853_at	NM_012686	Vsnl1	Visinin-like 1	2.077806	4.221647	0.004903
1387873_at	BI279661	Wfdc1	WAP four-disulfide core domain 1	1.449292	2.730739	0.003744
1370221_at	BF419320	Wisp1	WNT1 inducible signaling pathway protein 1	1.058567	2.082861	0.000154
1393613_at	BE117871	Zfp462	Zinc finger protein 462	1.088387	2.126361	0.004351
1383462_at	BF566263	Znf294	Zinc finger protein 294	1.010246	2.014255	0.000352

Supplementary Table 2 Downregulated genes in miR-146a-transfected hepatic stellate cell-2

Probe ID	Representative public ID	Gene symbol	Gene title	Log2	Fold change	*P*-value
1374345_at	AI111707			-3.40046	-10.55940	1.80E-07
1397317_at	BI296984			-3.25476	-9.54513	3.34E-07
1397400_at	BM391846			-3.11991	-8.69335	5.36E-05
1382027_at	BI296880			-3.05526	-8.31237	3.85E-07
1380245_at	AI411847			-2.90249	-7.47715	8.46E-07
1381129_at	BF392367			-2.52142	-5.74147	0.018162
1383211_at	BE109736			-2.46677	-5.52805	0.006284
1380057_at	BE097091			-2.33498	-5.04543	0.000685
1381048_at	BF398435			-2.31590	-4.97916	0.000443
1381064_at	AI137604			-2.26573	-4.80896	1.32E-07
1373583_at	BF396317			-2.26180	-4.79589	1.23E-07
1377240_at	AW526305			-2.19645	-4.58351	0.001977
1394468_at	BF287020			-2.12335	-4.35705	0.000157
1377678_at	BI283757			-2.12273	-4.35519	0.000117
1375101_at	BI292651			-2.05823	-4.16476	0.000442
1380712_at	AI406475			-2.04987	-4.14068	0.006175
1372110_at	BE113148			-1.95331	-3.87261	0.000463
1394517_at	AW522148			-1.88952	-3.70513	0.006671
1390530_at	AI169239			-1.87482	-3.66755	0.001510
1376463_at	AA955579			-1.87022	-3.65587	0.001028
1384743_at	BF418132			-1.86231	-3.63589	2.42E-14
1377114_at	AI410861			-1.83910	-3.57788	8.88E-06
1377161_at	BG378317			-1.83484	-3.56731	0.039081
1383220_at	BE114231			-1.73514	-3.32911	0.000179
1393143_at	AI045866			-1.69968	-3.24828	0.005065

1376324_at	BF406329			-1.68113	-3.20679	0.004765
1390429_at	BF398114			-1.62771	-3.09021	6.83E-05
1382431_at	AI103530			-1.61690	-3.06716	1.15E-06
1391251_at	BI290666			-1.60415	-3.04016	1.73E-05
1383580_at	AA859643			-1.58751	-3.00530	0.000305
1372011_at	BI292028			-1.57490	-2.97915	0.000487
1377232_at	BF406608			-1.54704	-2.92217	0.003334
1398457_at	AI146156			-1.50417	-2.83662	0.005925
1381161_a_at	BI301117			-1.47549	-2.78078	0.008417
1382142_at	AI029975			-1.45644	-2.74431	3.98E-05
1382472_at	AI502459			-1.44328	-2.71939	8.76E-05
1375266_at	BG380633			-1.43613	-2.70595	0.010349
1381862_at	AW524296			-1.39645	-2.63253	0.004828
1393235_at	AI059968			-1.39173	-2.62392	0.000172
1385181_at	AI029337			-1.39139	-2.62332	1.30E-07
1377686_at	AA859337			-1.37593	-2.59535	3.53E-07
1372449_at	AW253616			-1.37158	-2.58753	0.009120
1377556_at	AW535380			-1.36924	-2.58334	0.004323
1372637_at	AI169241			-1.36639	-2.57825	0.000419
1375957_at	AW434654			-1.30351	-2.46828	4.46E-06
1383936_at	BM386842			-1.29935	-2.46118	1.09E-05
1384724_at	AA850766			-1.28208	-2.43190	3.20E-05
1379510_at	BF546306			-1.20859	-2.31112	9.04E-05
1382296_at	BF291041			-1.20836	-2.31075	1.53E-05
1382544_at	AI058746			-1.20774	-2.30975	3.79E-06
1376816_at	BF284903			-1.19854	-2.29507	0.000155
1383019_at	BF558478			-1.18776	-2.27798	0.000454
1374558_at	AI010316			-1.17751	-2.26186	8.04E-06
1389250_at	AW915115			-1.16975	-2.24972	9.96E-05
1377328_at	BI290012			-1.16378	-2.24044	0.009615
1380602_at	AI764190			-1.16264	-2.23867	0.001006
1384812_at	AI229409			-1.15815	-2.23172	0.006986
1396217_at	BF542447			-1.13720	-2.19954	0.000345
1397668_at	H34328			-1.13718	-2.19950	3.26E-07
1374932_at	BI282731			-1.13055	-2.18943	0.001048
1385381_at	AA996491			-1.12644	-2.18320	0.000161
1394047_at	BE107848			-1.10947	-2.15767	0.035398
1397452_at	AI112776			-1.10848	-2.15618	0.000172
1393730_at	BI277836			-1.10819	-2.15575	0.033820
1381975_at	BG371767			-1.10049	-2.14427	0.001469
1380699_at	BE110761			-1.09332	-2.13364	0.000497
1374920_at	AI228955			-1.07203	-2.10239	2.65E-05
1375473_at	BI296644			-1.05345	-2.07548	0.001418
1373914_at	BM389075			-1.05092	-2.07186	0.001887
1383436_at	BG376768			-1.04917	-2.06933	0.000656
1390405_at	AA942765			-1.04540	-2.06394	0.003275
1376911_at	BM386385			-1.04165	-2.05858	0.000165
1384183_at	AA996869			-1.03133	-2.04391	0.004095
1377469_at	AI103161			-1.02160	-2.03016	0.006072
1393030_at	BE115641			-1.01825	-2.02547	0.000652
1377113_at	BF415786			-1.01680	-2.02343	0.002724
1382960_at	BE108047			-1.01496	-2.02084	9.24E-05
1377955_at	AI137602			-1.01226	-2.01707	4.55E-07
1391853_at	AA998997			-1.00175	-2.00243	0.000388
1384086_at	BG671196			-1.00024	-2.00033	0.031992
1385235_at	AA818804	A2bp1	Ataxin 2 binding protein 1	-1.24533	-2.37073	4.71E-05
1383130_at	BF555795	A2bp1	Ataxin 2 binding protein 1	-1.07712	-2.10983	0.000947
1394490_at	AI502114	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.80354	-3.49076	3.72E-07
1384381_at	BF284523	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.27132	-2.41382	0.000215
1383355_at	AW918387	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.21129	-2.31545	5.71E-07
1369928_at	NM_019212	Acta1	Actin, α 1, skeletal muscle	-3.10676	-8.61449	4.42E-05
1370856_at	AA800705	Actc1	Actin, α, cardiac muscle 1	-1.26863	-2.40933	2.02E-05
1394483_at	AW535310	Adamts5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	-1.53094	-2.88975	1.96E-05
1390383_at	BI285616	Adfp	Adipose differentiation related protein	-2.49834	-5.65037	3.30E-08
1382680_at	BG673602	Adfp	Adipose differentiation related protein	-2.22186	-4.66496	1.20E-05
1387395_at	NM_017161	Adora2b	Adenosine A2B receptor	-2.08666	-4.24763	0.000258
1395695_at	BE126420	Aebp1	AE binding protein 1	-1.59720	-3.02555	0.005916
1372301_at	BI278482	Aebp1	AE binding protein 1	-1.55735	-2.94313	0.011670
1368342_at	NM_031544	Ampd3	Adenosine monophosphate deaminase 3	-1.65434	-3.14779	0.004546
1377783_at	BI294141	Angpt4	Angiopoietin 4	-1.64644	-3.13060	0.001964
1397579_x_at	BI294552	Apc2	Adenomatosis polyposis coli 2	-1.91631	-3.77455	0.001510

1395461_at	BI294552	Apc2	Adenomatosis polyposis coli 2	-1.30834	-2.47656	0.002146
1391083_at	BM384457	Arhgap22	Rho GTPase activating protein 22	-1.09826	-2.14096	0.007774
1377385_at	BE100015	Arhgap27	Rho GTPase activating protein 27	-1.30775	-2.47556	6.57E-07
1387959_at	AB009372	Aspg	Asparaginase homolog (S. cerevisiae)	-1.29918	-2.46089	0.000534
1380726_at	BI290633	Aspn	Asporin	-2.13124	-4.38092	0.032454
1381504_at	AI639412	Aspn	Asporin	-1.90669	-3.74947	0.029180
1368477_at	NM_012914	Atp2a3	ATPase, Ca++ transporting, ubiquitous	-1.60464	-3.04119	0.000593
1369664_at	NM_053019	Avpr1a	Arginine vasopressin receptor 1A	-2.07931	-4.22605	1.62E-05
1375941_at	BI292120	Baiap2l1	BAI1-associated protein 2-like 1	-1.64069	-3.11816	1.70E-06
1369807_at	NM_030851	Bdkrb1	Bradykinin receptor B1	-1.11926	-2.17236	0.000155
1391345_at	BI293047	Bmper	BMP-binding endothelial regulator	-1.85131	-3.60828	3.59E-06
1387540_at	NM_012514	Brca1	Breast cancer 1	-1.02475	-2.03461	0.000898
1381995_at	AW530502	Brunol4	Bruno-like 4, RNA binding protein (Drosophila)	-2.20211	-4.60152	7.21E-08
1387893_at	D88250	C1s	Complement component 1, s subcomponent	-1.26395	-2.40152	0.048179
1375569_at	BM386267	Ccdc92	Coiled-coil domain containing 92	-1.03224	-2.04520	0.000197
1367973_at	NM_031530	Ccl2	Chemokine (C-C motif) ligand 2	-2.02826	-4.07912	0.002595
1379935_at	BF419899	Ccl7	Chemokine (C-C motif) ligand 7	-1.07909	-2.11271	0.006482
1370810_at	L09752	Ccnd2	Cyclin D2	-1.31667	-2.49091	0.005098
1389490_at	BI274335	Cd248	CD248 molecule, endosialin	-3.64068	-12.47250	1.26E-06
1389755_at	BM391858	Cdca7l	Cell division cycle associated 7 like	-1.44180	-2.71661	0.000141
1369425_at	NM_138889	Cdh13	Cadherin 13	-3.44410	-10.88370	4.56E-08
1375719_s_at	BG381748	Cdh13	Cadherin 13	-3.20680	-9.23301	2.00E-08
1373102_at	BI282750	Cdh13	Cadherin 13	-3.03097	-8.17360	6.92E-06
1373054_at	AA801076	Cdw92	CDW92 antigen	-1.00321	-2.00445	0.000362
1396034_at	BF402373	Ces7	Carboxylesterase 7	-2.24147	-4.72879	7.58E-05
1389179_at	BF284899	Cidea	Cell death-inducing DNA fragmentation factor, α subunit-like effector A	-1.13966	-2.20329	1.12E-05
1367740_at	M14400	Ckb	Creatine kinase, brain	-1.40456	-2.64738	0.000293
1392672_at	AI576758	Clec11a	C-type lectin domain family 11, member a	-1.45028	-2.73262	0.004619
1368571_at	NM_021997	Clip2	CAP-GLY domain containing linker protein 2	-1.09569	-2.13715	4.77E-07
1372584_at	BG672517	Cnrip1	Cannabinoid receptor interacting protein 1	-1.11448	-2.16517	1.59E-08
1379345_at	BM386752	Col15a1	Collagen, type XV, α 1	-6.09695	-68.44850	3.18E-06
1388939_at	AA800298	Col15a1	Collagen, type XV, α 1	-4.48588	-22.4071	2.71E-05
1384969_at	BE109107	Col24a1	Collagen, type XXIV, α 1	-1.82710	-3.54824	0.002725
1371349_at	AI598402	Col6a1	Collagen, type Ⅵ, α 1	-1.66097	-3.16228	0.001251
1371369_at	BI287851	Col6a2	Collagen, type Ⅵ, α 2	-1.80919	-3.50445	9.31E-05
1372818_at	BI284441	Colec12	Collectin sub-family member 12	-2.38156	-5.21100	3.25E-07
1372774_at	AI170570	Coq6	Coenzyme Q6 homolog (yeast)	-1.57329	-2.97583	1.94E-06
1369964_at	NM_130411	Coro1a	Coronin, actin binding protein 1A	-1.66410	-3.16916	7.57E-05
1392996_at	BG668435	Cpeb1	Cytoplasmic polyadenylation element binding protein 1	-1.42707	-2.68901	3.55E-05
1368293_at	NM_031766	Cpz	Carboxypeptidase Z	-1.17127	-2.25210	0.020505
1376051_at	BI293393	Cryl1	Crystallin, lambda 1	-2.8568	-7.24406	4.30E-05
1383575_at	BG376561	Ctnnd2	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	-2.38434	-5.22105	9.56E-05
1369947_at	NM_031560	Ctsk	Cathepsin K	-1.00677	-2.00941	0.001608
1387969_at	U22520	Cxcl10	Chemokine (C-X-C motif) ligand 10	-2.22859	-4.68674	4.90E-05
1368738_at	D11354	Cyp11b1	Cytochrome P450, subfamily 11B, polypeptide 1	-1.73100	-3.31958	0.003738
1387305_s_at	NM_012539	Cyp11b1 /// Cyp11b2	Cytochrome P450, subfamily 11B, polypeptide 1 /// cytochrome P450, subfamily 11B, polypeptide 2	-1.73027	-3.31790	4.54E-05
1387276_at	NM_021584	Dclk1	Doublecortin-like kinase 1	-1.05016	-2.07076	0.000907
1384971_at	BI289108	Depdc7	DEP domain containing 7	-1.10481	-2.15070	0.000468
1371732_at	BI285485	Dpt	Dermatopontin	-1.89757	-3.72585	0.009977
1383853_at	BE103067	Dyrk3	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	-1.76949	-3.40934	1.66E-07
1383641_at	BF414702	Ednra	Endothelin receptor type A	-2.19922	-4.5923	0.014670
1378342_at	BF284819	Ednra	Endothelin receptor type A	-1.78129	-3.43733	0.004867
1393415_at	BF548891	Ednra	Endothelin receptor type A	-1.51332	-2.85467	0.007430
1391442_at	AA957585	Ehd3	EH-domain containing 3	-1.71662	-3.28665	3.90E-05
1367905_at	NM_019370	Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	-1.33397	-2.52096	9.07E-05
1382434_at	AI059015	Entpd5	Ectonucleoside triphosphate diphosphohydrolase 5	-1.63931	-3.11518	2.50E-06
1370503_s_at	AB032828	Epb4.1l3	Erythrocyte protein band 4.1-like 3	-1.94250	-3.84372	0.005443
1368515_at	NM_053927	Epb4.1l3	Erythrocyte protein band 4.1-like 3	-1.52656	-2.88098	0.000173
1369422_at	NM_138850	Fap	Fibroblast activation protein, α	-1.69721	-3.24274	0.001039
1376561_at	AW523739	Fbxo16	F-box protein 16	-1.11142	-2.16058	6.95E-06
1367850_at	NM_053843	Fcgr2a /// LOC498276 /// LOC498277	Fc fragment of IgG, low affinity IIa, receptor (CD32) /// Fc γ receptor Ⅱ β /// similar to Low affinity immunoglobulin γ Fc region receptor Ⅲ precursor (IgG Fc receptor Ⅲ) (Fc-γ RⅢ) (FcRⅢ)	-1.23098	-2.34726	0.007866
1392865_at	BG371594	Fgf9	Fibroblast growth factor 9	-2.37371	-5.18274	0.002748
1373882_at	AI170324	Figf	C-fos induced growth factor	-2.48251	-5.58869	0.000198
1387709_at	AY032728	Figf	C-fos induced growth factor	-2.21994	-4.65873	5.44E-07

1374726_at	AI411941	Fndc1	Fibronectin type Ⅲ domain containing 1	-2.18189	-4.53749	0.005581
1370248_at	AA851939	Fxyd6	FXYD domain-containing ion transport regulator 6	-1.91583	-3.77331	0.004165
1385636_at	AI029226	Fzd3	Frizzled homolog 3 (Drosophila)	-1.30152	-2.46488	6.39E-06
1388395_at	AI406939	G0s2	G0/G1switch 2	-1.69782	-3.24410	6.68E-05
1382314_at	BE096523	G1p2	Interferon, α-inducible protein (clone IFI-15K)	-1.43064	-2.69565	0.012403
1370963_at	AJ131902	Gas7	Growth arrest specific 7	-2.12746	-4.36946	0.001866
1387221_at	NM_024356	Gch1	GTP cyclohydrolase 1	-1.03450	-2.04840	0.001025
1368085_at	NM_133595	Gchfr	GTP cyclohydrolase I feedback regulator	-1.09278	-2.13284	1.23E-05
1368770_at	NM_022276	Gcnt1	Glucosaminyl (N-acetyl) transferase 1, core 2	-1.35748	-2.56237	0.000674
1387659_at	AF245172	Gda	Guanine deaminase	-1.54413	-2.91629	0.000209
1377761_at	BI296057	Gfpt2	Glutamine-fructose-6-phosphate transaminase 2	-2.38870	-5.23685	0.003422
1387007_at	NM_012959	Gfra1	GDNF family receptor α 1	-1.49726	-2.82306	0.000202
1367954_at	U59486	Gfra1	GDNF family receptor α 1	-1.10817	-2.15573	0.000600
1397461_at	BF416400	Glt8d2	Glycosyltransferase 8 domain containing 2	-1.11036	-2.15899	8.28E-06
1386870_at	BI275294	Glul	Glutamate-ammonia ligase (glutamine synthetase)	-1.18385	-2.27182	0.000113
1367632_at	NM_017073	Glul	Glutamate-ammonia ligase (glutamine synthetase)	-1.11961	-2.17288	0.003082
1369302_at	NM_133573	Gper	G protein-coupled estrogen receptor 1	-1.10018	-2.14381	0.001057
1387241_at	NM_031696	Gpr88	G-protein coupled receptor 88	-1.11226	-2.16184	0.002865
1369926_at	NM_022525	Gpx3	Glutathione peroxidase 3	-2.05946	-4.16829	1.35E-06
1374488_at	AI175700	Gramd1b	GRAM domain containing 1B	-1.12881	-2.18678	0.001788
1368180_s_at	NM_017013	Gsta2 /// LOC494499	Glutathione S-transferase A2 /// LOC494499 protein	-2.31111	-4.96266	6.68E-05
1371298_at	BF284168	H19	H19 fetal liver mRNA	-1.87119	-3.65833	0.000303
1391575_at	BG380566	Hapln4	Hyaluronan and proteoglycan link protein 4	-1.12321	-2.17831	0.002326
1368255_at	NM_017354	Hnt	Neurotrimin	-2.33813	-5.05648	0.004300
1367816_at	NM_133621	Hopx	HOP homeobox	-1.32323	-2.50226	0.003008
1393592_at	AA998087	Hs3st5	Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	-1.19206	-2.28479	0.004675
1368578_at	NM_017265	Hsd3b1	Hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1	-2.13200	-4.38324	0.000250
1387282_at	NM_053612	Hspb8	Heat shock protein 8	-1.47457	-2.77901	2.14E-07
1388721_at	BG380282	Hspb8	Heat shock protein 8	-1.28154	-2.43098	1.12E-06
1376908_at	AW531805	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	-1.35357	-2.55544	0.019235
1382220_at	AI180454	Igf2bp2	Insulin-like growth factor 2 mRNA binding protein 2	-1.40203	-2.64272	0.007370
1387180_at	NM_053953	Il1r2	Interleukin 1 receptor, type II	-2.39783	-5.27012	0.000126
1387273_at	NM_013037	Il1rl1	Interleukin 1 receptor-like 1	-3.65216	-12.57210	0.013461
1370692_at	U04317	Il1rl1	Interleukin 1 receptor-like 1	-1.22822	-2.34278	0.004082
1387504_at	NM_133575	Il1rl2	Interleukin 1 receptor-like 2	-1.52580	-2.87946	7.08E-05
1377163_at	BM385741	Inhbb	Inhibin β-B	-1.23015	-2.34591	0.014339
1369043_at	NM_012971	Kcna4	Potassium voltage-gated channel, shaker-related subfamily, member 4	-4.17720	-18.09100	8.01E-08
1390404_at	BF556962	Lama2	Laminin, α 2	-2.54141	-5.82159	9.68E-05
1370138_at	NM_130429	Lef1	Lymphoid enhancer binding factor 1	-1.10908	-2.15708	0.000657
1378179_a_at	AW524864	Lhfpl2	Lipoma HMGIC fusion partner-like 2	-1.03801	-2.05339	0.002170
1371094_at	L06804	Lhx2	LIM homeobox 2	-1.06797	-2.09648	0.000653
1389885_at	BI294855	Limd2	LIM domain containing 2	-1.05098	-2.07194	6.73E-05
1376871_at	AA891475	LOC680910 /// LOC681069 /// LOC681182 /// LOC681196 /// LOC685030 /// LOC685048 /// LOC685111 /// LOC685262 /// LOC685305 /// LOC686848 /// LOC686899 /// RGD1559588 /// RGD1561143 /// RGD1561730 /// RGD1562525 /// RGD1563400 /// RGD1566006	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β	-1.06939	-2.09855	2.14E-06
1385047_x_at	AI012782	LOC685048 /// LOC685111 /// RGD1559588 /// Vom2r61	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// vomeronasal 2 receptor, 61	-2.82049	-7.06400	5.90E-06
1393688_at	AI012782	LOC685048 /// LOC685111 /// RGD1559588 /// Vom2r61	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// vomeronasal 2 receptor, 61	-2.75933	-6.77080	2.26E-07

1371293_at	AI103218	LOC688228	Similar to Myosin light polypeptide 4 (Myosin light chain 1, atrial isoform)	-1.30459	-2.47013	6.68E-07
1398732_at	BF553297	LOC688273	Hypothetical protein LOC688273	-3.37961	-10.40790	0.000276
1384540_at	BE101066	Lrfn3	Leucine rich repeat and fibronectin type III domain containing 3	-1.05498	-2.07769	0.000430
1388347_at	AI233210	Ly6e	Lymphocyte antigen 6 complex, locus E	-2.76880	-6.81539	1.82E-05
1376184_at	BG381127	Lynx1	Ly6/neurotoxin 1	-1.98858	-3.96846	8.46E-08
1393645_at	BI288003	Mageb16	Melanoma antigen family B, 16	-1.53459	-2.89706	0.000131
1388152_at	BG374290	Map2	Microtubule-associated protein 2	-1.77330	-3.41836	0.008563
1382046_at	AA963495	Map3k3	Mitogen activated protein kinase kinase kinase 3	-1.24191	-2.36511	1.42E-06
1392547_at	AI716211	MGC105649	Hypothetical LOC302884	-2.31654	-4.98136	0.004157
1388300_at	AA892234	Mgst3	Microsomal glutathione S-transferase 3	-1.00700	-2.00973	0.017898
1393836_at	BE097933	Mitf	Microphthalmia-associated transcription factor	-1.09560	-2.13702	0.010323
1368590_at	NM_080776	Mmp16	Matrix metallopeptidase 16	-1.01729	-2.02411	0.000829
1370301_at	U65656	Mmp2	Matrix metallopeptidase 2	-3.98031	-15.78310	0.000568
1382190_at	BF405725	Mrgprf	MAS-related GPR, member F	-3.60572	-12.17390	3.94E-07
1368441_at	NM_031658	Msln	Mesothelin	-1.36403	-2.57403	0.001477
1376648_at	BI275570	Mycn	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-1.62357	-3.08136	0.002089
1368415_at	NM_012604	Myh3	Myosin, heavy chain 3, skeletal muscle, embryonic	-1.24039	-2.36262	0.003414
1387787_at	NM_012605	Mylpf	Myosin light chain, phosphorylatable, fast skeletal muscle	-2.12252	-4.35453	0.000567
1398655_at	AA955902	Myod1	Myogenic differentiation 1	-2.39143	-5.24677	9.65E-05
1373839_at	BG372386	Nope	Neighbor of Punc E11	-1.24338	-2.36752	0.009522
1371036_at	BG671431	Nrcam	Neuronal cell adhesion molecule	-1.60969	-3.05187	2.40E-06
1384112_at	BI289470	Nt5e	5' nucleotidase, ecto	-1.12050	-2.17423	0.004069
1392780_at	BF283270	Nxf7	nuclear RNA export factor 7	-3.39721	-10.53570	0.005424
1377497_at	BF419319	Oasl	2'-5'-oligoadenylate synthetase-like	-1.36533	-2.57636	2.34E-05
1369008_a_at	NM_053573	Olfm1	Olfactomedin 1	-3.58878	-12.0318	0.000169
1368940_at	NM_017255	P2ry2	Purinergic receptor P2Y, G-protein coupled 2	-1.21116	-2.31523	0.009814
1383273_a_at	AA956005	Pcbp3	Poly(rC) binding protein 3	-2.10678	-4.30729	4.21E-06
1383274_at	AA956005	Pcbp3	Poly(rC) binding protein 3	-1.86620	-3.64572	0.000552
1385116_at	BF386807	Pcdhb21	Protocadherin β 21	-1.13304	-2.19321	0.007625
1373368_at	BI279680	PCOLCE2	Procollagen C-endopeptidase enhancer 2	-1.80500	-3.49430	7.61E-07
1368145_at	NM_013002	Pcp4	Purkinje cell protein 4	-4.28374	-19.47750	0.000401
1370941_at	AI232379	Pdgfra	Platelet derived growth factor receptor, α polypeptide	-1.62538	-3.08523	0.011837
1377100_at	AI172172	Pds5b	PDS5, regulator of cohesion maintenance, homolog B (S. cerevisiae)	-1.14971	-2.21869	1.40E-05
1388634_at	BI277505	Pgm1	Phosphoglucomutase 1	-1.66935	-3.18071	9.06E-09
1369473_at	NM_017033	Pgm1	Phosphoglucomutase 1	-1.50694	-2.84207	3.02E-06
1383749_at	AI112954	Phospho1	Phosphatase, orphan 1	-1.16307	-2.23934	0.003657
1370445_at	D88666	Pla1a	Phospholipase A1 member A	-1.35014	-2.54937	0.001836
1390190_at	BI293691	Plac1	Placenta-specific 1	-2.19087	-4.56582	0.000174
1384558_at	BI276313	Plac9	Placenta-specific 9	-1.07069	-2.10043	0.007797
1367800_at	NM_013151	Plat	Plasminogen activator, tissue	-1.15024	-2.21951	0.004677
1391187_at	BI303019	Ppl	Periplakin	-1.11393	-2.16434	0.000262
1368259_at	NM_017043	Ptgs1	Prostaglandin-endoperoxide synthase 1	-2.42323	-5.36370	3.32E-05
1381806_at	BF418208	Ptgs1	Prostaglandin-endoperoxide synthase 1	-1.06169	-2.08737	5.08E-05
1372084_at	AI104546	Ptp4a3	Protein tyrosine phosphatase 4a3	-1.02120	-2.02961	2.54E-05
1368350_at	NM_013080	Ptprz1	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	-1.04165	-2.05858	0.022268
1373646_at	BM384841	Rab15	RAB15, member RAS oncogene family	-1.17275	-2.25440	7.21E-05
1374035_at	BI296482	Rem2	RAS (RAD and GEM) like GTP binding 2	-1.00521	-2.00723	0.017185
1368080_at	NM_054008	Rgc32	Response gene to complement 32	-1.00087	-2.00121	0.032818
1392883_at	AI013730	RGD1305269	Similar to hypothetical protein	-1.04588	-2.06463	1.81E-05
1373226_at	BF400995	RGD1308019	Similar to hypothetical protein FLJ20245	-1.10844	-2.15613	0.015649
1381757_at	AA965058	RGD1309501	Hypothetical LOC305552	-1.28517	-2.43712	2.17E-05
1373596_at	AI230766	RGD1310423	Similar to hypothetical protein FLJ31737	-2.01177	-4.03277	6.86E-08
1398577_at	BI297744	RGD1310507	Similar to RIKEN cDNA 1300017J02	-1.72535	-3.30661	0.000821
1390397_at	BF413152	RGD1310753	Similar to chromosome 20 open reading frame 39	-2.10856	-4.31261	5.11E-05
1393191_at	BF554733	RGD1561205	Similar to RIKEN cDNA 2610200G18	-1.39361	-2.62736	0.000183
1376693_at	AA998964	RGD1563091	Similar to OEF2	-1.00045	-2.00063	0.047169
1395145_at	BF544481	Rgl1	Ral guanine nucleotide dissociation stimulator,-like 1	-1.54730	-2.92270	0.000329
1394472_at	BF282814	Rgl1	Ral guanine nucleotide dissociation stimulator,-like 1	-1.20268	-2.30166	0.005425
1391075_at	AI179271	Rgs17	Regulator of G-protein signaling 17	-1.50436	-2.83699	0.004883
1368373_at	NM_019343	Rgs7	Regulator of G-protein signaling 7	-3.29254	-9.79836	1.82E-05
1370142_at	NM_022175	Rhox5	Reproductive homeobox 5	-3.12793	-8.74178	2.44E-09
1383554_at	AW142796	Rnf128	Ring finger protein 128	-1.47300	-2.77598	1.47E-05
1389735_at	BE107296	Rps6ka6	Ribosomal protein S6 kinase polypeptide 6	-1.18452	-2.27288	0.013943
1384707_at	AI600020	Scara5	Scavenger receptor class A, member 5 (putative)	-1.46379	-2.75833	9.19E-06
1392856_at	AI549470	Serf1	Small EDRK-rich factor 1	-1.52508	-2.87802	0.000710
1375084_at	BF419780	Serinc2	Serine incorporator 2	-1.64207	-3.12113	0.000591

1377034_at	BF411331	Serpinb1a	Serine (or cysteine) proteinase inhibitor, clade B, member 1a	-1.17504	-2.25799	8.45E-05
1369547_at	NM_130404	Serpinb7	Serine (or cysteine) peptidase inhibitor, clade B, member 7	-1.40940	-2.65626	0.001746
1393620_at	AI113325	Sesn3	Sestrin 3	-1.17758	-2.26198	0.001718
1390119_at	BF396602	Sfrp2	Secreted frizzled-related protein 2	-1.64679	-3.13135	0.011642
1367881_at	NM_013016	Sirpa	Signal-regulatory protein α	-1.88267	-3.68756	1.79E-06
1392789_at	BI296353	Slc25a36	Solute carrier family 25, member 36	-2.25714	-4.78042	4.14E-05
1372341_at	AI233213	Slc25a36	Solute carrier family 25, member 36	-2.07958	-4.22685	0.000633
1369237_at	NM_053996	Slc6a7	Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	-1.12269	-2.17753	0.001140
1368322_at	NM_012880	Sod3	Superoxide dismutase 3, extracellular	-2.20485	-4.61028	1.15E-05
1368254_a_at	AB049572	Sphk1	Sphingosine kinase 1	-1.09627	-2.13802	0.004730
1368655_at	NM_020074	Srgn	Serglycin	-2.51283	-5.70737	0.006656
1373146_at	AI716240	Ssx2ip	Synovial sarcoma, X breakpoint 2 interacting protein	-1.23239	-2.34956	0.001168
1387174_a_at	AB006007	Star	Steroidogenic acute regulatory protein	-3.29954	-9.84599	0.000194
1368406_at	NM_031558	Star	Steroidogenic acute regulatory protein	-2.92085	-7.57291	7.29E-07
1377672_at	BI300997	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	-3.45192	-10.94290	3.19E-06
1369531_at	NM_133547	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	-2.77927	-6.86504	2.07E-05
1369627_at	L10362	Sv2b	Synaptic vesicle glycoprotein 2b	-2.39744	-5.26866	0.000178
1385637_at	AI029494	Svep1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	-1.08524	-2.12172	0.000482
1383686_at	BE111537	Syngr1	Synaptogyrin 1	-1.73204	-3.32197	0.005240
1384202_at	BI287326	Tesc	Tescalcin	-1.69209	-3.23124	0.004681
1371913_at	BG379319	Tgfbi	Transforming growth factor, β induced	-1.05453	-2.07704	0.023537
1369652_at	AI145313	Thy1	Thy-1 cell surface antigen	-3.93458	-15.29070	2.03E-07
1369651_at	NM_012673	Thy1	Thy-1 cell surface antigen	-3.67268	-12.75220	7.68E-08
1392980_at	AI716456	Tiam1	T-cell lymphoma invasion and metastasis 1	-1.56449	-2.95774	7.40E-06
1382222_at	BI293607	Tmem163	Transmembrane protein 163	-2.11820	-4.34153	8.73E-06
1376106_at	AI010157	Tmem178	Transmembrane protein 178	-3.28823	-9.76912	0.007054
1377554_at	BF394106	Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	-1.95931	-3.88877	1.88E-05
1370332_at	AF159356	Unc13d	Unc-13 homolog D (C. elegans)	-2.36452	-5.14980	0.000313
1368474_at	NM_012889	Vcam1	Vascular cell adhesion molecule 1	-3.24587	-9.48643	1.90E-05
1388142_at	AA850991	Vcan	Versican	-1.54149	-2.91096	0.000288
1388054_a_at	AF072892	Vcan	Versican	-1.53261	-2.89308	0.000536
1371232_a_at	AF084544	Vcan	Versican	-1.47614	-2.78204	0.000764
1388265_x_at	AF084544	Vcan	Versican	-1.45114	-2.73423	0.001864
1389253_at	BI289085	Vnn1	Vanin 1	-1.15392	-2.22518	0.000172
1382283_at	BF283711	Wipf1	WAS/WASL interacting protein family, member 1	-1.01562	-2.02176	3.13E-05
1387227_at	NM_057192	Wipf1	WAS/WASL interacting protein family, member 1	-1.00508	-2.00706	8.16E-05
1389119_at	AI105018	Xirp1	Xin actin-binding repeat containing 1	-1.17137	-2.25226	9.90E-05
1372989_at	BI296586	Zdhhc14	Zinc finger, DHHC-type containing 14	-3.38007	-10.4112	1.23E-07

ACKNOWLEDGMENTS

The authors would like to thank the Histopathology Unit at the Biopolis Shared Facilities for the expert hematoxylin and eosin and SMAA staining.

COMMENTS

Background

miRNAs are a relatively new and exciting tool to control the expression of multiple genes. During liver injury and subsequent wound healing involving hepatic stellate cells (HSCs), complex regulatory processes occur and have to be tightly regulated in this cell type. miRNAs could be one tool to control these processes, and therefore, it is of interest to the research community to gain information about the expression of miRNAs during liver fibrosis in HSCs.

Research frontiers

Liver fibrosis and subsequently cirrhosis are common outcomes of chronic injuries to the liver. HSCs are involved in liver fibrosis and repair. The tools for the treatment of liver fibrosis are limited and are still under development. In this study, the authors aimed to gain information for the possible role of miRNAs in liver fibrosis and whether they could become a future tool to develop a treatment for liver fibrosis by addressing the changes in HSCs.

Innovations and breakthroughs

Different publications have analyzed the miRNA expression in HSCs *in vitro* and studied the effect of various differentially regulated miRNAs in HSCs. The authors analyzed the miRNA expression in an *in vivo* model of hepatic fibrosis, namely choline-deficient ethionine supplemented diet. Furthermore, they studied the transcriptome changes upon overexpression of miR-146a and found that, in particular, tissue inhibitor of metalloproteinase-3 showed robust up-regulation, a hitherto unreported effect, which emphasizes its involvement in inflammation. Another important finding was the dynamics of miRNA regulation during the *in vitro* activation of HSCs.

Applications

miRNAs are becoming a promising tool for the regulation of gene expression. In order to use this tool, it is necessary to understand the role and regulation of the targeted miRNA. In this study, the authors describe the dynamic regulation of specific miRNAs. The results of this study show clearly that the use of miRNAs as target molecules will have to take this dynamic component into consideration. The same is valid for the use of miRNAs as therapeutic agents.

Terminology

miRNAs are small non-coding RNAs that are about 23 nucleotides long. The versatility of miRNAs depends on the imperfect binding (seed region) to the 3'-UTR of mRNAs. This imperfect binding results in many different binding partners. The regulation by miRNAs leads to a translational repression and/or mRNA destabilization.

Peer review

The field of miRNA research as well as HSC activation mechanisms are very up to date and important areas of research, in order to find new strategies against liver fibrosis. The methods used are comprehensive and convincing. In all, the study was fairly well conducted and interesting.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i22.2774

World J Gastroenterol 2011 June 14; 17(22): 2774-2780
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Influence of chitosan nanofiber scaffold on porcine endogenous retroviral expression and infectivity in pig hepatocytes

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Supported by The Natural Science Foundation of Jiangsu Province, No. BK2006008; the foundation of Medical Center of Jiangsu Province, No.ZX200605
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Received: September 15, 2010 Revised: November 15, 2010
Accepted: November 22, 2010
Published online: June 14, 2011

Abstract

AIM: To investigate the influence of chitosan nanofiber scaffold on the production and infectivity of porcine endogenous retrovirus (PERV) expressed by porcine hepatocytes.

METHODS: Freshly isolated porcine hepatocytes were cultured with or without chitosan nanofiber scaffold (defined as Nano group and Hep group) for 7 d. The daily collection of culture medium was used to detect reverse transcriptase (RT) activity with RT activity assay kits and PERV RNA by reverse transcription-polymerase chain reaction (PCR) and real time PCR with the PERV specific primers. And Western blotting was performed with the lysates of daily retrieved cells to determine the PERV protein gag p30. Besides, the *in-vitro* infectivity of the supernatant was tested by incubating the human embryo kidney 293 (HEK293) cells.

RESULTS: The similar changing trends between two groups were observed in real time PCR, RT activity assay and Western blotting. Two peaks of PERV expression at 10H and Day 2 were found and followed by a regular decline. No significant difference was found between two groups except the significantly high level of PERV RNA at Day 6 and PERV protein at Day 5 in Nano group than that in Hep group. And in the *in-vitro* infection experiment, no HEK293 cell was infected by the supernatant.

CONCLUSION: Chitosan nanofiber scaffold might prolong the PERV secreting time in pig hepatocytes but would not obviously influence its productive amount and infectivity, so it could be applied in the bioartificial liver without the increased risk of the virus transmission.

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Key words: Chitosan nanofiber scaffold; Porcine hepatocyte; Porcine endogenous retrovirus; Bioartificial liver

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Han B, Shi XL, Xiao JQ, Zhang Y, Chu XH, Gu JY, Tan JJ, Gu ZZ, Ding YT. Influence of chitosan nanofiber scaffold on porcine endogenous retroviral expression and infectivity in pig hepatocytes. *World J Gastroenterol* 2011; 17(22): 2774-2780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i22/2774.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i22.2774

INTRODUCTION

Although liver transplantation is currently recognized as the most effective treatment for acute liver failure and end-stage liver diseases, its application has been seriously limited because of the lack of donor organs[1,2]. Therefore, bioartificial liver (BAL) has been proposed as a temporary liver support for patients awaiting liver transplantation[3-5].

At present, porcine hepatocytes were still a major cell source because of their adequate resources, accessibility and characters similar to human hepatocytes[6-8]. However, the clinical application of BAL based on porcine hepatocytes was not very optimistic[4]. So how to increase the *in-vitro* function of hepatocytes with a suitable scaffold was always an attractive issue and some previous reports showed the enhanced function of procine hepatocytes with their scaffold, including the chitosan nanofiber scaffold developed in our institute[9-11].

Nevertheless, as xenogeneic cells, there were some problems with porcine hepatocytes. The security of the transmission of porcine endogenous retrovirus (PERV) has been one of the most essential concerns that can not be ignored, since PERV infection of human cells *in vitro* was widely reported[12]. Therefore, it raised new questions that whether the production and infectivity of PERV would be influenced and whether the risk of PERV infection in BAL would be increased when the function of porcine hepatocytes was enhanced with certain scaffolds.

Previously, we had proved the chitosan nanofiber scaffold could enhanced hepatocytes adhesion, viability and function *in vitro*[13]. This study was focused on the influence of chitosan nanofiber scaffold on PERV expression and infectivity in pig hepatocytes.

MATERIALS AND METHODS

Animals and reagents

Outbred white pigs with an average weight of 15-20 kg received humane care and all animal procedures were performed according to institutional and national guidelines and approved by the Animal Care Ethics Committee of Nanjing University and Nanjing Drum Tower Hospital. All cell culture-related reagents were purchased from GIBCO (USA). Lactobionic acid (LA) and chitosan (low molecular weight, brookfield viscosity 20 000 cps, 85% deacetylation) were purchased from Sigma-Aldrich (Saint Louis, USA). N-Hydroxysuccinimide was purchased from Thermo-Pierce (Rockford, USA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide and N,N,N0N0-tetramethylethylenediamine were obtained from TCI (Tokyo, Japan). Poly(ethylenoxide) (PEO, MWz1_106) was supplied by Guoren Chemical Co. (Beijing, China). All other reagents were of analytical reagent grade.

Preparation of chitosan nanofiber scaffold via electrospinning

The chitosan nanofiber scaffold was prepared according to the previous reference[13]. In brief, Chitosan (Sigma) and PEO powders (9:1 w/w) were dissolved in formic acid/ethanol (7:3 v/v) to give 2.6% (w/v) at room temperature. The stock solution was then filled into a 5 mL glass syringe fitted with a 20 G needle and then expressed at 5 mL/h using a syringe pump. The nanofibers were collected on 24 mm diam. coverslips located at a fixed distance of 10-20 mm from the needle tip. A non-coated cover slip was also prepared and tested in the same manner as control.

Hepatocytes isolation, characterization and culture

Primary pig hepatocytes were harvested by a two-step *in situ* collagenase perfusion technique[14,15]. First, the prewarmed (37℃) Ca^2+ and Mg^2+ free Hanks balanced salt solution was perfused into the livers of the anesthetized pigs *in vivo via* the portal vein at a flow rate of 80-100 mL/min for 30-40 min, followed by 0.05% Type IV collagenase (37℃) at a rate of 10 mL/min for 40-50 min. The released cells were filtered through nylon mesh with 100-mm openings and washed *via* three centrifugations (50 g). The viability of the isolated primary hepatocytes determined by trypan blue exclusion was more than 95%. Nonparenchymal cells, as judged by their size (< 10 mm in diameter) and morphology (nonpolygonal or stellate), were less than 1%, which was also verified by immunocytochemical analysis of albumin and cytokeratin 18 (data not shown). Freshly isolated hepatocytes were seeded at a density of 10^6 cells/mL in the substratum of 2 mL RPMI-1640 without serum and incubated in 6-well microtiter plates with or without chitosan nanofiber scaffold (defined as Nano group and Hep group) at 37℃ and 5% CO_2. Culture medium was replenished daily without the growth medium containing low-glucose Dulbecco's modified Eagle's medium (DMEM-LG) supplemented with 10% fetal bovine serum (FBS) for 7 consecutive days. The daily collection of culture medium and cells were deposited at -80℃ for later use.

Reverse transcription-polymerase chain reaction

Total RNA was extracted respectively from the centrifuged supernatant with Trizol (Invitrogen, US) and then treated with DNase I (Invitrogen, US) according to the manufacturer's instructions. For each sample, 60 ng extracted RNA with the value of OD 260/280 among 1.60 and 2.00 was reversed transcribed to cDNA using the reverse transcriptase (RT) kits (Biouniquer, China) in accordance with the instructions. Then polymerase chain reaction (PCR) was completed with the protease-specific primers (forward, 5'-GCTACAACCATTAG-GAAAACTAAAAG-3'; and reverse, 5'-AACCAG-GACTGTATATCTTGATCAG-3'), polymerase-specific primers (forward, 5'-CTACAACCA TTAGGAAAACTA-AAAG-3'; and reverse, 5'-AACCAGGACTGTATATCTT-GATCAG-3'), and porcine glyceraldehyde 3-phosphate dehydrogenase-specific primers (forward, 5'-CATCAC-CATCTTCCAGGAG-3'; and reverse, 5'-TGCCCA-CAGCCTTGGCAGC-3') and its conditions were as follows: 50℃ for 30 min and then 95℃ for 5 min followed

by 35 cycles of 94℃ for 30 s, 55℃ for 45 s, 72℃ for 30 s, and a final extension step of 72℃ for 5 min[16,17]. Amplified production was detected by 2% agarose gel electrophoresis and ethidium bromide (EB) staining.

PERV-specific real time PCR

Quantitative real time PCR was performed with the obtained cDNA, the Roche SuperScript Ⅲ platinium system, the MX3000P thermocycler (Stratagene), and the primers specific for PERV protease gene (GenBank accession number U77599) (forward, 5'-AGTGCTGCTACAACCATTAGGAAA-3'; and reverse, 5'-AGGGATGACCAGAAACGAGTG-3') and for porcine β-actin gene (GenBank accession number DQ845171) (forward, 5'-GGACTTCGAGCAGGAGATGG-3', and reverse, 5'-AGGAAGGAGGGCTGGAAGAG-3').The conditions were as follows: 95℃ for 10min followed by 40 cycles of 95℃ for 15 s, 60℃ for 1 min and a final cycle of 95℃ for 1 min, 65℃ for 30 s, 95℃ for 30 s. PERV expression was normalized to the amount of the expression of the house-keeping genes β-actin and denoted with the relative value of protease/β-actin.

Western blotting analysis

The lysates of the cultured cells harvested every day were analysed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and Western blotting with polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). The membranes were then incubated with a 1:1500 dilution of mouse anti-FeLV p27 IgG antibody (ABcam, San Francisco, US) which had been proven cross-reactivity with protein gag p30 of PERV[18] overnight at 4℃, followed by a 1:10000 dilution of goat protein anti-mouse IgG conjugated horseradish peroxidase (KeyGEN, Nanjing, China) for 1 h at 37℃. Immunoreactive proteins on membranes were detected with the enhanced chemiluminescence system and exposured for 15 to 20 s on hyperfilm ECL (Kodak, US) Meanwhile, The protein β-actin was detected as controls. Software ImageJ was used to measure the lightness of each band.

In vitro infection experiments

The *in vitro* infection experiments were performed according to the reference with some modification[19,20]. Human embryo kidney 293 (HEK293) cells (as gifts from Professor Hua, Nanjing University) were passed overnight in 24-well plates and then incubated in the supernatant of the cell culture (0.5 mL/well) for 6 h in the presence of 8 mg/mL of polybrene after the supernatant was centrifuged at a speed of 5000 r/min for 5 min to remove the cells and cell debris. Meanwhile, the supernatant of porcine kidney 15 (PK15) cells and 0.8 g/mL polybrene was inoculated into the culture of HEK293 cells as positive control. After 4 h of exposure at 37℃, the incubating medium was removed and the cell monolayer was washed with phosphate buffered solution for two times. Then the cells were cultured with the DMEM-HG supplemented with 10% FBS and passed upon confluence for 1 mo before collection.

DNA extraction and PCR

Total DNA was extracted from the treated HEK293 cells with the DNA extracting kits (Axygen, California, US) according to their instructions. PCR was completed with the human β-actin primers (forward, 5'-GCTCGTCGTCGACAACGGCTC-3'; and reverse, 5'-CAAACATGATCTGGGTCATCTTCTC-3'), Sus scrofa cytochrome B (SsCytB) primers (forward, 5'-CATTGGAGTAGTCCTACTATTTACCG-3'; and reverse, 5'-GTAGGATTAGTATTATAAATAAGGCTCCT-3'), above mentioned protease-specific primers and polymerase-specific primers and its conditions consisted with the proceeding of PCR in reverse transcription-PCR (RT-PCR). Amplified production was detected by 2% agarose gel electrophoresis and EB staining.

RT activity assay

The RT activity of the supernatant from the cultured hepatocytes in both group and the treated HEK293, was detected by the C-type RT activity kits (Cavidi-Tech, Uppsala, Sweden) according to the quantitative and qualitative protocols of the manufacturer's instructions, respectively. The RT activity was examined twice for each collected supernatant sample.

Statistics

Both culture conditions of this study were completed in quintuplicate. All values were expressed as mean ± SD. The two-tailed unpaired Student's *t*-test was used to evaluate the statistical significance of differences which was set with a *P*-value less than 0.05.

RESULTS

PERV RNA in the supernatants

From the results of electrophoresis (shown in Figure 1A and B, representatively), it could be found that the changing trend of the protease sequence and the polymerase sequence was accordant. In real time PCR, the relative value of protease/β-actin was defined as the normalized amount of PERV RNA. Two PERV secreting peaks at 10H and Day 2 was observed, and then the amount of PERV RNA declined gradually after Day 2. No PERV expression was found after Day 6 in Hep group and Day 7 in Nano group, respectively (Figure 1C). There were significant differences at Day 6 between two groups (0.071 ± 0.0348 *vs* 0.014 ± 0.0193, $P < 0.05$).

Western blotting

Figure 2A demonstrated a representative result in Western blotting. In Hep group, positive gag p30 proteins were found at 10H and from Day 2 to Day 4, while in Nano group positive bands were observed at 10H and from Day 2 to Day 5. The lightness of each band was measured by the software ImageJ and the amount of the expressed

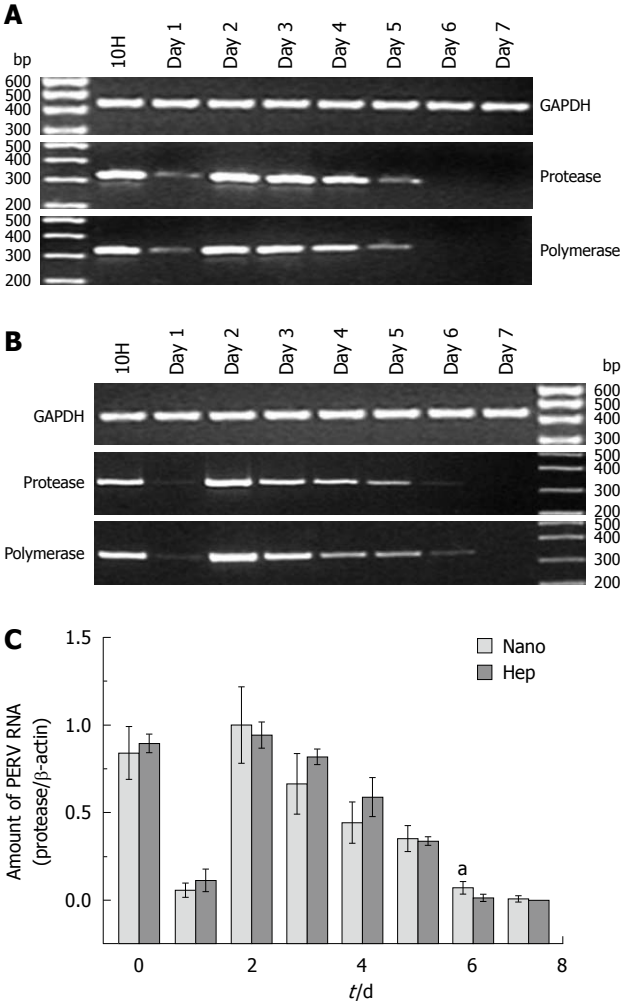

Figure 1 Porcine endogenous retrovirus RNA in the supernatants. A and B: The representative results of reverse transcription-polymerase chain reaction (PCR) electrophoresis with the RNA extracted from the supernatants in Hep group and Nano group, respectively; C: The results of real time PCR. $^{a}P < 0.05$. PERV: Porcine endogenous retrovirus; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

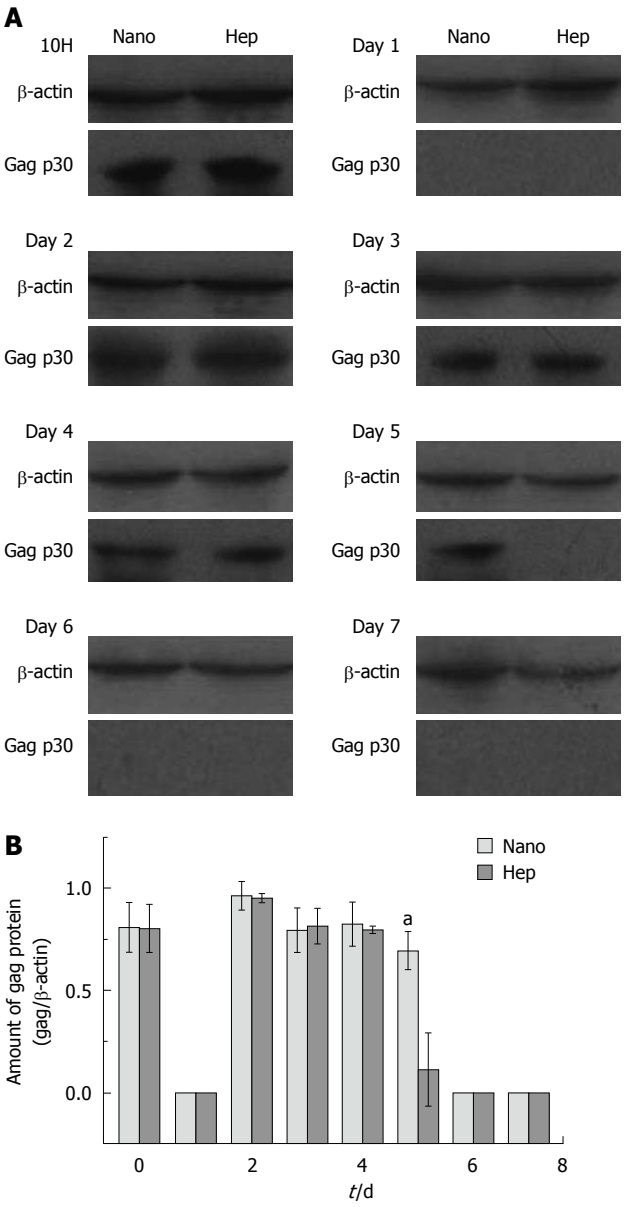

Figure 2 Western blotting of the porcine endogenous retrovirus gag protein in the cell lysates. A: Representative results of Western blotting with the cell lysates in both groups; B: The normalized protein amount in different days. $^{a}P < 0.05$.

gag p30 protein was normalized with the relative value of gag/β-actin, which was depicted in Figure 2B. Except for the significant differences found at Day 5 (0.70 ± 0.0929 *vs* 0.11 ± 0.180, $P < 0.01$), there were no remarkable differences between two groups in other days.

Infection of HEK293 cells in vitro

No PERV DNA sequence including protease and polymerase genes and SsCytB sequence were found in the HEK293 DNA (Figure 3). The DNA extracted from PK15 infected HEK293 cells and the pure water was used as positive control and negative control, respectively. And the RT activity in the culture supernatant of the treated HEK293 cells was all negative.

RT activity assay

The RT activity in the supernatants from the cultured hepatocytes was demonstrated in Figure 4. No significant difference was found between two groups in each day. And there was no positive RT activity in the supernatant of the HEK293 cells incubated with the supernatant from Hep group or from Nano group.

DISCUSSION

Porcine hepatocytes were still a major cell source for BAL at present[6-8]. How to enhance their function was always an attractive issue and some progress has been made[9-11]. The chitosan nanofiber scaffold fabricated in our previous study had been proved its superior abilities on cell adhesion and biocompatibility, thereby greatly improved the functions of hepatocytes[13]. However, there had been a new and un-ignorable issue that whether the porcine hepatocytes with improved function would secrete more

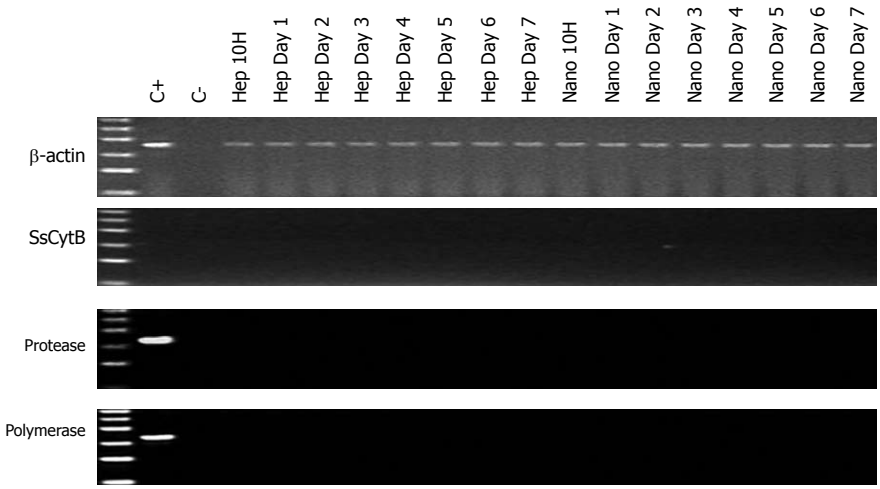

Figure 3 Representative results of polymerase chain reaction electrophoresis with the DNA extracted from human embryo kidney 293 cells of *in vitro* infection experiments. C+: Porcine endogenous retrovirus-infected human embryo kidney 293 cells; C-: Pure water. The ladder ranged from 100 to 600 bp. "Hep" and "Nano" meant the incubating supernatant from the simple culture of hepatocytes and the hepatocyte culture on chitosan nanofiber scaffolds. SsCytB: Sus scrofa cytochrome B.

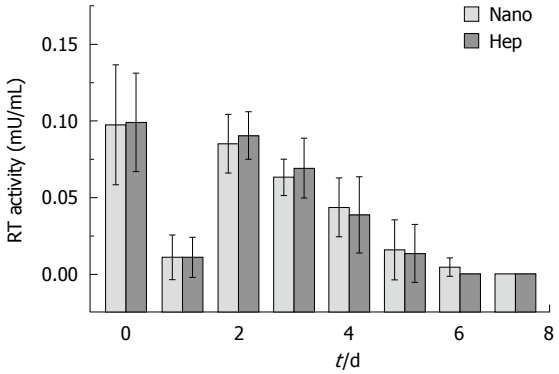

Figure 4 Reverse transcriptase activity of the cultured porcine hepatocytes on different substrata. RT: Reverse transcriptase.

PERV with enhanced infectivity, which might limit its application in BAL.

PERV was first discovered in 1971 in porcine kidney (PK15) cells[21]. In 1997, Patience *et al*[22] found for the first time that PERV released from PK15 could infect HEK293 cells *in vitro*. And then PERV is known to exist regularly in the porcine genome and various porcine cells can excrete PERV particulates[12]. Meanwhile, it was found that PERV successfully infected a variety of human cells *in vitro*, such as endothelial cells, fibroblasts and bone marrow stromal cells and so on, and virus replication was observed in some of these cells as well[23-28]. Nyberg *et al*[19] reported that freshly isolated porcine hepatocytes secreted the PERV which didn't infect HEK293 cells *in vitro*, at the same time, the production of PERV by cultured pig hepatocytes was unaffected by exposure to growth factors and cytokines present in human FHF sera. Fortunately, there was no evidence of PERV transmission into the patients treated with BAL so far[20,29-32]. However, a recent article[33] claimed PERV released from a BAL infected primary human cells by short-term contact of primary porcine liver cell supernatants with primary human cells, which increased the anxiety about the problem of PERV infection in the application of BAL.

In this study, we sought to find the influence of chitosan nanofiber scaffold on the production and infectivity of PERV expressed by porcine hepatocytes. PERV RNA, RT activity and PERV gag protein was detected for analysis of PERV production by porcine hepatocytes. At present, RT-PCR was the most specific and sensitive method for PERV detection[12]. And it had been identified that the RT activity was related with the retrovirus particles[34], so the result of low RT activity implied a small number of secreted PERV particles. In addition, in previous reports[18,35], sucrose gradient-purified PERV from the culture supernatant was used to qualitatively detect the protein gag p30 by Western blotting, but the condensed PERV was not suitable for hemi-quantitative analysis of the protein. Then we considered the supernatant as the samples directly and attempted to do all the test again. Much to our regret, no positive bands were observed. The possible reason may lay on the microamount of the virus. Therefore, the cell lysate was chosen as samples in Western blotting and the expressing level of the protein in cell lysate was detected to reflect the level of virus replication indirectly.

The results of real time PCR, RT activity assay and Western blotting presented a similar changing trend with some minor differences. Positive PERV RNA but no gag protein at Day 6 might result from less sensitivity of Western blotting. From these results, it could be seen that two peaks of PERV expression at 10H and Day 2 were followed by a regular decline in both groups, and no obviously increased expression level with chitosan nanofiber scaffold was found in first 5 d. The first secreting peak might be regarded as the expression of fresh

isolated hepatocytes with some contents of the ruptured non-adherent cells. In order to reduce the influence of the ruptured non-adherent cells, 10H was chosen as the first time point for detecting. But the second peak should be the real secreting peak and the low PERV level in Day 1 might be attributed to the short supernatant collecting interval between 10H and Day 1. On the other hand, the level of PERV RNA at Day 6 and the amount of PERV protein at Day 5 in Nano group was significantly more than that in Hep group, implying a prolonged expression time, which might be due to the superior activity maintaining of hepatocytes cultured on chitosan nanofiber scaffold[13].

The most important concern was no doubt the effect of chitosan nanofiber scaffold on the infectivity of PERV secreted by hepatocytes. Nyberg *et al*[19] had reported PERV released from hepatocytes didn't infect the HEK293 cells *in vitro* and its infectivity would not influenced by human fulminant hepatic failure sera. Likewise, our *in vitro* infection experiments demonstrated no PERV gene sequence and even no porcine specific SsCytB gene existed in the DNA of HEK293 cells, and no positive RT activity could be detected in the supernatant of treated HEK293 cells either, which implied no PERV transmission into the HEK293 cells and no microchimerism. So it could be concluded that there was no obvious infectivity of the PERV secreted by porcine hepatocytes.

In conclusion, porcine hepatocytes could express PERV for 5 d with normal culture condition, but the secreting time might be prolonged to 6 d when cells were cultured with chitosan nanofiber scaffold. Nevertheless, the productive amount and infectivity of the PERV expressed by porcine hepatocytes would not be obviously influenced by chitosan nanofiber scaffold. Therefore, it could be applied in BAL without enhanced risk of the virus infection.

COMMENTS

Background

Bioartificial liver (BAL) carrying porcine hepatocytes which were still a major cell source at present has been proposed as a temporary liver support for patients waiting for liver transplantation or even as a treatment for liver failure. However, the clinical application of BAL was not very optimistic. As a result of that, much progress had been made on cellular function improvement.

Research frontiers

Some scaffold materials were reported to be capable enhancing the *in vitro* function of hepatocytes. And chitosan nanofiber scaffold fabricated in our previous study had demonstrated superior abilities on cell adhesion and biocompatibility, thereby greatly enhanced the functions of hepatocytes. So it became a potential material which could be applied in BAL and a new bioreactor based on chitosan nanofiber scaffold was developed in our institute.

Innovations and breakthroughs

Although much progress had been made cellular function improvement, there were few researches on the influence of the materials on the expression and infectivity of porcine endogenous retrovirus (PERV) in porcine hepatocytes. So this study was focus on the safety of chitosan nanofiber scaffold, finding probable prolonging of PERV expression within chitosan nanofiber scaffold but no increased productive amount and infectivity.

Applications

The study identified no obvious influence of chitosan nanofiber scaffold on PERV expression and infectivity in porcine hepatocytes, and concluded that it could be applied in BAL without enhanced risk of the virus infection.

Terminology

PERV was a porcine specific C-type retrovirus which was capable infecting human cells *in vitro*. Chitosan nanofiber scaffold was a scaffold material made with chitosan by nanotechnology, and it was proved to enhance the hepatocyte function *in vitro*.

Peer review

Generally speaking, the paper presents a laboratory-based study in which a number of highly sophisticated procedures took place, especially in terms of the methods utilized to demonstrate the presence and infectivity of retroviral agent. In this sense, efforts made by the authors deserve particular attention, making this manuscript very interesting.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
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World J Gastroenterol 2011 June 14; 17(22): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 June 14; 17(22): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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

March 21-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

June 14-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, 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 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 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 J Gastroenterol 2011 June 14; 17(22): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Instructions to authors

GENERAL INFORMATION

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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 systemically 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 consensuses 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

ISSN and EISSN

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 Directory of Open Access Journals. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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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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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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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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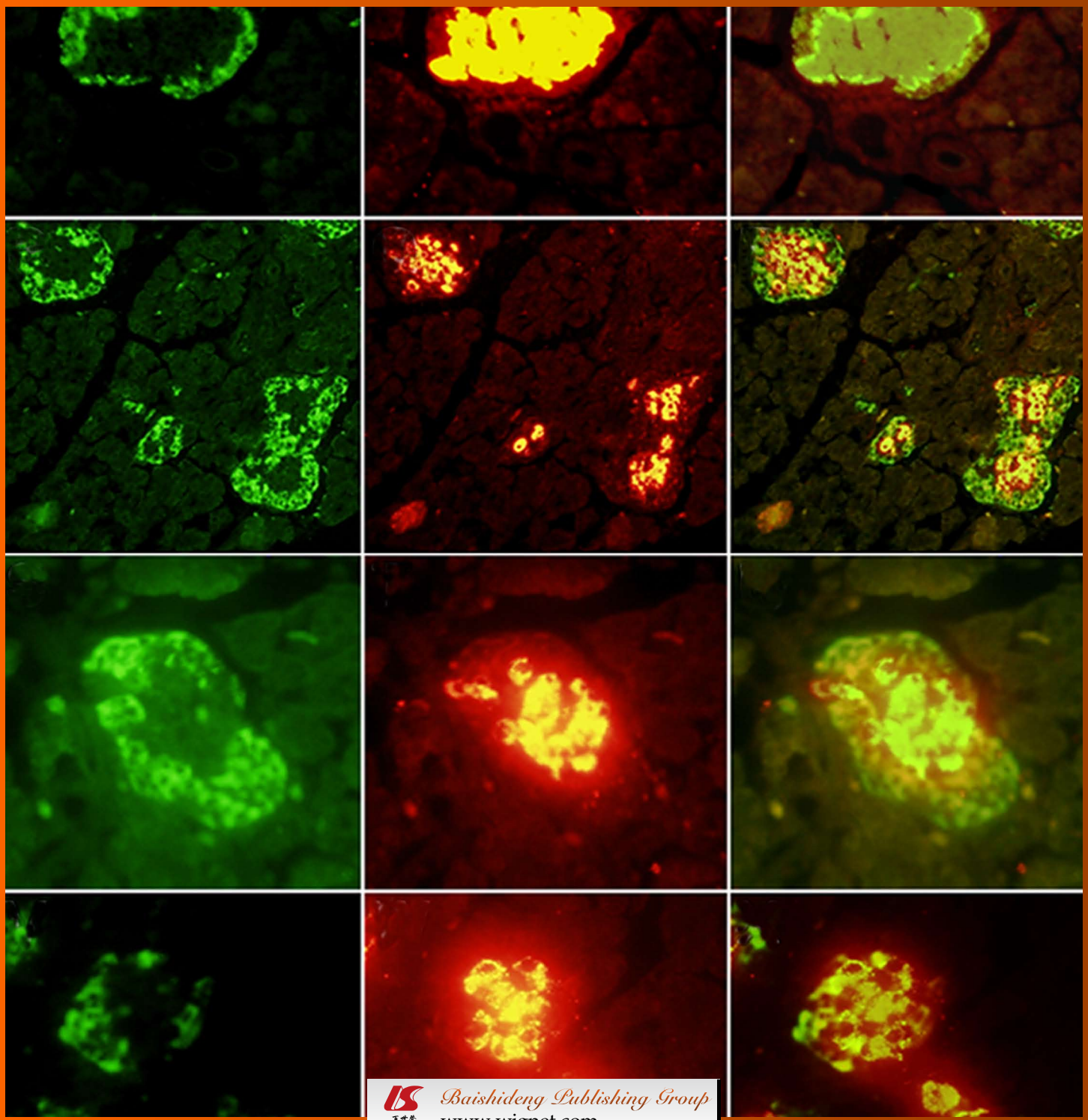

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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 23 June 21, 2011

EDITORIAL

2781 Targeting key signalling pathways in oesophageal adenocarcinoma: A reality for personalised medicine?
Keld RR, Ang YS

REVIEW

2791 Need for a comprehensive medical approach to the neuro-immuno-gastroenterology of irritable bowel syndrome
Katiraei P, Bultron G

2801 Adiponectin, a key adipokine in obesity related liver diseases
Buechler C, Wanninger J, Neumeier M

ORIGINAL ARTICLE

2812 Streptozotocin-induced expression of Ngn3 and Pax4 in neonatal rat pancreatic α-cells
Liang XD, Guo YY, Sun M, Ding Y, Wang N, Yuan L, De W

2821 Pathological and MR-DWI study of the acute hepatic injury model after stem cell transplantation
Shang QL, Xiao EH, Zhou QC, Luo JG, Wu HJ

BRIEF ARTICLE

2829 *NOD2* and *ATG16L1* polymorphisms affect monocyte responses in Crohn's disease
Glubb DM, Gearry RB, Barclay ML, Roberts RL, Pearson J, Keenan JI, McKenzie J, Bentley RW

2838 Is the schatzki ring a unique esophageal entity?
Müller M, Gockel I, Hedwig P, Eckardt AJ, Kuhr K, König J, Eckardt VF

2844 Factors influencing lower esophageal sphincter relaxation after deglutition
Tibbling L, Gezelius P, Franzén T

2848 Effects of sargentgloryvine stem extracts on HepG-2 cells *in vitro* and *in vivo*
Wang MH, Long M, Zhu BY, Yang SH, Ren JH, Zhang HZ

2855 Sonographic features of duodenal lipomas in eight clinicopathologically diagnosed patients
Chen HT, Xu GQ, Wang LJ, Chen YP, Li YM

Contents

World Journal of Gastroenterology
Volume 17 Number 23 June 21, 2011

2860 Association between *ITGA2* C807T polymorphism and gastric cancer risk
Chen J, Liu NN, Li JQ, Yang L, Zeng Y, Zhao XM, Xu LL, Luo X, Wang B, Wang XR

2867 Log-normal censored regression model detecting prognostic factors in gastric cancer: A study of 3018 cases
Wang BB, Liu CG, Lu P, Latengbaolide A, Lu Y

CASE REPORT

2873 Diaphragm disease compared with cryptogenic multifocal ulcerous stenosing enteritis
Chung SH, Jo Y, Ryu SR, Ahn SB, Son BK, Kim SH, Park YS, Hong YO

LETTERS TO THE EDITOR

2877 Time for the world to move beyond the percutaneous endoscopic gastrostomy
Pang AS

World Journal of Gastroenterology
Volume 17 Number 23 June 21, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Liang XD, Guo YY, Sun M, Ding Y, Wang N, Yuan L, De W. Streptozotocin-induced expression of Ngn3 and Pax4 in neonatal rat pancreatic α-cells. *World J Gastroenterol* 2011; 17(23): 2812-2820 http://www.wjgnet.com/1007-9327/full/v17/i23/2812.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
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LAUNCH DATE
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PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

PUBLICATION DATE
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ISSN 2219-2840 (online)

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doi:10.3748/wjg.v17.i23.2781

World J Gastroenterol 2011 June 21; 17(23): 2781-2790
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Targeting key signalling pathways in oesophageal adenocarcinoma: A reality for personalised medicine?

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Author contributions: Keld RR reviewed and searched the literature; Ang YS conceived the idea and revised this paper.
Supported by UK National Institute of Health Research/Cancer Research Network (UK NIHR/UKCRN) and Research and Development Department of Wrightington Wigan and Leigh NHS Foundation Trust (to Ang YS); R Keld Wrightington Wigan and Leigh NHS Foundation Trust Cancer Therapy Fund (to Keld RR, in part)
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Received: April 17, 2010 Revised: July 20, 2010
Accepted: July 27, 2010
Published online: June 21, 2011

Abstract

Cancer treatments are rapidly changing. Curative treatment for oesophageal adenocarcinoma currently involves surgery and cytotoxic chemotherapy or chemoradiotherapy. Outcomes for both regimes are generally poor as a result of tumor recurrence. We have reviewed the key signalling pathways associated with oesophageal adenocarcinomas and discussed the recent trials of novel agents that attempt to target these pathways. There are many trials underway with the aim of improving survival in oesophageal cancer. Currently, phase 2 and 3 trials are focused on MAP kinase inhibition, either through inhibition of growth factor receptors or signal transducer proteins. In order to avoid tumor resistance, it appears to be clear that targeted therapy will be needed to combat the multiple signalling pathways that are in operation in oesophageal adenocarcinomas. This may be achievable in the future with the advent of gene signatures and a combinatorial approach.

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Key words: Oesophageal adenocarcinoma; Signalling pathways; MAP and PI3 Kinase pathways; Wnt signalling; Transforming growth factor-β pathway; Nuclear factor-κB pathways; Transcription factors; Tyrosine kinase receptors

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Keld RR, Ang YS. Targeting key signalling pathways in oesophageal adenocarcinoma: A reality for personalised medicine? *World J Gastroenterol* 2011; 17(23): 2781-2790 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2781.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2781

INTRODUCTION

Oesophageal adenocarcinoma is the 10th commonest malignancy in the UK yet it is the 5th commonest cause of cancer death[1]. This poor prognosis is partly attributable to a disease afflicting an elderly population. All too often the disease presents with symptoms of dysphagia which usually heralds advanced disease, typically with lymph node or distant metastases[2]. The 5 year survival, despite recent advances in neo-adjuvant chemotherapy, radiotherapy and surgery is approximately 25%. The incidence has been steadily increasing over the past 30 years[3-5]; this is thought to be due to the trend of an aging and increasingly obese population in combination with *Helicobacter pylori* eradication[1,6,7]. Barrett's oesophagus has been established

as a clear risk factor for oesophageal adenocarcinoma[8]. It has been demonstrated that surveillance of patients with Barrett's oesophagus can identify early stage adenocarcinomas[9,10]. If diagnosed at an early stage, with the disease confined to the submucosa, 5 year survival rates are as high as 90%[11]. Unfortunately current strategies for surveillance of Barrett's oesophagus are insufficient to reduce the incidence of oesophageal adenocarcinoma and most cases are diagnosed in patients that are not on Barrett's surveillance programs[10]. This may be accounted for by the fact that a significant proportion of patients with Barrett's oesophagus are asymptomatic. Currently, it is not economically viable to screen the whole population for Barrett's oesophagus[12]. Until this is addressed, there does not seem to be a solution to providing an early diagnosis of oesophageal adenocarcinoma for the majority of patients. This indicates the importance of developing improved treatments for advanced disease.

CURRENT MEDICAL TREATMENTS

The medical therapies in mainstream use for the treatment of oesophageal and junctional adenocarcinomas are cytotoxic and antimetabolite agents. They target rapidly dividing cells in an non cancer cell specific manner[13]. 5-Fluorouracil (5-FU) inhibits DNA synthesis through inhibition of thymidylate synthetase[14]. The platinum agents cisplatin and oxaliplatin form DNA adducts and cross-links which prevents DNA transcription and replication[15]. The anthracyclines epirubicin and doxorubicin induce DNA damage and inhibit DNA transcription through inhibition of topoisomerase Ⅱ and DNA helicase activity[16]. The cytotoxic action of taxanes are predominantly due to disruption of microtubules[17].

Cytotoxic chemotherapy is generally not very effective and side effects are common. The agents are usually contraindicated in severe cardiac and liver disease, a common occurrence in the affected elderly population. Recent advances have been made with the route of administration. A tablet form is now available, capecitabine, which is an effective alternative to infusing 5-FU. This reduces the morbidity associated with central venous catheterisation. Furthermore, oxaliplatin appears to be less toxic and more potent than cisplatin, and it can be infused over a shorter period of time[18,19]. Approximately 30% of patients with oesophageal adenocarcinoma are offered palliative chemotherapy and radiotherapy[1,20]. Prognosis is only 6-11 mo[21,22], with a 5 year survival of 4%[22]. Surgery is beneficial in patients that present with disease localised to the oesophagus or with localised lymph node metastases. Neo-adjuvant chemotherapy modestly improves survival compared to surgery alone; 5 year survival is 23% compared to 36% with neo-adjuvant chemotherapy[23]. On subgroup analysis, patients with tumors at the gastro-oesophageal junction seemed to benefit the most and this regimen is offered in the UK[24]. In the USA, the protocol of neo-adjuvant chemo-radiotherapy is favoured[25]. 5 year survival is 8%-20% in selected patients. Curative chemo-radiation is an alternative strategy to surgery and prognosis is similar[2]. This may be due to the avoidance of postoperative mortality and morbidity. Whatever regimen is used, the poor prognosis for oesophageal adenocarcinoma is largely a result of disease recurrence and the morbidity surrounding major surgery[2]. Treatment failure is thought to be a consequence of the blanket therapy approach due to the nature of the non-specific or non-targeted mechanism of action of the medical agents described earlier. Recent evidence suggests that standard chemotherapy and radiotherapy activate signalling pathways that stimulate growth and resistance of cancer cells[26]. Prognosis may improve with agents that specifically target mitogenic signalling pathways and intense research is currently underway.

The aim of this review is to explore the key signalling pathways that are associated with oesophageal adenocarcinoma and review the clinical trials of novel therapeutic agents that are in progress. We will draw parallels from breast and colon cancer. We will address the question: by targeting key signalling pathways is personalised medicine a reality in oesophageal adenocarcinoma?

DEFINING SIGNALLING PATHWAYS

Signalling pathways are essential components in all cells; they are important to stimulate cell growth, proliferation, differentiation, invasion and apoptosis. Certain pathways are specifically important in embryonic development, inflammation and carcinogenesis. Signalling pathways have common mechanisms of action. They convey extra-cellular stimuli, usually *via* cell surface receptors, onto a chain of signal transducer proteins which subsequently enter the nucleus. In the nucleus the signalling proteins activate transcriptional machinery on gene promoters. Gene expression and cell phenotype are altered. In the context of cancer cells, phenotypic change may include cell growth, cell division, increased cell motility, evasion of apoptosis and sustained angiogenesis. These changes constitute the hallmarks of cancer[27]. Signalling pathways in cancer cells are usually unregulated and resistant to feedback inhibition, and this usually occurs as a consequence of sustained activation from their components. The components are commonly known as oncogenes or tumor suppressor genes[28]. Oncogenes and tumor suppressor genes are usually expressed as a result of genetic mutations. In oesophageal cancer, mutations occur as a consequence of DNA damage from bile or acid reflux, nitric oxide, alcohol and cigarette smoking. Mutations usually involve chromosomal translocations of oncogenes or tumor suppressor genes onto housekeeping genes or other genes undergoing active transcription[29,30]. This culminates in persistent activation or inhibition of specific signalling pathways. Components of signalling pathways can be potentially inhibited at a variety of levels. Inhibitors can target the cell surface receptor, signal transducer proteins or even transcription factors. Unfortunately multiple pathways and receptors are associated with oesophageal cancer and the complexity that exists between different pathways is likened to a computer circuit (Figure 1). Blockade of one pathway or component may not be sufficient.

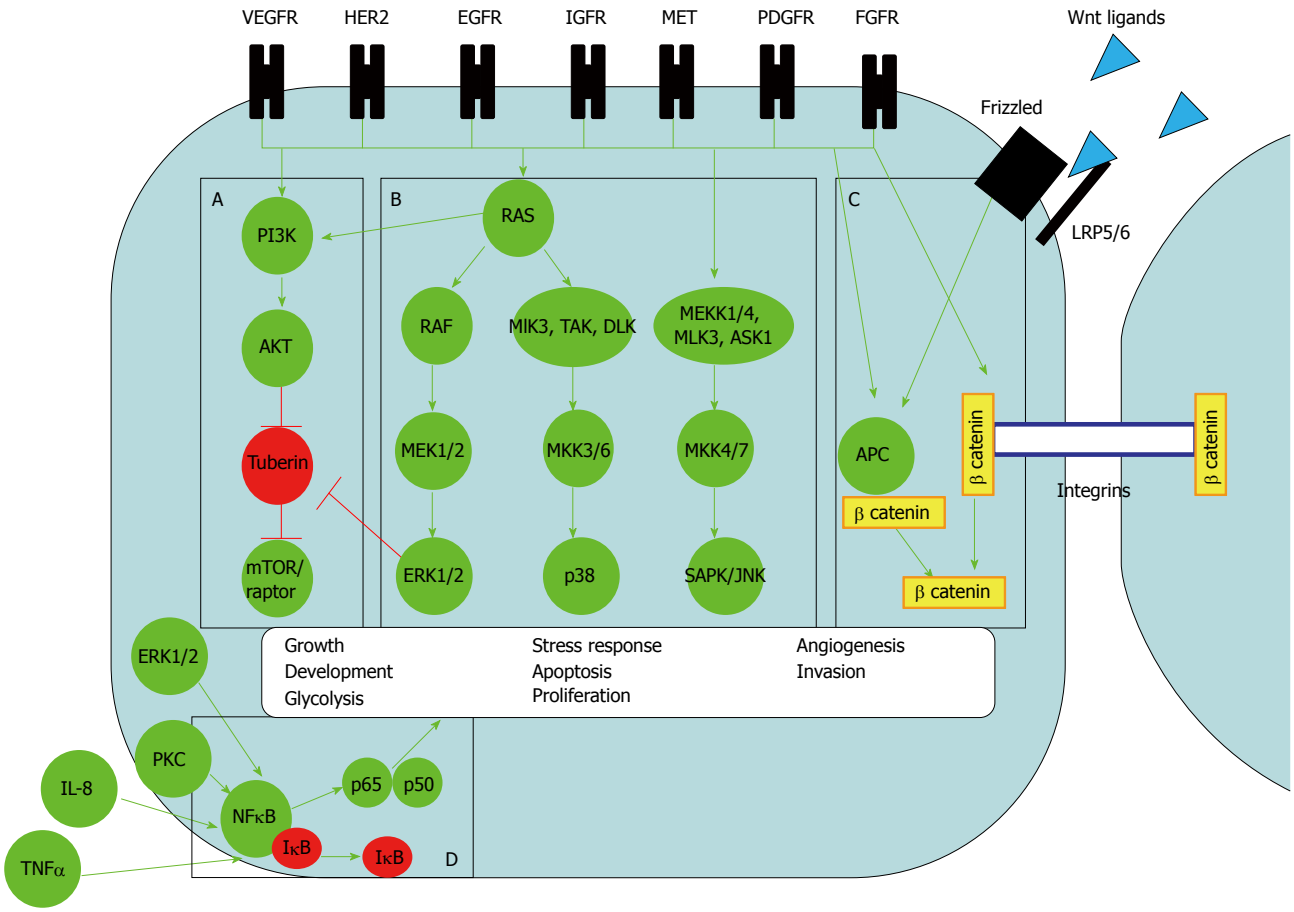

Figure 1 Signalling pathways in oesophageal adenocarcinoma. The pathways known to be operative in oesophageal adenocarcinoma are A: PI3-Kinase, B: Mitogen activated protein (MAP) Kinase, C: Wnt signalling and D: Nuclear factor-κB (NF-κB). The green arrows indicate activation, the red arrows indicate inhibition. Vascular epidermal growth factor (VEGF), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor 1 (EGFR), insulin growth factor receptor (IGFR), hepatocyte growth factor receptor (MET), platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) are the known receptor tyrosine kinase (RTK) associated with oesophageal adenocarcinomas. The pathways are complex and inter-connected. For example RTKs can activate all 4 known pathways. The ultimate biological response varies from cell proliferation, development, apoptosis, differentiation, inflammatory response, angiogenesis and invasion depending on which specific pathway or receptor is activated. A: PI3-Kinase pathway. RTK activates PI3-Kinase directly or through RAS. As a consequence of phosphorylation, PI3K recruits AKT. AKT inhibits tuberin, this allows activation of the Raptor/mTOR complex to activate transcriptional machinery. mTOR/raptor activates transcription factors and co-activator proteins cMyc and hypoxia inducible factor (HIF)-1α that drive the biological response of growth, proliferation, glycolysis, angiogenesis and invasion; B: MAP Kinase pathways. The 3 main MAP Kinase pathways are extracellular related kinase (ERK), p38 and JUN kinase (JNK). They are all activated by similar RTKs. The signalling pathways have a common feature of a cascade of phospho-proteins. A MAPKK Kinase (e.g. RAF), a MAPK Kinase [e.g. MAP ERK kinase (MEK)1/2] and a MAP Kinase (eg. ERK) transfer the signal onto multiple transcription factors on gene promoters[31]. ERK signalling for example can alter the gene expression of over 200 genes[32]. The subsequent biological response varies from cell proliferation, development, apoptosis, differentiation and inflammatory response, depending on the specific pathway activated; C: Wnt signalling. Wnt signalling exerts a biological response through release of β catenin into the nucleus with subsequent action on gene promoters. β catenin is released directly from RTK phosphorylation of Axin. Alternatively Wnt ligands bind to Frizzled receptors and form a complex with the LRP5/6 membrane receptor. The membrane receptor complex recruits dishevelled and axin from a cytoplasmic complex of dishevelled, Axin, adenomatous polyposis coli protein (APC) and β catenin. This allows release of β catenin; D: NF-κB pathway. NF-κB (p65p50) activates gene promoters only when released from IκB. Interleukin-8 (IL-8), tumour necrosis factor (TNF)α and radiation activate the pathway. Adding to complexity, ERK MAP Kinase and Wnt, through protein Kinase C, can also potentially activate NF-κB although this link has not been investigated in oesophageal adenocarcinoma.

KEY SIGNALLING PATHWAYS IN OESOPHAGEAL ADENOCARCINOMA

MAP-Kinase and PI3 kinase pathways

MAP-Kinase (MAPK) pathways are the most well described pathways in carcinogenesis. They are made up of three distinct pathways: ERK, SAP/JNK and p38[31] (Figure 1[31,32]). The pathways are normally activated by growth factors, temperature changes, cytokines and hypoxia *via* a variety of cell surface receptors[33]. In oesophageal cancer cells, gastric and bile acid[34,35] and the cytotoxic agent etoposide[36] are known to activate MAPK pathways. Cell surface receptors known to activate MAPK include receptor tyrosine kinase (RTK), G protein linked receptors and integrins[33]. Following activation of cell surface receptors a cascade of phospho-proteins is initiated *via* GTPase signal transducer proteins. RAS and RAF are examples of GTPase signal transducer proteins which act as a hub, receiving signals from many different cell surface receptors[28] (Figure 1). GTPase signal transducer proteins also amplify signals onward through multiple signalling pathways (Figure 1). MAPK pathways are often up-regulated in breast[37,38], ovarian[39] and prostate cancer[40]; however the impact on prognosis of MAPK signalling is sometimes conflicting. ERK MAPK is active in 60% of ERK MAPK oesophageal adenocarcinomas[36]. Tumors with active ERK MAPK signalling frequently have metastases and a worse prognosis. This suggests that blockade of ERK MAPK in oesophageal adenocarcinomas may have an important therapeutic role.

The PI3-Kinase (PI3K) pathway is activated by RTKs and RAS (Figure 1). Following RAS and/or RTK activation, AKT is phosphorylated by PI3K. Activation of the PI3K pathway stimulates cell growth, glycolysis, and proliferation[41] mainly through cMyc and hypoxia inducible factor 1α (HIF-1α) stimulation. Components of the PI3K pathway are up-regulated in oesophageal adenocarcinoma. The expression of phosphorylated AKT is increased in oesophageal adenocarcinoma tissue compared to normal epithelial and Barrett's tissue[34]. PI3K pathway mutations are thought to occur in 6% of oesophageal adenocarcinomas[42]. Crosstalk exists between the MAPK and PI3K pathways at the levels of RAS and ERK (Figure 1). This is likely to play a role in drug resistance seen in therapies that target signal transducer proteins. Crosstalk indicates that inhibition of multiple pathways may be needed for effective anti-cancer therapy.

Mechanisms of sustained MAPK and PI3K activation

RTKs on the cell surface are key activators of MAPK and PI3K pathways. RTKs can be activated constitutively by dimerisation, by ligand activation or by receptor over-expression[43]. Alternatively RAS mutations can render the GTPase in its active form so that the signal is permanently switched on, resistant to the activity of cell surface receptors. RAS mutations occur in only 10% of oesophageal adenocarcinomas. Aberrant expressions of RTK are frequently associated with oesophageal adenocarcinoma and there are many different family members (Figure 1). Receptor over-expression is usually associated with disease recurrence and a poor prognosis. Human epidermal growth factor receptor 1 (EGFR) and human epidermal growth factor receptor 2 (HER2) are over-expressed in 50% of oesophageal adenocarcinomas and positive expression is associated with a poor prognosis and cytotoxic drug resistance[30,44]. High expression of the hepatocyte growth factor receptor (Met) predicts metastases and recurrence in resectable oesophageal adenocarcinoma[45]. Vascular endothelial growth factor receptors (VEGF) are commonly associated with oesophageal adenocarcinoma, VEGF is thought to be important in angiogenesis and correlates with tumor microvessel density, crucial for tumor growth. VEGF A and C expression indicates a poor prognosis[46]. The significance of insulin like growth factor receptors (IGFR) has not been studied in oesophageal adenocarcinoma[47,48], however low IFGR expression correlates with an improved survival in metastatic gastric adenocarcinoma. The platelet derived growth factor receptor (PDGF) has also been shown to be expressed in oesophageal adenocarcinoma[49]. In oesophageal squamous cell carcinoma, fibroblast growth factor receptors[50] and tropomyosin-related kinase receptors[51] are indicators of tissue invasion and chemo-resistance respectively. Each member of an RTK family may have up to 20 subtypes[52] and it has been demonstrated that oesophageal adenocarcinoma cells often co-express the different subtypes of RTKs[49]. Therefore a therapy that targets only one receptor may not be effective. Furthermore cancers are known to be heterogeneous, made up of a population of genetically different cells. Gene expression at the invasive site of a tumor is different from the centre[53] and the gene expression of primary tumors is different to those at metastatic sites[54]. The variety of RTKs and downstream MAPK signalling pathways indicates that complete blockade of such a complex and diverse system may be impossible. Growth inhibition is more pronounced in oesophageal adenocarcinoma cells treated with combined inhibition of the EGFR and IGFR compared to inhibition of either receptor in isolation[55]. Furthermore patients with co-expression of HER-2 and EGFR also have a worse outcome[56]. Even if drug inhibition of both IGFR and EGFR is successful, resistance will prevail if alternative receptors or signal transducer proteins are active. Tumor heterogeneity may explain the modest improvement in response and survival seen with agents directed towards a solitary receptor, discussed in more detail below.

Targeting the epidermal growth factor receptor 1

Gefitinib and Erlotinib: Gefitinib and Erlotinib are small molecular inhibitors of tyrosine kinase phosphorylation of EGFR (Figure 2). Gefitinib therapy has been investigated in metastatic oesophageal adenocarcinoma[57]. Two thirds of patients had prior standard cytotoxic chemotherapy of which half had received surgery. Partial response and stable disease (according to Response Evaluation Criteria of Solid Tumors) was achieved in 37% and the median survival was 4.5 mo[57]. It is difficult to compare small phase 2 clinical trials but results were not significantly different compared to treatment with combined cytotoxic chemotherapy in a similar cohort of patients. Partial response was 12.5% and a median survival of 5 mo was seen in patients treated with irinotecan with docetaxel[58]. Partial response was 29% and median survival was 6.4 mo in patients treated with irinotecan with 5-FU/leucovorin[59]. To understand the poor results seen with gefitinib, ERK MAPK and PI3K pathway activation was determined by immunohistochemistry. Staining for phospho-ERK and phospho-AKT was assessed before and after treatment in 7 patients. No differences in staining patterns were seen in tumors treated with gefitinib, suggesting that the two pathways were not inhibited by the drug. This result is mirrored in a larger study of 70 gastric adenocarcinomas treated with gefitinib[60]. This indicates ERK MAPK and PI3K pathway resistance to EGFR blockade. A further study conducted in 43 metastatic adenocarcinomas at the gastro-oesophageal junction treated with gefitinib[61] also had similar survival and response rates to the study by Ferry *et al*[57]. Trends for favourable outcome were more likely in tumors with expression of EGFR, ERK MAPK and PI3K signalling activation. This was assessed by immunohistochemistry prior to treatment although assessment was not made post treatment. The differences in outcome did not meet statistical significance, but this is likely due to the small sample size. Of the poor responders, 2 (9%) had k-RAS mutations. This study indicates the importance of patient selection with targeted therapy. On the contrary in gastric and oesophageal adenocarcinomas treated with a similar EGFR inhibitor, erlotinib (Figure 2), EGFR expression

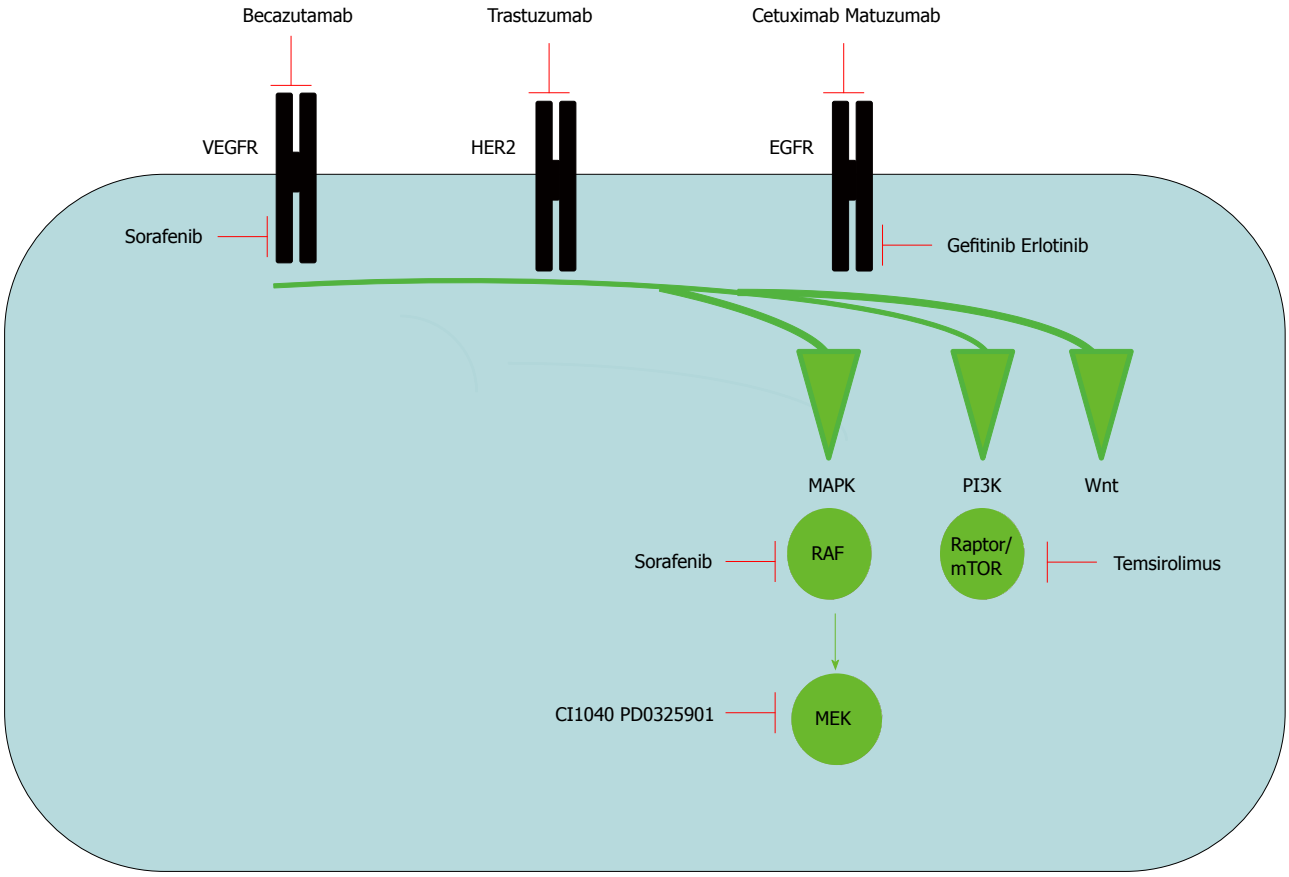

Figure 2 Drug inhibition of signalling pathways. Drugs that target signalling pathways can be divided into two groups. Antibodies [Becazutumab, trastuzumab, cetuximab and matuzumab are antibodies that target the receptor tyrosine kinase (RTK), vascular epidermal growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor 1 (EGFR) respectively] and small molecular inhibitors (The small molecular inhibitors gefitinib and erlotinib target EGFR. Temsirolimus specifically inhibits the PI3-kinase pathway at the level of Raptor/mTOR. CI1040 and PD0325901 inhibits ERK MAP kinase pathway at the level of MEK. Sorafenib is a dual inhibitor of the VEGF receptor and ERK MAP kinase at the level of RAF).

and PI3K signalling activation was not found to influence drug response[62]. The difference may be explained by different receptor specificity between gefitinib and erlotinib or it may indicate the activity of additional RTKs or other cell surface receptors

Matuzumab: Matuzumab is a humanised monoclonal antibody that binds with the EGFR (Figure 2). Phase 1 trials have been conducted in metastatic oesophageal adenocarcinoma treated with conventional therapy with matuzumab[63]. EGFR was evident in 80%-100% of tumor specimens; however MAPK activity was not measured post treatment. This makes it difficult to assess the efficacy of the medication in the absence of survival data from this phase 1 study.

Cetuximab: Cetuximab is a monoclonal antibody directed against the EGFR (Figure 2), utilised in the treatment of advanced colorectal adenocarcinoma[64]. Trials have shown an improvement in average survival to 9 mo. RAS mutations occur commonly in colon cancer and account for resistance seen with cetuximab. When taken into account, colorectal carcinoma patients without k-RAS mutations have a significantly improved response to cetuximab compared to patient with k-RAS mutations which have survival times comparative to that of supportive care alone[65]. RAS mutations are less commonly seen in oesophageal adenocarcinomas and occur in less than 10% of cases so this is unlikely to account for the poor response seen with tyrosine kinase receptor inhibition[66]. Phase 2 clinical trials with cetuximab in advanced oesophageal adenocarcinoma have shown modest results similar to that seen with gefitinib[67].

Targeting the epidermal growth factor receptor 2

A Phase Ⅱ trial has been conducted with trastuzumab, a monoclonal antibody targeted to targeting the epidermal growth factor receptor 2 (HER2) (Figure 2). Trastuzumab was tested in combination with cisplatin, paclitaxel and radiotherapy in locally advanced oesophageal adenocarcinoma[68]. Patients were selected and included those with HER2 expression on immunohistochemistry. 74% of patients had positive HER2 expression. Median survival was 24 mo and 50% survived for 2 years. The patient population was different to the patients treated with gefitinib and cetuximab. None of the patients had organ metastases and distant lymph node metastases were present in only 37%, which makes it difficult to make direct comparisons.

Targeting the vascular epidermal growth factor receptor

Becazutumab is a monoclonal antibody directed against

the targeting of vascular epidermal growth factor receptor (VEGFR) (Figure 2). A phase 2 trial in metastatic gastric adenocarcinomas with 23 oesophageal junctional adenocarcinomas showed a response rate of 65% and median survival time of 12.3 mo[69]. Most patients were inoperable and the results were an improvement on standard cytotoxic therapies. Although VEGFR is frequently over-expressed in oesophageal adenocarcinomas[49], an assessment of VEGFR expression was not made prior to treatment. This may suggest that an improved outcome could be achieved by selecting tumors with high VEGFR expression.

Taken together, this may indicate that tailored RTK inhibition has a role in the treatment of selected patients with oesophageal adenocarcinomas. Initial trials have yet to make a significant impact and this may be down to poor patient selection and the use of growth factor receptor inhibitors in isolation.

Wnt signalling

Wnt signalling is important in cell growth, motility, angiogenesis, differentiation and other important phenotypic characteristics of cancer cells. Wnt ligands activate the Frizzled cell membrane receptor; Wnt is under feedback control from Wnt ligand inhibitors. Once activated, Frizzled forms a complex with another receptor LRP5/6 and recruits Dishevelled and Axin. The complex of APC, Axin and GSK and β-catenin is disrupted releasing unphosphorylated β-catenin (Figure 1). β-catenin can then enter the nucleus, and activate genes that stimulate growth, angiogenesis, invasion and cell cycle progression (*c-Myc, COX2, MMP7* and *Cyclin D1)*. Alternatively β-catenin can also be released by RTK phosphorylation of E-cadherin or Axin (Figure 1). Furthermore Wnt ligands can also directly activate calmodium kinase Ⅱ and protein kinase C in turn releasing intracellular calcium or increasing JNK. Components of the pathway are altered in oesophageal adenocarcinoma. APC, Axin and Wnt ligand inhibitors are silenced by loss of heterozygosity or DNA methylation and collectively these events increase β-catenin activity. Although APC mutations are less commonly seen than in colorectal cancer, β-catenin or Wnt ligands are over-expressed in up to 77% of oesophageal adenocarcinomas[70,71]. This makes components of this pathway a potential target for drug inhibition.

Transforming growth factor-β pathway

Transforming growth factor-β (TGF-β) is a tumor suppressor gene and a potent inhibitor of cell growth. TGF-β binds to serine/threonine kinase type 1 and type 2 receptors. Upon binding to receptors, TGF-β forms a complex and phosphorylates intracellular signalling mediators called SMAD2/3. SMAD2/3 dissociates from the receptors and forms a complex with SMAD4 allowing it to enter the nucleus and regulate a large number of target genes. One target is *SMAD7* which targets ubiquitin to the membrane receptor complex resulting in feedback inhibition of the pathway. Down regulation of SMAD4 has been shown in the progression of Barrett's oesophagus to adenocarcinoma. TGF-β is anti-proliferative in some oesophageal cancer cell lines[72]. In contrast TGF-β expression has been demonstrated at the invasive margin of oesophageal adenocarcinomas and promotes cell invasion[73]. This may be explained by cross talk between the TGF-β pathway with PI3K, Wnt, PKC and the MAP-Kinase pathways. One potential mechanism is *via* SMAD7 inhibition leading to loss/diminished feedback inhibition of the pathway.

Nuclear factor-κB pathways

Nuclear factor-κB (NF-κB) is a proinflammatory transcription factor. It exists as a heterodimer p50/p65, situated in the cytoplasm under inhibitory control by IκB (Figure 1). Numerous activators have been identified including ERK MAPK signalling, cytokines (IL-8, TNF-α) and radiation. Specifically for oesophageal adenocarcinoma, bile salts and gastric acid have been shown to activate NF-κB. Gastrin has been shown to activate NF-κB through PKC signalling in gastric cancer cells[74]. Once activated, NF-κB enters the nucleus and through chromatin re-modelling it becomes a central regulator of many genes including cell cycle regulators (*cyclin D1*, *cMyc*, *p53*), inhibitors of apoptosis (*Bcl-2*), cytokines (*interleukins*, *TNF-α*), angiogenic mediators (*COX2*) and the growth factor receptor *EGFR*. Increased NF-κB expression is seen in Barrett's oesophagus and adenocarcinoma. In oesophageal adenocarcinoma the expression correlates with chemo-radiation resistance[75].

Wnt signalling, TGF-β pathway and NF-κB pathways all activate important mediators in oesophageal adenocarcinoma. However there are no trials investigating the impact of specific inhibitors of these pathways outside the laboratory setting. RTK inhibitors have been investigated in oesophageal adenocarcinoma; however their role in Wnt signalling inhibition of β catenin has not been evaluated. The development of agents that inhibit alternative components of the Wnt, TGF-β and NF-κB signalling pathways are needed to avoid resistance and improve the modest responses seen with current therapies in clinical trials in oesophageal adenocarcinoma that focus on RTKs and MAPK.

POTENTIAL FUTURE TREATMENT TARGETS IN OESOPHAGEAL ADENOCARCINOMA

ERK MAPK inhibition by targeting MEK

An alternative approach is to target signal transducer proteins which may be downstream of many different RTKs. Theoretically this may reduce resistance of RTK co-expression. MEK is a downstream signal transducer protein of the ERK MAP Kinase pathway (Figure 1). No clinical trials have explored the role of MEK inhibition in oesophageal adenocarcinoma but lessons may be learned from trials in other cancers. Phase 2 clinical trials of the MEK inhibitor CI1040 (Figure 2) in advanced pancreatic, breast and non small cell lung cancer failed to make an impact on tumor progression[76]. Parallels can be drawn from colon cancer where RAS mutations reduced the efficacy of ce-

tuximab. Patients treated with MEK inhibition were tested for ERK MAPK and PI3K signalling activation in archived samples, sometimes many months preceding treatment. This did not influence recruitment into the study and patients were enrolled if ERK or PI3K activation was judged to be low. Better patient selection may have resulted in a better response to treatment. ERK MAPK signalling activity was not assessed post treatment which may mean that the dosage was insufficient. More potent MEK inhibitors, such as PD0325901, have been evaluated in hepatocellular carcinomas[77]. Alternatively the poor responses with MEK inhibition may be explained by "cross talk" between different signalling pathways (Figure 2). Resistance to MEK inhibition may be explained by PI3-Kinase activation. The combination of MEK inhibition and PI3-Kinase inhibition is superior to treatments in isolation for inhibiting the growth of breast cancer cells[78]. This suggest that dual therapy is needed to combat both pathways.

ERK MAPK inhibition by targeting RAF and VEGF

A combined targeted approach may be beneficial in oesophageal adenocarcinoma. Sorafenib, a multifunctional kinase inhibitor, which acts on several growth regulatory pathways including VEGF and RAF (Figure 2), has been shown to be of benefit in renal cell carcinoma and hepatocellular carcinomas[78,79]. Sorafenib has been shown to inhibit key signalling pathways in SEG-1 lung adenocarcinoma cells[80]. This method of inhibition of both receptor and signalling protein such as RAF may prove beneficial due the diversity of growth factor receptors displayed by tumors and this approach may have a future role in the treatment of oesophageal adenocarcinoma.

PI3 kinase by targeting mTOR

No inhibitors of the PI3 kinase have been evaluated in oesophageal adenocarcinoma. Cell line studies in oesophageal adenocarcinoma have identified that the PI3 kinase pathway is important for cell growth. Mutations of the PI3 kinase pathway occur infrequently in oesophageal adenocarcinoma. However, activation of the pathway is known to occur from RTK, a common occurrence in oesophageal adenocarcinomas. Indeed in breast cancer, the PI3 kinase pathway has been proposed as a mechanism of drug resistance to MEK inhibition[55]. Inhibition of the PI3 kinase pathway has been used with success in metastatic renal cell carcinoma. Analogues of rapamycin have been developed to target mTOR (Figure 2). The agent temsirolimus has been evaluated in stage 3 clinical trials[81] (Figure 2). In this trial, 626 patients were divided into 3 groups; temsirolimus alone, interferon alone, and interferon in combination with temsirolimus. Overall survival was 10.9, 7.3 and 8.4 mo respectively in favour of temsirolimus. In view of this, a strategy of mTOR inhibition may have a future role in oesophageal adenocarcinoma.

Targeting transcription factors

Targeting an activated transcription factor or central regulator such as NF-κB would theoretically reduce the chance of the development of drug resistance from the activity of multiple surface receptors and multiple signalling pathways. This is not without problems. Firstly transcription factors are difficult to target. Interference RNA technology involves the insertion of an oligonucleotide into the nucleus of a cancer cell, usually using a viral vector. Oligonucleotides can be manufactured to complement the sequence and therefore dimerize with any RNA of interest such as NF-κB. This allows the targeting of transcription factor RNA with the prevention of protein translation. Interference RNA technology may be the answer to transcription factor inhibition but the technology remains in its infancy. The major hurdle appears to be the development of an efficient delivery system of oligonucleotides into cancer cells. Phase 1 trials are currently underway targeting VEGF in macular degeneration using direct ocular injection[82,83]. If this technology is developed in oesophageal cancer then gene expression profiling of tumors would be required to ensure that specific targeted therapy is delivered. The identification of more central regulators of carcinogenesis, such as HIF-1α and PEA3/ETV4 transcription factors, is likely to increase treatment options. The advent of gene expression profiling will certainly increase the number of potential targets.

CONCLUSION

An international effort is underway with the aim of improving survival in oesophageal adenocarcinoma by targeting key signalling pathways. Clinical trials using receptor tyrosine kinase inhibitors in oesophageal adenocarcinomas have so far only recruited patients with advanced or metastatic cancer. Studies utilised agents that inhibit solitary receptor tyrosine kinase, sometimes in an unselected manner. This strategy is problematic. At an advanced stage the heterogeneity within the tumor is extensive, which increases the likelihood of alternative signalling pathways resistant to receptor blockade. Secondly the pathway or receptor of interest may not be active or expressed, culminating in ineffective treatment. Tumor growth is immensely complex and this is emphasised in a study of 75 oesophageal adenocarcinoma specimens. Micro array studies identified 4 genes important in disease progression[84]. The genes independently predicted prognosis independent from traditional radiological methods. Unfortunately a further 115 genes were also indicators of survival. It is not clear what role the genes play in carcinogenesis; however this study indicates the complexity and diversity of the factors implicated in oesophageal adenocarcinoma development. Taken together, this suggests that a tailored combinatorial approach for treatment that inhibits multiple genes may be useful; therapies targeting either receptors, hub signalling proteins or even transcription factors, is likely to be necessary to deliver effective responses. By tailoring therapy to tumors that express specific gene and protein signatures and prescribing a regimen of treatments that act in fundamentally different

mechanisms, then further improvements in survival are likely to be possible in oesophageal adenocarcinoma.

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S- Editor Wang JL **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2791

World J Gastroenterol 2011 June 21; 17(23): 2791-2800
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Need for a comprehensive medical approach to the neuro-immuno-gastroenterology of irritable bowel syndrome

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Supported by A Generous Grant from the Riverside Community Health Foundation
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Received: December 23, 2010 Revised: March 8, 2011
Accepted: March 15, 2011
Published online: June 21, 2011

Abstract

Irritable bowel syndrome (IBS) is defined by the Rome Ⅲ criteria as symptoms of recurrent abdominal pain or discomfort with the onset of a marked change in bowel habits with no evidence of an inflammatory, anatomic, metabolic, or neoplastic process. As such, many clinicians regard IBS as a central nervous system problem of altered pain perception. Here, we review the recent literature and discuss the evidence that supports an organic based model, which views IBS as a complex, heterogeneous, inter-dependent, and multi-variable inflammatory process along the neuronal-gut axis. We delineate the organic pathophysiology of IBS, demonstrate the role of inflammation in IBS, review the possible differences between adult and pediatric IBS, discuss the merits of a comprehensive treatment model as taught by the Institute of Functional Medicine, and describe the potential for future research for this syndrome.

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Key words: Irritable bowel syndrome; Abdominal pain; Inflammation; Probiotics; Stress

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Katiraei P, Bultron G. Need for a comprehensive medical approach to the neuro-immuno-gastroenterology of irritable bowel syndrome. *World J Gastroenterol* 2011; 17(23): 2791-2800 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2791.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2791

INTRODUCTION

Functional abdominal pain (FAP) and irritable bowel syndrome (IBS) are debilitating and common conditions. IBS is defined by the Rome Ⅲ criteria as, "symptoms of recurrent abdominal pain or discomfort and a marked change in bowel habit for at least six months, with symptoms experienced on at least three days of at least three months, with two of the three following findings: (1) Pain is relieved by a bowel movement; (2) Onset of pain is related to a change in frequency of stool; and (3) Onset of pain is related to a change in the appearance of stool"[1].

FAP occurs in 10%-15% of school-aged children, of which 17%-24% have pain significant enough to disrupt their activity, and 13%-15% awaken from their sleep due to the pain[2,3]. Up to 53% of children with abdominal pain continue to have abdominal pain as adults, and 18% are ultimately diagnosed with IBS[4].

Chronic abdominal pain is associated with significant morbidity, including depression[2], decreased quality of life measures[5], and disability leading to inability to work[6]. Adults with abdominal pain have higher rates of potentially unnecessary surgeries[7-10]. Patients with IBS and FAP are costly to the medical system[11,12]. Both children and adults with IBS frequently visit the offices of primary care physicians and gastroenterologists[13]. Adults with IBS

have significantly more hospitalizations, outpatient visits, diagnostic testing, and overall medication use than well patients[14]. A large percentage of the medical costs associated with IBS are related to hospitalizations and inpatient diagnostic testing, such as endoscopies[15]. Antidepressants and other neuropharmacological agents help the symptoms of IBS[16], but these treatments have their own limitations and potential adverse effects.

IBS is thought to be just a functional problem that is "without demonstrable evidence of a pathological condition such as an anatomic, metabolic, infectious, inflammatory, or neoplastic disorder"[17]. IBS is seen as a non-organic syndrome, primarily involving altered perception and processing of pain. As a result, the majority of current therapies for IBS revolve around stress reduction, alteration of pain pathways, and alleviation of symptoms[16].

In this literature review, we delineate the gastrointestinal-neuro-immune pathophysiology of IBS and discuss the link between inflammation and pain. We believe that more effective treatment models are possible through a patient-centered approach that simultaneously treats the multiple variables that lead to IBS, as addressed in this review. The integration of this IBS treatment model may improve patient outcomes while reducing the medical cost burden of IBS. This paper will also discuss the possible differences between adult and pediatric IBS and present potential areas of future research.

STRESS AND THE GASTROINTESTINAL-NEURO-IMMUNE AXIS

Stress in various forms predisposes individuals to developing IBS[18-20] and increases IBS symptoms in children[21]. Abuse or other significant stressors change the neurobiology of stress and alters the levels of corticotropin-releasing factor (CRF) or hormone (CRH)[22], a hypothalamic stress hormone. CRF activates the pituitary-adrenal axis and mediates behavioral, autonomic, immune, and visceral responses to stress[23]. Patients with IBS have enhanced stress responses and release higher amounts of CRF in response to stress[24].

Stress changes the physiology of the gastrointestinal tract. Maternal separation of rat pups causes CRF-mediated mucosal barrier dysfunction with macromolecular permeability and increased bacterial adherence/penetration of the gastrointestinal mucosa with translocation to the spleen[25]. These animals also have mitochondrial swelling of the gut epithelial cells, immune cell infiltration, mucus depletion, and mast cell degranulation[23,26-29]. Stressed human beings show similar findings[30].

Stress compromises the integrity of the gut and induces inflammation through numerous pathways, as demonstrated by several published papers[22,28,31]. CRF released from the hypothalamus can directly influence human colonic mast cells[32,33], which then induce intestinal epithelial pathophysiology and mucosal barrier defects[34-37]. Substance P (SP) and calcitonin gene-related peptide (CGRP)-containing gastrointestinal efferent neurons can also influence mast cells[38-41] and result in degranulation[42] and release of TNF-α[43]. These compounds, in turn, result in gut inflammation and intestinal permeability[44].

These stress-induced changes in the gastrointestinal tract "persist after the stressor is removed from the animal"[37]. This is likely to be due to the ability of mast cells to influence their environment. In rats, inflammation results in increased mast cell-neuronal contacts and mucosal nerve cell density that last well beyond the initial insult[43,45]. Gastrointestinal inflammation in humans also results in neuron proliferation[46-48]. Stress and inflammation modulate nerve growth factor (NGF), which then affects mucosal nerve remodeling[49,50], sprouting, and synaptogenesis[51]. Mast cells, in close contact with neurons, synthesize and release NGF, and thus, can alter neuronal density and synaptogenesis[49,52].

Furthermore, inflammation preceding a psychological stress can alter the epithelial response to stress signals and make the gut more susceptible to stress[53]. In addition, inflammation can change the morphology of mast cells and their intracellular contents, further changing the susceptibility of the gut to various future stressors[54-57].

Inflammation can play an important part in the manifestation of IBS symptoms[58]. Once the inflammatory cascade is activated, this immune response can create a vicious cycle of self-perpetuating inflammation. Activated mast cells can directly release CRF[59]. Patients with inflammatory bowel disease (IBD) and IBS have CRF-immunoreactive macrophages, enterochromaffin cells, lymphocytes, neutrophils, and eosinophils, which are present in higher concentrations than in healthy controls[60-63]. CRF induces lymphocyte proliferation[64] and macrophage release of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6)[65]. These activated immune cells, in turn, locally release CRF and other immune peptides[61,66], which then activate mast cells[22]. Mast cell-derived tryptase is another compound that recruits lymphocytes, eosinophils, and macrophages[67], and can further perpetuate inflammation.

INFLAMMATION INDUCED NEUROLOGICAL TONE

Patients with IBS have central processing abnormalities associated with the perception of pain[68-71]. Colonic irritation can lead to visceral hypersensitivity[72]. Patients with IBS have inflammatory changes in their gut mucosa, which can only be identified by quantitative histopathology, immunohistochemistry, and electron microscopy[73]. These patients have increased numbers of mast cells in the mucosa of the colon[30,74,75]. Mast cell concentrations and their distance from mucosal nerve cells are positively associated with various IBS symptoms[75]. The tryptase released from these mast cells can directly activate gastrointestinal neurons in animals and humans, and can cause visceral hypersensitivity[76-78]. Tryptase cleaves and activates transmembrane proteins called proteinase-activated receptor-2 (PAR2), which are found on the primary afferent neurons of the gastrointestinal tract[79]. Activation of PAR2 receptors leads to neuronal activation, which then creates the experience of

chronic pain.

In addition to central nervous system activation, patients with IBS also have sensitization and upregulation of the dorsal horn[70,71,80], which explains the cutaneous hyperalgesia found in the lower extremities, rather than upper extremities, due to viscerosomatic convergence of nociceptive afferent neurons from the colon/rectum and lower extremities[81]. Seybold *et al*[82] review the mechanisms by which gastrointestinal inflammation leads to gastrointestinal primary afferent neuronal activation and spinal cord activation/sensitization and inflammation.

If true, the perpetual mild mast-cell mediated inflammation can trigger the "excessive or prolonged stimulation of extrinsic afferents (that) may also result in the development of neuronal sensitization, at peripheral, spinal, or higher CNS levels, such that perception of sensations from the bowel is heightened, resulting in symptoms of urgency, bloating, and pain"[83]. This subclinical inflammation may also influence gastrointestinal serotonin pathways.

IBS, NEUROLOGICAL TONE, AND SEROTONIN

Serotonin (5-HT) can influence the motor function and sensitivity of the gastrointestinal tract[84-89]. Serotonin exerts a range of effects *via* its seven receptor subtypes (5-HT_1 to 5HT-7). Serotonin receptor $5HT_5$, $5HT_6$, $5HT_7$ are found in the brain, whereas $5HT_1$, $5HT_2$, $5HT_3$, $5HT_4$, and $5HT_7$ are the gastrointestinal serotonin receptors[90]. A large majority of the body's serotonin is stored in gastrointestinal enterochromaffin cells (EC)[85]. Patients with diarrhea predominant IBS have increased EC cells[91-93], which are activated by inflammation to release serotonin and may result in the elevated serotonin levels found in patients with IBS[94,95]. Tegaserod, a partial $5HT_4$ agonist has been used for constipation dominant IBS and Alosetron, a $5HT_3$ antagonist, in diarrhea dominant IBS.

Serotonin reuptake transporters (SERT) in the gut epithelial cells terminate the effects of serotonin[96,97] and influence serotonin concentrations and symptoms of IBS[85]. Patients with IBS have genetic polymorphisms that lead to lower expression of transport proteins and less serotonin reuptake[83,98,99]. The noted inflammation may also alter SERT expression and decrease its function in patients with IBS[100]. Further studies on the modulation of the gastrointestinal tract serotonin pathways may help further define and treat IBS.

INTESTINAL PERMEABILITY, CHRONIC INFLAMMATION, AND ANTIGENS

The presence and activity of mast cells, along with other inflammatory cells, alone are not likely result in chronic inflammation. Other intestinal antigens, such as food, bacteria, and fungi, are likely to be needed to perpetuate the inflammation in the presence of an impaired gastrointestinal epithelial barrier.

Healthy individuals have tight junctions that help to form the gastrointestinal epithelial barrier along with mucous, SIgA, and other peptides. This epithelial barrier controls the interaction between luminal bacteria and antigens and the mucosal immune system[22,101]. It also allows immune tolerance of food antigens and bacteria. Activation of PAR2 not only leads to neuronal activation, but also to epithelial barrier defects in patients with IBS[102,103].

Low level PAR2 activation of the myosin light chain kinase (MLCK), causes phosphorylation of the myosin light chain, which then leads to contraction of the actin-myosin ring. Tight junction protein zona occludens-1 (ZO-1) relocalizes into the cytoplasm and disrupts the tight junctions, which increases paracellular permeability. High level PAR2 activation in the rat colon results in localized inflammation and increased production of TNF-α and IFN-γ. INF-γ decreases ZO-1 expression and alters the actin cytoskeleton organization[104]. TNF-α activates MLCK and results in tight junction protein relocation[105,106]. A more detailed discussion of these pathways can be found in articles by Gareau *et al*[23] and Cenac *et al*[103].

Children and adults with IBS have increased intestinal permeability[107,108]. Increased intestinal permeability results in "mucosal barrier defects (that) allow the passage of an increased load of luminal antigens of dietary and bacterial origin which, in turn, elicit the activation of mucosal immune responses"[109].

Various triggers can activate mast cells. Bacteria are powerful antigens for the gastrointestinal immune system[110-115]. Stress can result in increased bacterial adherence and penetration into the gastrointestinal mucosa[23,25-27], which may increase the interaction between the luminal bacteria and local immune response. This may explain why patients with IBS have higher antibody titers to specific bacterial flagella than healthy controls[116]. The DNA of these bacteria can interact with toll-like receptors[117], which then influence the immune system through regulation of tumor necrosis factor alpha and interferon gamma[118].

Escherichia coli, Campylobacter, and other bacteria can negatively influence the GI immune system and result in gastrointestinal inflammation and intestinal permeability[46,91,119-121]. Conversely, commercially available beneficial bacteria, in the form of probiotics, can reduce gastrointestinal inflammation[122-125], reverse or prevent intestinal permeability[120], and stop bacterial adhesion[126] and translocation[27,127]. Probiotics can also reverse visceral hypersensitivity from various causes[128,129], including stress[130]. Probiotics attenuate the upregulation of pain pathways at the spinal and supraspinal levels[131], and induce epithelial cells to express micro-opiate receptor 1 (MOR1) and cannabinoid 2 (CB2) opioid receptors[132]. Probiotics can reduce the symptoms of IBS[133,134].

Adults with IBS have gastrointestinal microflora that are significantly different than those of healthy populations[135]. Children with IBS are also likely to have significant alterations in their gastrointestinal microflora. We speculate that there may be a subset of children who are predisposed to developing IBS through repeated or

prolonged exposure to antibiotics for various reasons (recurrent otitis media, sepsis, meningitis, osteomyelitis, vesicoureteral reflux, acne, *etc*). Various antibiotics, including Augmentin, the macrolides, and amoxicillin significantly alter the composition of the bacteria in the GI tract[136-138]. Antibiotic use has been related to increased rates of IBS and functional abdominal pain[139,140].

Gastrointestinal bacteria are also influenced by the diet. Dietary soluble fiber encourages the growth of beneficial species like lactobacilli and bifidobacteria[141-143]. In mice, a white bread diet significantly prolonged antibiotic induced bacterial perturbations[136]. It is common knowledge that the standard American diet lacks fiber, and thus may predispose human beings to have prolonged antibiotic induced bacterial perturbations.

Prebiotics are short chain carbohydrates that help some of the beneficial bacteria or probiotics in the intestines to grow more effectively[142,143]. Prebiotics may decrease IBS symptoms[144-146]. Prebiotics are fermented by probiotics and metabolized into short chain fatty acids (SCFA). SCFAs can decrease inflammation and are used in maintaining the intestinal epithelial lining[147]. While breast milk naturally contains prebiotics[148], up until a few years ago, most infant formulas did not contain prebiotics. Thus, there may be a population of children who were formula fed and required several courses of antibiotics that now have perturbed gastrointestinal flora, as well as intestinal epithelial barriers. We believe that these children may be at risk of developing IBS.

Food proteins are other significant antigens for the gut immune system. Food antigens induce mast cell activation[149] and degranulation, which can lead to visceral hypersensitivity. In children, certain foods may exacerbate intestinal permeability and the elimination of the foods help resolve the IBS symptoms[150]. Elimination of certain foods may decrease immune activation by removing the allergic antigenic load to the local immune system. In patients with IBS, sodium cromoglycate can eliminate IBS symptoms[151-153] by preventing the degranulation of mast cells and inhibiting the release of inflammatory mediators, following contact with an allergen[154].

Over 60% of patients believe that certain foods worsen their IBS symptoms and that elimination of these foods can reduce their symptoms[155-157]. Some believe that these food reactions are psychological in origin[158-160]. Blinded food challenges have raised many questions about the validity of elimination diets for IBS treatment[161-163]. There is also a growing body of evidence to support the use of elimination diets as part of a treatment protocol for IBS[164-167]. Milk, wheat, and eggs are the most commonly identified food triggers[163].

Another potential antigen for the gastrointestinal immune system is *Candida albicans*. Adult studies have shown that *Candida* does not play a significant role in patients with IBS[168,169]. To our knowledge, the role of candida in pediatric IBS has not been determined. Some children who have received numerous courses of antibiotics, such as amoxicillin, can have disruption of the bacterial balance and have overgrowth of the commensal *Candida*[137,170-173]. *Candida* induces inflammation. It produces alcohols and glycoproteins that stimulate mast cells to produce histamine and prostaglandins[174,175]. *Candida* also produces inflammatory prostaglandins that affect mammalian cells[176], as well as proteases that degrade the gastrointestinal IgA and, thus, allow candida to overcome the local immune defense mechanisms[177]. Candidal proteases can induce a B-cell response and result in increased inflammation[174]. In animals and humans, *Candida* perpetuates intestinal inflammation[169].

SMALL INTESTINAL BACTERIAL OVERGROWTH

Another possible contributing factor to IBS signs and symptoms is small intestinal bacterial overgrowth (SIBO), defined as bacterial counts greater than 10^5 cf/mL from small intestinal aspirates[178]. Controversy exists over the ideal method of assessing SIBO[178-181]. A significant number of patients with IBS complain of bloating and pain. SIBO may explain this bloating and pain, as well as other IBS-like-symptoms[182-184]. Several studies have shown antibiotics to be helpful in reducing the symptoms of IBS[185-188].

Patients with IBS who have delayed gastric emptying have a higher risk of developing SIBO[189-192]. Stress is one cause of delayed gastric emptying[193-196]. Once SIBO is present, it can trigger an inflammatory response. SIBO, through abnormal gastrointestinal flora fermentation, may be another cause of IBS symptoms and must be considered in the evaluation of the patient. Furthermore, proton pump inhibitors can also increase the risk of SIBO by decreasing gastric acidity and further perturbations of the gastrointestinal flora species[170,197-200]. We speculate that SIBO may play a larger role in adults with IBS than in children. Further studies are required to elucidate the various differences between adult and pediatric IBS.

CONCLUSION

The evidence presented in our review suggests that IBS is an organic disease with a complex pathophysiology (Figure 1) that is difficult to identify by standard diagnostic tools. The pathophysiology of IBS varies from person to person and from children to adults. The underlying mast cell mediated inflammation of IBS, along with serotonin signaling, can drive the chronic nociceptive input from the periphery to dynamically maintain the altered central processing defects and perception of pain[70,80,201].

In addition to the pathophysiology, clinicians must focus more attention on the well known and less well characterized risk factors that may predispose individuals to developing IBS (Table 1). It is our belief that clinicians should further use the field of neurogastroenterology to better understand the effects of stress on the gastrointestinal tract. Clinicians and researchers must work to develop and adopt models to help us better predict and prevent this condition in susceptible individuals. For chil-

Table 1 Risk factors for irritable bowel syndrome

Genetics/family history
Stress/high academic performance/parental psychiatric disorders
Recurrent or chronic antibiotic use
Bacterial or viral enteritis
Unrecognized food sensitivities
Low fiber diet/diet high in simple carbohydrates
Formula feeding
Chronic acid suppression

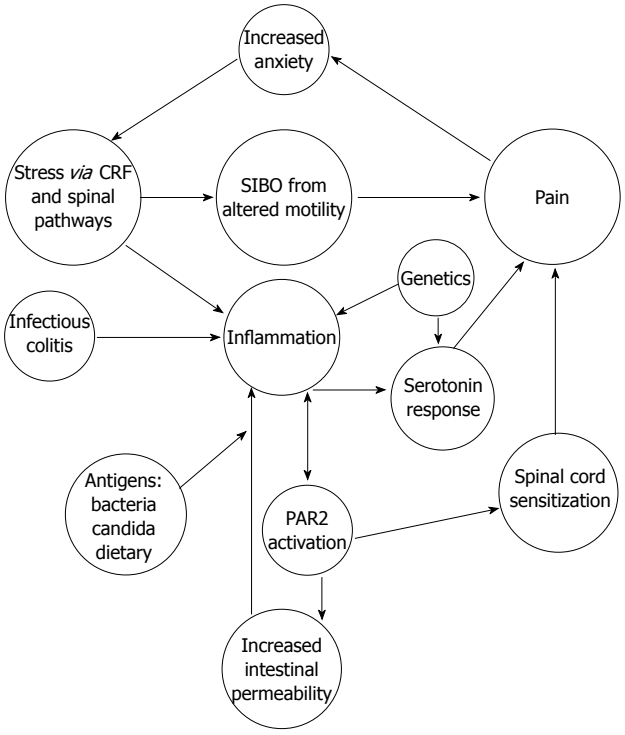

Figure 1 Proposed pathophysiology of irritable bowel syndrome. CRF: Corticotropin-releasing factor; SIBO: Small intestinal bacterial overgrowth; PAR2: Proteinase-activated receptor-2.

dren, these models will require additional studies to evaluate the impact of recurrent antibiotic use and resultant overgrowth of candida on the development of IBS.

Effective treatment models for IBS must reflect the complex physiology of IBS and simultaneously address multiple pathophysiological factors to break the vicious cycle of inflammation and ultimately allow for cessation of symptoms. The Institute of Functional Medicine (IFM)[202] has created such a model of care for IBS. The IFM model has the potential to provide significant improvement in patient care, while reducing healthcare costs and deserves further consideration and evaluation. Please refer to the IFM website and various publications for a more detailed discussion on treatment options.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2801

World J Gastroenterol 2011 June 21; 17(23): 2801-2811
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Adiponectin, a key adipokine in obesity related liver diseases

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Author contributions: Buechler C wrote the review article and Neumeier M and Wanninger J revised the manuscript and participated in the design of the figures.
Supported by The Faculty of Medicine of the University of Regensburg (ReForM C); The Deutsche Forschungsgemeinschaft
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Received: August 17, 2010 Revised: November 17, 2010
Accepted: November 24, 2010
Published online: June 21, 2011

Abstract

Non-alcoholic fatty liver disease (NAFLD) comprising hepatic steatosis, non-alcoholic steatohepatitis (NASH), and progressive liver fibrosis is considered the most common liver disease in western countries. Fatty liver is more prevalent in overweight than normal-weight people and liver fat positively correlates with hepatic insulin resistance. Hepatic steatosis is regarded as a benign stage of NAFLD but may progress to NASH in a subgroup of patients. Besides liver biopsy no diagnostic tools to identify patients with NASH are available, and no effective treatment has been established. Visceral obesity is a main risk factor for NAFLD and inappropriate storage of triglycerides in adipocytes and higher concentrations of free fatty acids may add to increased hepatic lipid storage, insulin resistance, and progressive liver damage. Most of the adipose tissue-derived proteins are elevated in obesity and may contribute to systemic inflammation and liver damage. Adiponectin is highly abundant in human serum but its levels are reduced in obesity and are even lower in patients with hepatic steatosis or NASH. Adiponectin antagonizes excess lipid storage in the liver and protects from inflammation and fibrosis. This review aims to give a short survey on NAFLD and the hepatoprotective effects of adiponectin.

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Key words: Hepatic steatosis; Non-alcoholic steatohepatitis; Adiponectin; Obesity; Adipose tissue

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Buechler C, Wanninger J, Neumeier M. Adiponectin, a key adipokine in obesity related liver diseases. *World J Gastroenterol* 2011; 17(23): 2801-2811 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2801.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2801

INTRODUCTION

Obesity is associated with insulin resistance, a common risk factor for type 2 diabetes, cardiovascular disease, hepatic steatosis and non-alcoholic steatohepatitis (NASH)[1,2]. Hypertrophied adipocytes in obesity fail to appropriately store excess triglycerides and excessive ectopic accumulation of lipids in skeletal muscle and liver disturbs insulin signalling[3]. Body fat distribution appears to be even more important than the total amount of adipose tissue, and visceral fat mass is strongly linked to insulin resistance and non-alcoholic fatty liver disease (NAFLD)[4]. Visceral fat released free fatty acids are transported to the liver by the portal vein and may contribute to hepatic steatosis, production of triglyceride rich very low density lipoproteins (VLDL) and elevated β-oxidation[5,6] (Figure 1). Metabolically healthy but obese (MHO) individuals are insulin sensitive and hepatic fat accumulation is significantly lower compared to similarly overweight subjects that develop

insulin resistance[7,8]. Despite comparable fatness between MHO and control cohorts that develop insulin resistance, MHO subjects have 49% less visceral fat which further emphasizes the unfavourable characteristics of this fat depot[9]. Lean body mass may be associated with a higher insulin sensitivity and is significantly lower in MHO subjects[9]. A recent study even describes an independent association of lean body mass with impaired glucose disposal and systemic C-reactive protein (CRP) levels in centrally obese postmenopausal women that may exacerbate the harmful effects of visceral fat mass[10]. These studies further point to the highly complex interplay of various factors associated with metabolic diseases like the metabolic syndrome.

Various epidemiological studies have identified central obesity as an independent risk factor for metabolic diseases and highlight the crucial role of impaired production or activity of adipose tissue released proteins[6,11]. Most of the adipokines identified so far are elevated in obesity and raised chemokine C-C motif ligand 2 (CCL2) contributes to the increasing number of adipose tissue resident macrophages[11,12]. They produce inflammatory proteins like interleukin-6 (IL-6) and tumour necrosis factor (TNF) whose circulating levels are increased in obesity, a state of low-grade, chronic inflammation[13]. TNF impairs insulin signalling and plays a crucial role in non-alcoholic steatohepatitis (NASH) progression[14,15]. Visceral fat released proteins are directly transported to the liver by the portal vein and the anatomical feature of this fat depot may explain the harmful metabolic effects of visceral adiposity[6]. IL-6 is preferentially released from visceral fat and upregulates suppressor of cytokine signalling 3 (SOCS3) in the liver that causes hepatic insulin resistance[16-18]. Furthermore, IL-6 is a well known inducer of CRP, a marker protein for systemic inflammation[19] (Figure 1). Leptin is mainly produced by adipocytes, and obesity is characterized by elevated systemic levels and central and peripheral leptin resistance[6]. Leptin prevents lipid accumulation in non-adipose tissues like the liver. Leptin lowers stearoyl-CoA desaturase that catalyzes the rate-limiting reaction of monounsaturated fatty acid synthesis and thereby may ameliorate hepatic insulin sensitivity[20]. Animal studies have proven that leptin directly promotes fibrogenesis. Leptin induces transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF) production in hepatic stellate cells through indirect effects on Kupffer cells[21]. In humans, a direct association of circulating leptin and liver fibrosis has not been confirmed yet and locally produced leptin and/or leptin resistance may have to be taken into account[22].

The adipokine adiponectin is highly abundant in human serum and is secreted by adipose tissue in inverse proportion to the body mass index[23]. Adiponectin circulates as trimer, hexamer and higher order multimer in serum and isoform-specific effects have been described[24-26]. Adiponectin may also form hetero-oligomers with additional members of the C1q/TNF-related protein (CTRP) family like the recently described CTRP9[27]. Early studies indicate

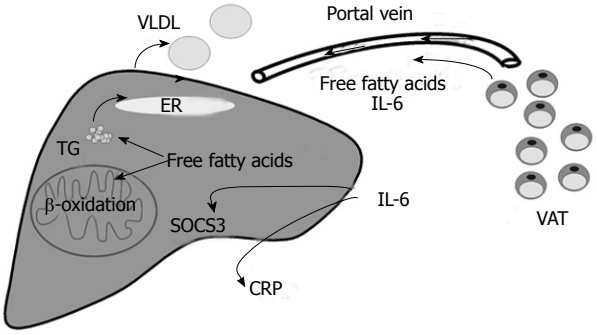

Figure 1 Crosstalk of visceral adipose tissue and the liver. Free fatty acids and Interleukin (IL)-6 released by visceral adipose tissue (VAT) are transported to the liver by the portal vein. Free fatty acids promote steatosis, enhance β-oxidation and the release of very low density lipoproteins (VLDL) contributing to dyslipidemia. IL-6 induces hepatic C-reactive protein (CRP) synthesis and suppressor of cytokine signalling 3 (SOCS3), and thereby is linked to systemic inflammation and hepatic insulin resistance, respectively (adapted from Schaffler A, Scholmerich J, Buchler C. Mechanisms of disease: adipocytokines and visceral adipose tissue--emerging role in nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005; 2: 273-280[6]). ER: Endoplasmatic reticulum; TG: Triglycerides.

that globular adiponectin, the globular C1q domain of this protein generated by proteolysis of the full-length protein, may also exist in serum[28]. However, circulating levels seem to be rather low, questioning the biological significance of this protein[29] that may nevertheless be of therapeutic relevance. Epidemiological studies revealed that low adiponectin levels are associated with NASH independent of insulin resistance and body mass index, and hepatoprotective effects of adiponectin have been identified in animal studies or with isolated liver cells[30-32]. MHO individuals are insulin sensitive and have adiponectin levels similar to normal-weight controls despite excessive weight and body fat, and this association may further underline the protective effects of this adipokine[33].

NAFLD not only compromises the hepatic manifestation of the metabolic syndrome but is linked to a higher risk of develop metabolic disorders like type 2 diabetes or cardiovascular disease[34,35]. Fatty liver is even associated with dyslipidemia, metabolic syndrome and low adiponectin independent of body mass index (BMI), waist to hip ratio and visceral fat mass[36]. NAFLD has been predicted to increase along with the growing epidemic of obesity[37] and understanding of its pathophysiology is a prerequisite to develop non-invasive diagnostic tools and to establishing effective treatment regimes. Rising adiponectin levels may be beneficial in liver disease and its protective effects in hepatic steatosis and NASH[38] are summarized in the current review article.

EPIDEMIOLOGY OF NAFLD

Diagnosis of NAFLD requires a careful anamnesis to exclude other liver diseases or drug-mediated liver damage. Moderate alcohol intake, defined by most physicians as 20 to 40 g/d in men and 20 g/d in women, has to be en-

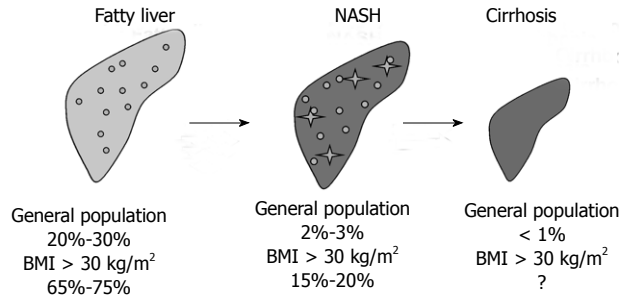

Figure 2 Epidemiology of non-alcoholic fatty liver disease. Current estimates of the prevalence of fatty liver, non-alcoholic steatohepatitis (NASH) and obesity-related liver cirrhosis in the general population and in obesity defined as body mass index (BMI) above 30 kg/m^2.

quired about[39]. Liver biopsy is essential for diagnosis and staging but the use of this invasive method is limited to a subgroup of patients[39].

When NAFLD is defined as elevation of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) and transferrin saturation of less than 50% the frequency is 5.4% in the general population of the United States[40]. When elevated gamma-glutamyltranspeptidase (GGT) is included and lower cut-off values are used the prevalence is 24%[41]. Studies performed in gastroenterology units restricted cohorts identified 11% of the patients as having NAFLD[42]. In bariatric surgery patients hepatic steatosis ranges from 65% to 90%[43,44]; NASH has been diagnosed in 15 to 55% and fibrosis in 34% to 47%[41,45].

Current estimates based on different studies in unselected and selected populations indicate that about 20% to 30% of adults in Western countries have excess fat accumulation in the liver, 2% to 3% of adults are thought to meet current diagnostic criteria for NASH and eventually up to one third of those with NASH suffer from progressive fibrosis or even cirrhosis[41,45,46]. In obesity defined as BMI above 30 kg/m^2 and in morbidly obese patients these values are much higher and patients with NASH are overrepresented in these populations[41,45] (Figure 2).

GENETICS OF NAFLD

Visceral adiposity and insulin resistance are clearly related to NAFLD[47] and genetic variations associated with obesity and disproportionate body fat distribution may predispose to development of steatotic liver. Chemerin is a recently identified adipokine and a common genetic variation is associated with increased visceral fat mass in non-obese subjects but epidemiological studies to link chemerin alleles with NAFLD are still lacking[48]. The adiponectin 45T →G variant contributes to overall fatness and abdominal obesity but is not an important determinant of NAFLD at least in Chinese people[49,50].

Gene variations may influence NAFLD stage, progression and even occurrence. NAFLD is much more likely in Hispanic Americans than among whites[51] and African Americans have a lower degree of hepatic steatosis relative to whites[52]. Familial clustering of NAFLD has been demonstrated, and fatty liver is more common in siblings and parents of children with NAFLD indicating that NAFLD, similar to type 2 diabetes, is a multifactorial disease[53]. Environmental and genetic factors define the individual risk of developing NAFLD and may also explain why only a subgroup of patients develop more progressive liver damage.

Studies in small cohorts have identified genetic associations of microsomal triglyceride transfer protein, an enzyme regulating hepatic VLDL release, the antioxidant mitochondrial enzyme superoxide dismutase 2, the inflammatory cytokine TNF and the main profibrotic cytokine TGF-β with NAFLD[54,55]. Genome-wide association studies find that variations of patatin-like phospholipase domain containing 3 (PNPLA3, adiponectin), a protein with close homology to adipose triglyceride lipase but so far unknown function, contributes to ethnic and inter-individual differences in hepatic steatosis and susceptibility to NAFLD[56,57].

This association has not been confirmed in a recent study in non-Hispanic, Caucasian, women with liver biopsy proven NAFLD, where an association between NASH activity score and single nucleotide polymorphisms (SNPs) within the squalene synthase (FDFT1) gene, a key regulator of cholesterol biosynthesis, is described. Polymorphisms of the pregnancy zone protein, a proteinase involved in clearance of TGF-β, are linked to systemic AST levels, and variants of platelet-derived growth factor α are linked to liver fibrosis[58].

Genetic variations of adiponectin are found to be associated with NAFLD[50,59], and single nucleotide polymorphisms in adiponectin receptor 1 (AdipoR1) and AdipoR2 contribute to variations in hepatic fat accumulation in humans[60,61].

SYSTEMIC ADIPONECTIN IN NAFLD

Systemic adiponectin concentrations are in the μg/mL-range indicating that adiponectin constitutes a substantial fraction of plasma proteins, and these high levels are remarkably constant. Despite its abundant presence in plasma, adiponectin is cleared rapidly by the liver with a half-life of about 75 min[62].

Visceral adiposity is associated with elevated circulating free fatty acids and higher concentrations of most adipose tissue released proteins[63]. Adiponectin differs from the adipokines described so far because its systemic levels are decreased in obesity[23]. In high fat diet induced obese rodents and in ob/ob mice adiponectin levels are reduced in plasma and clearance is significantly prolonged, indicating markedly impaired adiponectin synthesis in obesity[62,64,65]. Visceral fat accumulation is associated with hypoadiponectinemia and negative associations of visceral fat with systemic adiponectin have been identified[66,67].

Besides adiponectin, circulating levels of omentin predominantly released from the stromovascular cells of visceral fat are found reduced in obesity and serum omentin levels are increased in patients with NAFLD and independently predict hepatocyte ballooning[68].

In healthy Caucasians, BMI and adiponectin, but not insulin resistance, predict serum concentrations of both ALT and GGT[69]. Low adiponectin levels are even found associated with NASH independent of insulin resistance and BMI[30]. Multivariate regression analysis identifies decreased adiponectin as an independent predictor of liver steatosis and elevated ALT and GGT levels in healthy obese individuals[32]. In NAFLD patients low adiponectin levels are closely associated with the degree of hepatic steatosis, necroinflammation and fibrosis[30,32]. Shimada *et al*[70] reported that 90% of patients with early-stage NASH can be predicted by a combined evaluation of the serum adiponectin level, homeostasis assessment model-insulin resistance (HOMA-IR) score, and serum type Ⅳ collagen 7S level.

Circulating adiponectin levels in the μg/mL range by far exceed concentrations commonly required for receptor-dependent signalling. This may indicate receptor independent functions of adiponectin and binding to growth factors like platelet derived growth factor (PDGF), extracellular matrix proteins, low density lipoprotein (LDL) and opsonization of apoptotic cells to stimulate phagocytosis have been described[71-74].

Systemic adiponectin is about 20% to 60% lower in NAFLD than healthy controls[75-77] but considering the high levels in the circulation and a half-maximal effective dose of 0.85 μg/mL full-length adiponectin for AdipoR2 stimulated fatty acid oxidation[78] the question arises whether impaired receptor-mediated signalling due to reduced concentrations is a reasonable explanation for metabolic complications associated with hypoadiponectinemia. Therefore, it is likely that adiponectin receptor signal transduction pathways are also impaired in NAFLD.

ADIPONECTIN RECEPTORS IN NAFLD

Two 7-transmembrane proteins, AdipoR1 and AdipoR2, have been identified to function as adiponectin receptors[78]. Although initial studies using rodent tissues reveal preferential expression of AdipoR2 in the liver, in human tissues AdipoR1 and AdipoR2 mRNAs are most abundant in skeletal muscle and both are moderately expressed in the liver[78]. AdipoR1 protein is easily detected in human hepatocytes indicating that both receptors may play a role in liver physiology[79].

Although there is a well documented relationship between low adiponectin and liver disease, an association of NAFLD and reduced expression of hepatic adiponectin receptors is not consistently reported. Furthermore, mainly mRNA expression has been analysed and this may not necessarily predict protein abundance[80,81].

In animal models of obesity, hepatic adiponectin receptor mRNAs are found unchanged or even increased[65,82,83]. In human biopsies, hepatic adiponectin receptor mRNAs are increased in biopsy-proven NASH compared to steatotic livers[84]. Other studies, however, describe similar levels of adiponectin receptor mRNA in normal liver, steatotic liver and NASH[85,86]. There are also reports on reduced AdipoR2 mRNA in NASH compared to simple steatosis or lower AdipoR2 mRNA in fatty liver with no further reduction in NASH[87,88].

Data on AdipoR2 proteins are sparse and one study demonstrates reduced AdipoR2 protein in human NASH compared to steatotic liver[88]. Treatment of hepatocytes with palmitate is used as an in vitro model for hepatocyte steatosis and 200 μmol/L of this fatty acid reduce AdipoR2 protein in Huh7 cells[89]. Activating transcription factor 3 (ATF3) is induced upon endoplasmic reticulum stress and in the liver of ob/ob mice, and suppresses AdipoR2 in HepG2 cells[90]. Therefore, besides low circulating adiponectin, AdipoR2 may be reduced in hepatic steatosis and NASH indicating a possible adiponectin resistant state.

ANTISTEATOTIC EFFECTS OF ADIPONECTIN

Dyslipidemia is characterized by high circulating triglycerides[91] and low high density lipoprotein (HDL) cholesterol levels, and is frequently accompanied by hepatic steatosis[92]. Adiponectin negatively correlates with serum triglycerides and apolipoprotein B (ApoB), the main apolipoprotein of the triglyceride rich VLDL[93,94]. Hepatocyte ApoB and triglycerides are reduced by adiponectin indicating lower hepatic VLDL release[28,95,96]. Furthermore, VLDL catabolism is enhanced by an increased skeletal muscle lipoprotein lipase and VLDL receptor expression[97]. This more favourable lipid profile may be linked to lower hepatic lipid storage.

A choline and L-amino acid deficient diet induces more severe hepatic steatosis in adiponectin deficient mice compared to wild type animals[14]. Adenoviral expression of adiponectin ameliorates lipid deposition in the liver[95]. SREBP-1c is a central regulator of fatty acid synthesis, and is suppressed by adiponectin in hepatocytes and in the liver of db/db mice[95]. AMP-activated protein kinase (AMPK) is physiologically activated by low energy status, and switches on ATP-producing catabolic pathways (such as fatty acid oxidation and glycolysis), and switches off ATP-consuming anabolic pathways (such as lipogenesis)[98]. Adiponectin activates AMPK by binding to AdipoR1[78]. Suppression of SREBP-1c by adiponectin is mediated through AdipoR1/LKB1, an upstream kinase of AMPK, and AMPK pathway[95]. AMPK in addition phosphorylates acetyl-CoA carboxylase (ACC) and this is subsequently associated with a higher activity of carnitine palmitoyltransferase 1 (CPT-1), a rate limiting enzyme in fatty acid oxidation[98].

Signalling *via* AdipoR2 enhances peroxisome-proliferator activated receptor α (PPARα) activity[78,99]. PPARα upregulates CPT-1, stimulates β-oxidation, reduces lipid synthesis and thereby prevents excess triglyceride storage[100].

ANTIINFLAMMATORY AND ANTIAPOPTOTIC EFFECTS OF ADIPONECTIN

Lipopolysaccharide (LPS) is involved in the pathogenesis

of NAFLD and elevated levels of circulating LPS are found in obesity[101,102]. Increased gut permeability and a higher prevalence of small intestinal bacterial overgrowth correlates with the severity of steatosis but not with NASH[103]. Besides age, inflammation was identified as an independent predictor of progression to advanced fibrosis in NASH patients[104]. Hepatic steatosis may be accompanied by inflammatory cell infiltrates composed of neutrophils and mononuclear cells. In several mouse models of immune mediated hepatitis, adiponectin reduces TNF and induces interleukin-10 (IL-10) release from Kupffer cells[105]. Adiponectin lowers CRP synthesis in cytokine stimulated rat hepatocytes, and an inverse correlation of systemic adiponectin and CRP has been identified in obese patients[106,107]. Adiponectin may exert its anti-inflammatory activity by lowering nuclear factor kappa B (NFκB) action in preactivated cells or by inducing tolerance to inflammatory stimuli by a rapid and transient activation of NFκB that subsequently renders the cells inert to further activation[108-111].

Nevertheless, in patients suffering from chronic inflammatory diseases like inflammatory bowel disease or type 1 diabetes that are not associated with adiposity elevated circulating adiponectin levels that even correlate with inflammatory markers are found[112-114], and an induction of inflammatory proteins and activation of NFκB by recombinant adiponectin is described in several studies[25,108,113,115]. Therefore, adiponectin seems to be regulated in the opposite direction in classic versus obesity-associated chronic inflammatory diseases and may even exert opposite activities in resting compared to activated cells[116].

NFκB promotes cell survival and NEMO-mediated NFκB activation in hepatocytes has an essential physiological function to prevent the spontaneous development of steatohepatitis and hepatocellular carcinoma[117]. Adiponectin activates NFκB in human hepatocytes, and thereby may prevent hepatocyte apoptosis. Adiponectin further upregulates the chemokine interleukin 8 (CXCL8) *via* AdipoR1 and NFκB dependent pathways in primary human hepatocytes[118]. CXCL8 is an antiapoptotic protein[119] and overexpression of the rodent CXCL8 homologous protein protects the liver from galactosamine and endotoxin induced damage[120].

Adiponectin further antagonizes hepatocyte death by blocking fatty acid-induced activation of c-Jun NH2 terminal kinase[121], by reducing TNF levels[105] and by inhibiting fatty acid mediated upregulation of CD95[122].

ANTIOXIDATIVE EFFECTS OF ADIPONECTIN

Fatty liver is thought to represent the first incident towards the subsequent development of liver fibrosis[6]. Accelerated β-oxidation of fatty acids in hepatic steatosis is associated with excess reactive oxygen species (ROS), lipid peroxidation, the release of inflammatory cytokines, death of hepatocytes and activation of hepatic stellate cells[1]. ROS and lipid peroxidation are thought to contribute to the progression of liver injury partly by accelerating inflammation that in turn causes ROS production[1]. Oxidative stress is enhanced in human hypoadiponectinemia and in adiponectin knock-out mice fed a choline-deficient L-amino acid deficient diet[123,124]. Hepatic cytochrome P450 2E1 (CYP2E1) is elevated in these animals and in human NASH and may contribute to higher ROS levels[125,126].

Aldehyde oxidase 1 (AOX1) is a xenobiotic metabolizing protein whose physiological role has not been evaluated in detail so far[127]. AOX1 activity has been identified as an important source of ROS[128] and is reduced in hepatocytes by adiponectin *via* activation of PPARα[31] Adiponectin also increases ROS detoxifying enzymes and AdipoR2 is involved in the induction of superoxide dismutase 1 and catalase[129].

ANTIFIBROTIC EFFECTS OF ADIPONECTIN

Liver injury causes activation of otherwise "quiescent" hepatic stellate cells (HSC) and activated cells proliferate, synthesize CTGF and extracellular matrix proteins[130]. TGF-β is the main profibrotic factor in fibrosis and induces CTGF synthesis. CTGF stimulates binding of TGF-β to its receptor and thereby enhances TGF-β activity[130]. CTGF is induced by TGF-β indicating an autocrine or paracrine loop that mutually enhances synthesis of both proteins[130]. Knock-down of AdipoR2 in mice fed a methionine-choline deficient diet to cause progressive fibrosing steatohepatitis is associated with higher levels of steatosis, inflammation and fibrosis[131]. Overexpression of AdipoR2 is protective, and this mechanistically includes inhibition of TGF-β signaling and stimulation of PPARα activity[131].

Expression of recombinant adiponectin in activated HSC reduces proliferation and lowers α-smooth muscle actin that is induced in activated HSC[132]. Furthermore, apoptotic cell death of activated HSC is augmented[132]. Exogenously added recombinant adiponectin suppresses PDGF-stimulated HSC proliferation by activation of AMPK[133]. Adiponectin may also bind to growth factors like PDGF and thereby inhibits binding to their corresponding receptors[71]. Leptin is a well described profibrotic adipokine and several studies have shown that adiponectin antagonizes leptin bioactivity[134,135]. Adiponectin blocks leptin-induced STAT3 phosphorylation in activated HSC and leptin-mediated upregulation of TIMP-1 release and these in-vitro findings have been confirmed *in-vivo*[134].

DIET, EXERCISE AND PHARMACOLOGICAL INTERVENTIONS

Studies analysing the impact of changes in life style and medications in NAFLD have been performed in small patient groups sometimes even lacking suitable controls. Currently weight loss and exercise are recommended as initial strategies to improve NASH[136]. Diet and diet in conjunction with exercise for 6 mo cause a similar reduction in body weight and intrahepatic fat[137]. In 19 sedentary obese

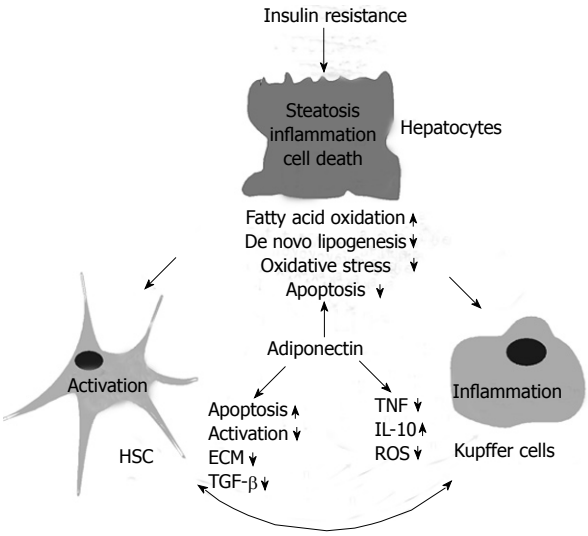

Figure 3 Hepatoprotective effects of adiponectin. Hepatic insulin resistance correlates with liver fat content, and is currently thought to represent the first incident in metabolic liver diseases. Insulin resistance and steatosis may also promote inflammation and fibrosis although the factors leading to advanced liver damage have not been identified so far. Major pathophysiological alterations of hepatocytes, hepatic stellate cells (HSC) and Kupffer cells in hepatic steatosis and/or non-alcoholic steatohepatitis are indicated. The protective activities of adiponectin are listed and arrows indicate an induction or repression of these pathways/proteins by adiponectin. IL: Interleukin; TGF: Transforming growth factor; TNF: Tumor necrosis factor; ECM: extracellular matrix; ROS: Reactive oxygen species.

men and women four weeks of aerobic exercise improved hepatic steatosis even in the absence of weight loss[138]. In a randomized controlled trial enrolling 31 patients with biopsy-proven NASH intensive changes in life style with the objective of at least 7% weight loss and educational training without weight reduction have been compared[139]. Weight loss significantly correlates with improvement in NASH histological activity score and weight loss of 7% or even more is recommended as a treatment strategy for these patients[139]. Another study also reports improvements of histological and laboratory parameters when body weight is reduced by 10% in NASH patients[140]. Adiponectin concentrations increase by about 36% in type 2 diabetic patients by 13% weight loss[141], and this may partly contribute to the metabolic improvements observed in these patients.

Clinical trials using fibrates have revealed inconsistent results so far. Treatment of sixteen NASH patients with clofibrate did not ameliorate biochemical or histological parameters[142], whereas a second study demonstrated biochemical and ultrasound improvements with fenofibrate[143]. Emerging data on thiazolidinediones have demonstrated improvement in both liver enzymes and histology[144,145]. These drugs activate PPARγ and thereby inhibit growth of HSC and TGF-β mediated induction of CTGF, respectively[146]. PPARγ is the main adipogenic transcription factor and its agonists stimulate adipogenesis[147]. Thiazolidinediones strongly stimulate adiponectin synthesis and elevate systemic adiponectin[147]. Increase of adiponectin by pioglitazone is related to histological improvement of steatosis, inflammation and fibrosis confirming the crucial role of adiponectin in NAFLD[148]. The PPARγ agonist rosiglitazone even induces AdipoR2 in hepatocytes[146]. A recent study reports that pioglitazone therapy improves adipose tissue insulin sensitivity and this correlates with a reduction in hepatic fat and necroinflammation[149]. Activation of PPARγ primes human monocytes into alternative M2 macrophages with anti-inflammatory properties and patients may also benefit from reduced inflammation[150]. In line with this hypothesis pentoxifylline with multiple pharmacological effects including antioxidant and anti-inflammatory activity[151] has been tested in small clinical trials, and biochemical and histological improvements have been reported[151,152]. Vitamin E therapy decreases AST and ALT levels and hepatic steatosis but does not improve necroinflammation and fibrosis[153]. Antioxidants may even prevent health-promoting effects of physical exercise namely insulin sensitivity and rise of systemic adiponectin in untrained and pre-trained individuals, and therefore, may be more effective in patients with low physical activity[154]. In summary to date no pharmacologic treatment has been reliably shown to be effective for the treatment of NASH patients.

CONCLUSION

Adiponectin has emerged as a protective adipokine in insulin resistance and obesity related liver diseases (Figure 3), and drugs that elevate systemic adiponectin may be useful as therapeutics for NAFLD. Adiponectin receptor signalling pathways and potential hepatic adiponectin resistance in NASH, however, have been poorly investigated so far. Identification of molecules downstream of AdipoR1/2 and strategies to enhance adiponectin receptor activity may constitute promising approaches towards treatment of NAFLD.

ACKNOWLEDGEMENTS

The authors thank Professor Charalampos Aslanidis for helpful suggestions.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2812

World J Gastroenterol 2011 June 21; 17(23): 2812-2820
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Streptozotocin-induced expression of Ngn3 and Pax4 in neonatal rat pancreatic α-cells

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Author contributions: De W and Liang XD contributed to the conception and design; Liang XD, Guo YY and Sun M contributed to the acquisition of data; Liang XD and Wang N analyzed and interrelated the data; Liang XD and Guo YY drafted the manuscript; Yuan L revised the manuscript critically for important intellectual content; all the authors have read and approved the final version of the manuscript.

Supported by The National Natural Science Foundation of China, No. 81070620

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Received: January 14, 2010 Revised: March 7, 2011
Accepted: March 14, 2011
Published online: June 21, 2011

Abstract

AIM: To investigate the mechanism behind β-cell regeneration in neonatal rat pancreas treated with streptozotocin (STZ).

METHODS: Neonatal Sprague Dawley rats were intraperitoneally injected with 70 mg/kg STZ. Body weight, pancreas weight and blood glucose were recorded every two days after the treatment. To identify the expression and location of transcription factors in the rat pancreas, double immunofluorescent staining was performed using antibodies to specific cell markers and transcription factors.

RESULTS: Expression of Neurogenin 3 (Ngn3), a marker for endocrine precursor cells, was observed by immunofluorescence in a few β-cells and many α-cells. The expression reached a peak 12 d after treatment. Pax4, a transcription factor that lies downstream of Ngn3 and plays an important role in β-cell differentiation, was also expressed in the α-cells of STZ-treated rats. We did not observe significant changes in Nkx6.1, which is essential for β-cell maturation in the treated rats.

CONCLUSION: α-cells dedifferentiated into endocrine precursor cells and acquired the ability to dedifferentiate in the neonatal rat pancreas after STZ treatment.

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Key words: Pancreatic remodeling; Dedifferentiation; Endocrine precursor cells; Streptozotocin; Transcription factors

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Liang XD, Guo YY, Sun M, Ding Y, Wang N, Yuan L, De W. Streptozotocin-induced expression of Ngn3 and Pax4 in neonatal rat pancreatic α-cells. *World J Gastroenterol* 2011; 17(23): 2812-2820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2812.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2812

INTRODUCTION

The pancreas originates from gut endoderm. During development, the rat pancreas undergoes two transitions in embryonic days[1]. After birth, many major developmental changes occur, including β-cell apoptosis, replication, and exogenesis[2]. This stage is referred to as the remodeling of pancreas. In our previous work, we found alpha-fetoprotein and Mesothelin in embryonic rat pancreases but not in adult rat pancreases, nevertheless, we observed the expression of these two proteins during the remodeling of the rat pancreas[3,4]. These studies indicate that during the remodeling phase, the neonatal pancreas is not fully matured. Furthermore, after treatment with Streptozotocin

(STZ) during this stage, the ontogeny of regeneration can be observed[5]. Conversely, after treatment with STZ during adulthood, little regeneration of β-cells was found[6]. Extensive studies have been reported on the model of STZ-induced depletion of β-cells in the neonatal rat pancreas, which showed that this model can be used to study β-cell replacement therapy for diabetes[5,7,8].

The development of β-cells is regulated by a series of transcription factors[9,10]. However, few studies have focused on the expression of these transcription factors during the regeneration of β-cells in STZ-treated neonatal rats. One of the most important upstream transcription factors is pancreas-duodenal homeobox 1 (Pdx1)[11]. The initial expression of Pdx1 (E8.5-E9.0) marks the prepancreatic endoderm before it is visibly thickened[12-14], and corresponds to the classically defined period of pancreatic specification[15]. Following the expression of Pdx1 is the Neurogenin 3 (Ngn3), a basic helix-loop-helix transcription factor[16] that marks endocrine pancreatic precursor cells. Among a series of transcription factors that differentiate endocrine precursors into β-cells, paired domain homeobox gene 4 (Pax4)[17] and NK family member Nkx6.1[18] lie downstream of Ngn3.

Although these factors are essential for the development and maturation of β-cells, it is unknown whether Ngn3, Pax4 and Nkx6.1 participate in the regeneration of β-cells after STZ treatment during the remodeling phase of the pancreas. Especially, there is little information on the differentiation factors that are involved in the remodeling of the rat pancreas. This study was designed to determine the expression and location of these transcription factors in the STZ-treated neonatal rat pancreas.

MATERIAL AND METHODS

Animals

Pregnant Sprague Dawley rats from the Animal Center of Nanjing Medical University, Nanjing, China, were kept under conventional conditions and provided with a 12:12 h light-dark cycle. Litters were reduced to 12 pups at birth. Four days after birth, half of the pups in each litter was intraperitoneally injected with 70 mg/kg STZ freshly dissolved in citrate buffer (0.05 mol/L, pH 4.5). The remaining pups received vehicle only. Blood glucose was measured with a OneTouch Ultra blood glucose meter (LifeScan Inc. Milpitas, CA, USA) in blood obtained by lancing the tail vein. Body weight was recorded every two days. On the day of treatment and days 4, 8, 12, 16 and 20 after treatment, animals were killed by decapitation or by overdose of anesthesia (sodium amobarbital, amytal sodium, Sigma-Aldrich 200 mg/kg body weight). Pancreases were collected immediately and frozen in liquid nitrogen or fixed. Three to five pups from at least three separate litters were studied at each time point. All experiments were conducted in accordance with the Chinese Law for Animal Protection and were approved by Nanjing Medical University Ethics Review Committee (approval No. 200913).

Fluorescence immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 24-36 h followed by a standard protocol of dehydration and paraffin embedding. Sections (5 μm) were cut and mounted on glass slides (Fisher Scientific, Pittsburgh, PA, USA). The paraffin sections were deparaffinized in xylene and dehydrated in graded ethanol and distilled water. The tissue sections were blocked in 1% bovine serum albumin for 1 h. For double fluorescence immunohistochemical localization of glucagon and insulin, the mouse anti-glucagon (1:100, Sigma-Aldrich, St. Louis, MO, USA) antibody was applied after blocking and revealed using goat anti-mouse IgG-FITC (1:400, Santa Cruz, Santa Cruz, CA, USA). Rabbit anti-insulin polyclonal antibody (1:100, Santa Cruz, Santa Cruz, CA, USA) was then applied and revealed by Cy3-labeled anti-rabbit IgG (1:400, Santa Cruz, Santa Cruz, CA, USA). For dual fluorescence immunohistochemical localization of Ngn3, Pax4 or Nkx6.1 and insulin, rabbit anti-neurogenin 3 (1:100, Santa Cruz, Santa Cruz, CA, USA), goat anti-Pax4 (1:100, Santa Cruz, Santa Cruz, CA, USA) or goat anti-Nkx6.1 (1:100, Santa Cruz, Santa Cruz, CA, USA) antibody were added, respectively, and revealed by rabbit anti-goat IgG-FITC (1:400, Chemicon, Temecula, CA, USA) or mouse anti-rabbit IgG-FITC (1:400, Chemicon, Temecula, CA, USA). Mouse anti-insulin (1:100, Sigma-Aldrich, St. Louis, MO, USA) was applied and revealed by Cy3 conjugated anti-mouse (1:400, Chemicon, Temecula, CA, USA) antibody. For co-localizations of Ngn3, Pax4 or Nkx6.1 and glucagon, rabbit anti-neurogenin 3 (1:100), goat anti-Pax4 (1:100) or goat anti-Nkx6.1 (1:100) antibodies were added and revealed by rabbit anti-goat IgG-FITC (1:400) or mouse anti-rabbit IgG-FITC (1:400). Mouse anti-glucagon (1:100) was applied and revealed by Cy3 conjugated anti-mouse (1:400) antibody. Sections were placed in Gel Mount Aqueous Mounting Medium (G0918, Sigma) with a cover glass, and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan) at a magnification of × 200 or × 400.

β-cell mass

β-cell mass was measured by point counting morphometry on the same stained sections as described above. Each section was covered systematically at a magnification of × 400 using a 48-point grid to obtain the number of grid intercepts over β-cells, endocrine non-β-cells, exocrine pancreatic tissues, and non-pancreatic tissues. The relative β-cell area was calculated by dividing the number of intercepts over β-cells by the number of intercepts over total pancreatic tissue; the β-cell mass was then estimated by multiplying the relative β-cell volume by the corrected pancreatic weight. Non-β-cell mass was similarly calculated. A correction factor for pancreas weight was obtained by multiplying the pancreas weight by the ratio of intercepts over non- pancreatic tissues to intercepts over total tissues. Actual pancreas weight was then calculated by subtracting this correction factor from total pancreas weight. A monogram related to the number of points, the

Table 1 Body and pancreas weight in control and streptozotocin-treated animals after streptozotocin treatment on postnatal day 4 (mean ± SD)

	Days after STZ treatment				
	4	8	12	16	20
Body weight (g)					
Control	13.6 ± 0.3	24.4 ± 1.5	30.5 ± 1.3	34.5 ± 1.5	50.0 ± 1.5
STZ	12.4 ± 0.3	22.3 ± 1.4	26.4 ± 1.3	32.4 ± 1.7	43.9 ± 1.5
Pancreas weight (g)					
Control	24.4 ± 2.3	49.8 ± 3.2	59.8 ± 1.4	60.5 ± 3.4	187.5 ± 6.9
STZ	18.7 ± 1.4	46.8 ± 3.3	57.1 ± 1.9	57.3 ± 3.2	162.5 ± 8.4

n = 3 litters/18 animals per group. STZ: Streptozotocin.

volume density and the expected relative standard error of the mean (< 10%) was used to determine the number of intercepts needed for a representative sampling.

Statistical analysis

The experimental data was analyzed by paired Student *t* test using the SPSS 17.0 software. $P < 0.05$ was considered statistically significant. Data were presented as mean ± SD.

RESULTS

Body and pancreatic weight, blood glucose and islets in STZ-treated neonatal rat pancreases

After STZ treatment, body and pancreas weight did not change significantly (Table 1). Blood glucose concentrations significantly increased within 2 d after STZ treatment (Figure 1A). However, on day 20 after treatment, there was no longer a difference in blood glucose concentrations between the two groups.

Histological analysis showed that approximately 60% of insulin immunoreactive cells within the islets were lost 4 d after STZ treatment (Figure 1B). On day 8 after treatment, an increased number of small islets was observed (Figure 1C). On day 20 after treatment, more large islets were found, which may indicate that islet function had also recovered. Similarly, calculation of β-cell mass showed a reduction in β-cell mass from 4 d after STZ treatment onwards (Figure 1D). While β-cell mass was still reduced in STZ-treated rats on day 20 after treatment, blood glucose levels were not significantly different.

Expression and location of Ngn3

We used double immunofluorescence to stain Ngn3 and insulin or glucagon at different time points after STZ treatment. We did not find Ngn3 co-located with insulin in either treated or control rats (Figure 2A). By analyzing the coexpression of Ngn3 and glucagon, we observed abundant expression of Ngn3 in the treated rat islet α-cells (Figure 2B). In the STZ group, expression of Ngn3 could be detected on day 8 and reached a peak on day 12 after treatment (Figure 2C). However, no significant changes were observed in the signal from Ngn3 in α-cells 20 d after treatment compared with the control rats. In contrast, few α-cells expressed Ngn3 in control rats at each time point.

Expression and location of Nkx6.1

We stained Nkx6.1 and glucagon or insulin by immunofluorescence. Consistent with previous work, we found coexpression of Nkx6.1 and insulin in both the controls and the treated group (Figure 3A), while no Nkx6.1 expression was found in α-cells at any time point (Figure 3B) when we studied the coexpression of glucagon and Nkx6.1.

Expression and location of Pax4

We studied the colocation of Pax4 and insulin or glucagon by dual immunofluorescence. Consistent with previous work, we observed coexpression of insulin and Pax4 in both the control group and the treated group (Figure 4A). We also found enhanced expression of Pax4 in STZ-treated rat pancreases compared with control rats (Figure 4A). Eight days after treatment, we observed expression of Pax4 in α-cells of the treated rats but little expression in the control rats. However, we found coexpression of glucagon and Pax4 in both treated and control rats on day 12 after treatment (Figure 4B). On day 20 after STZ treatment, we could still observe a signal of Pax4 in the α-cells. However, in the control rats, few α-cells expressed Pax4 on day 20.

DISCUSSION

It is established that neonatal β-cells are able to regenerate after subtotal β-cell damage by STZ treatment. Regeneration of neonatal β-cells after destruction mainly relies on replication of pre-existing β-cells and heterogenesis of new cells[19]. In this article, we demonstrated a series of transcription factors expressed in pancreatic α-cells, which suggested that α-cells may be a source of β-cells during the regeneration of the STZ-treated neonatal rat pancreas.

We found that β-cells were damaged 4 d after STZ treatment. On day 8 after treatment, β-cell numbers were recovered in STZ-treated rats. By day 20 after treatment, there was still a reduction in β-cell mass but the blood glucose concentrations had reverted to normal. Although the model resulted in transient hyperglycemia, no difference in the mean body weight or pancreatic weight was seen between the two groups.

The study of pancreatic development has focused on transcription factors and transcription factor hierarchies during development. A central and heavily studied transcription factor in pancreatic development is Pdx1. Although Pdx1 is a key component of pancreatic specification, we found no significant difference in α or β-cells between STZ-treated rats and control animals. This indicates that Pdx1 is not involved in the regeneration of β-cells in STZ-treated animals.

After pancreatic formation, which is mediated by Pdx1, Ngn3 regulates the differentiation of endocrine pancreas. Lack of Ngn3 leads to an absence of islets[20]; and the ectopic expression of Ngn3 in other cells converts these

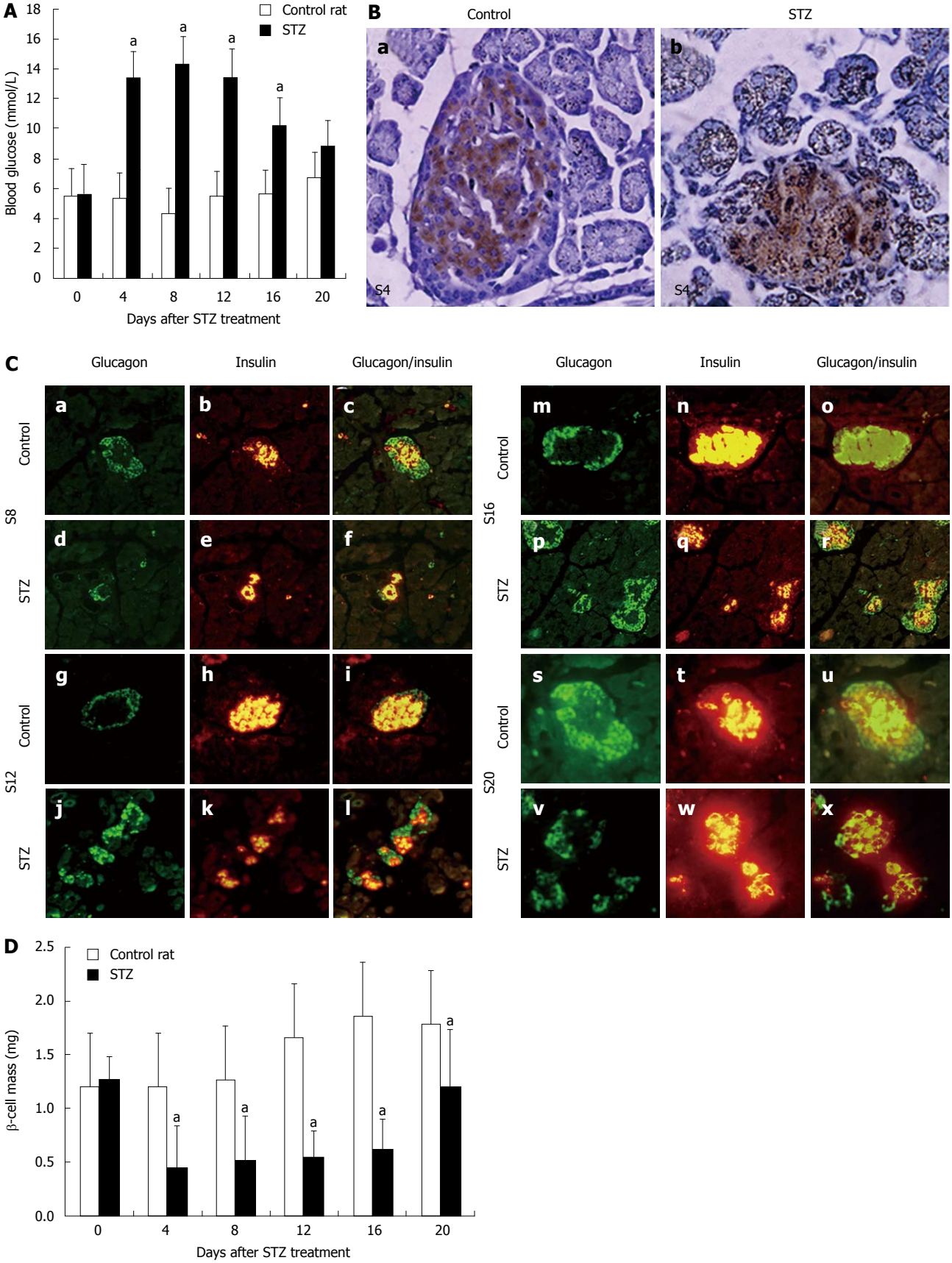

Figure 1 Body and pancreatic weight, blood glucose, islets and β-cell mass in streptozotocin-treated neonatal rat pancreas. A: Concentrations of fasting blood glucose in control rats or rats treated with streptozotocin (STZ) between day 0 and day 20 after STZ treatment. Data were obtained from 12-18 animals at each time point. $^{a}P < 0.005$ *vs* control; B: Immunohistochemical location of insulin in sections of rat pancreas in the control (a) and 4 d after STZ treatment; (b). Original magnification, × 400; C: Structure of islets in STZ-treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-glucagon antibody (green) and anti-insulin antibody (red). Original magnification, × 400. Mean ± SD; D: β-cell mass in control rats or rats treated with STZ between day 0 and day 20 after STZ treatment. Data were obtained from three rats per time point. $^{a}P < 0.05$ *vs* control rats.

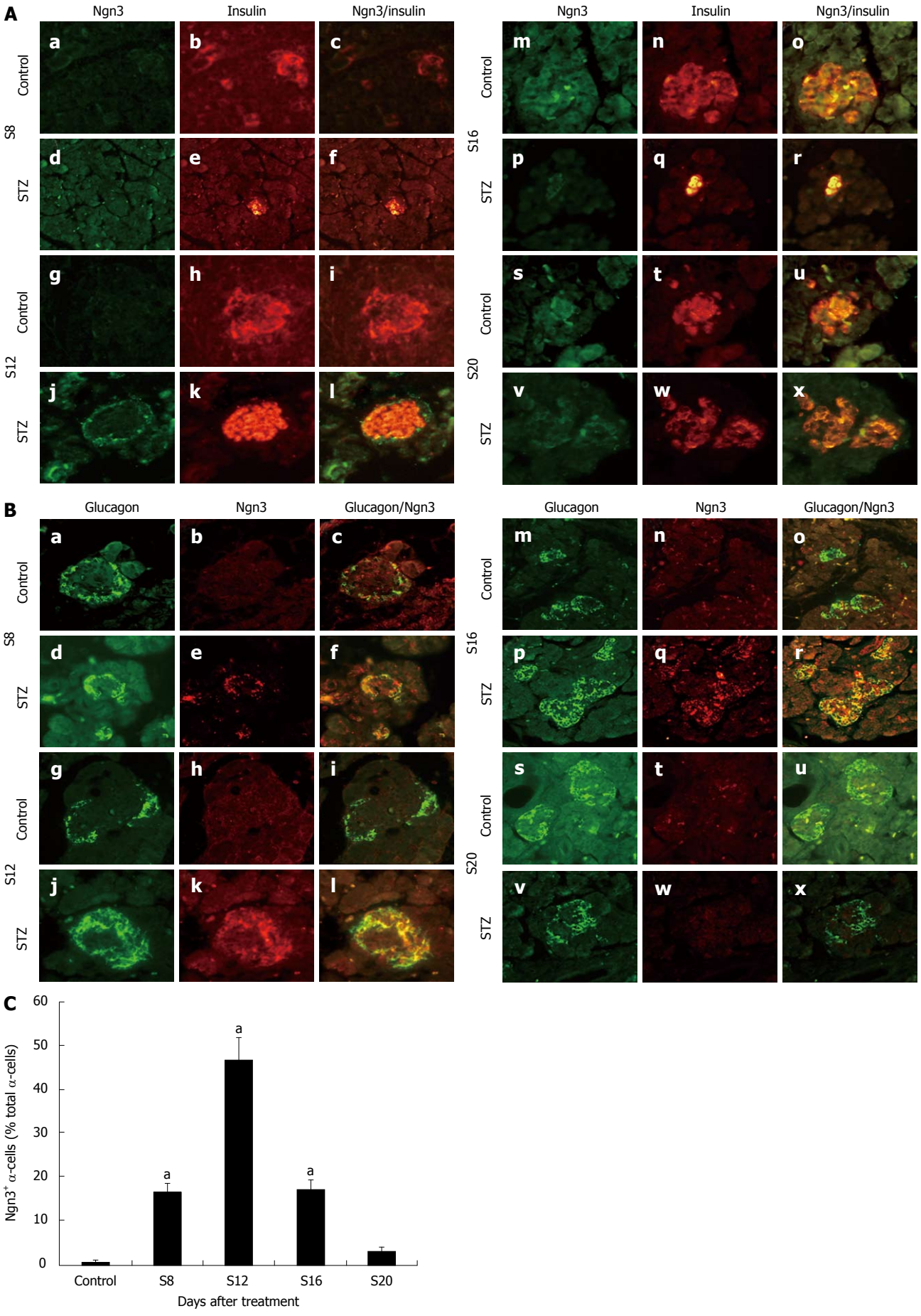

Figure 2 Expression and location of Ngn3. A: Immunofluorescent colocalization of Ngn3 and insulin in streptozotocin (STZ)-treated rats (d-f, j-l, p-r and v-x) and

control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-Ngn3 antibody (green) and anti-insulin antibody (red). Original magnification, × 400; B: Immunofluorescent colocalization of Ngn3 and glucagon in STZ-treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-glucagon antibody (green) and anti-Ngn3 antibody (red). Original magnification, × 400; C: Proportion of Ngn3+ / glucagon+ cells in total α-cells after treatment (Y). $^{a}P < 0.05$ *vs* control rats.

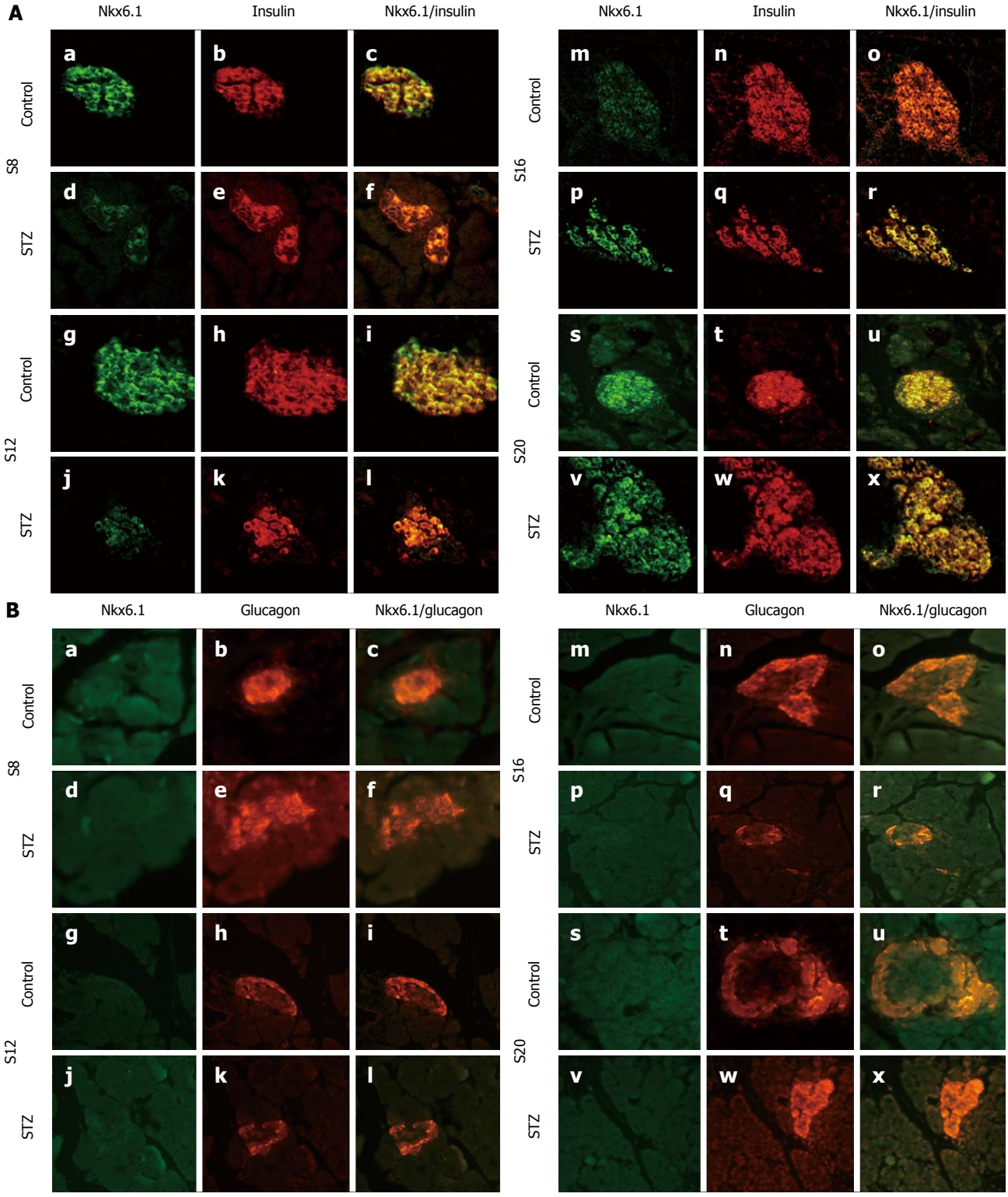

Figure 3 Expression and location of Nkx6.1. A: Immunofluorescent colocalization of Nkx6.1 and insulin in streptozotocin (STZ)- treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-Nkx6.1 antibody (green) and anti-insulin antibody (red); B: Immunofluorescent colocalization of Nkx6.1 and glucagon in STZ-treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-Nkx6.1 antibody (green) and anti-glucagon antibody (red). Original magnification, × 400.

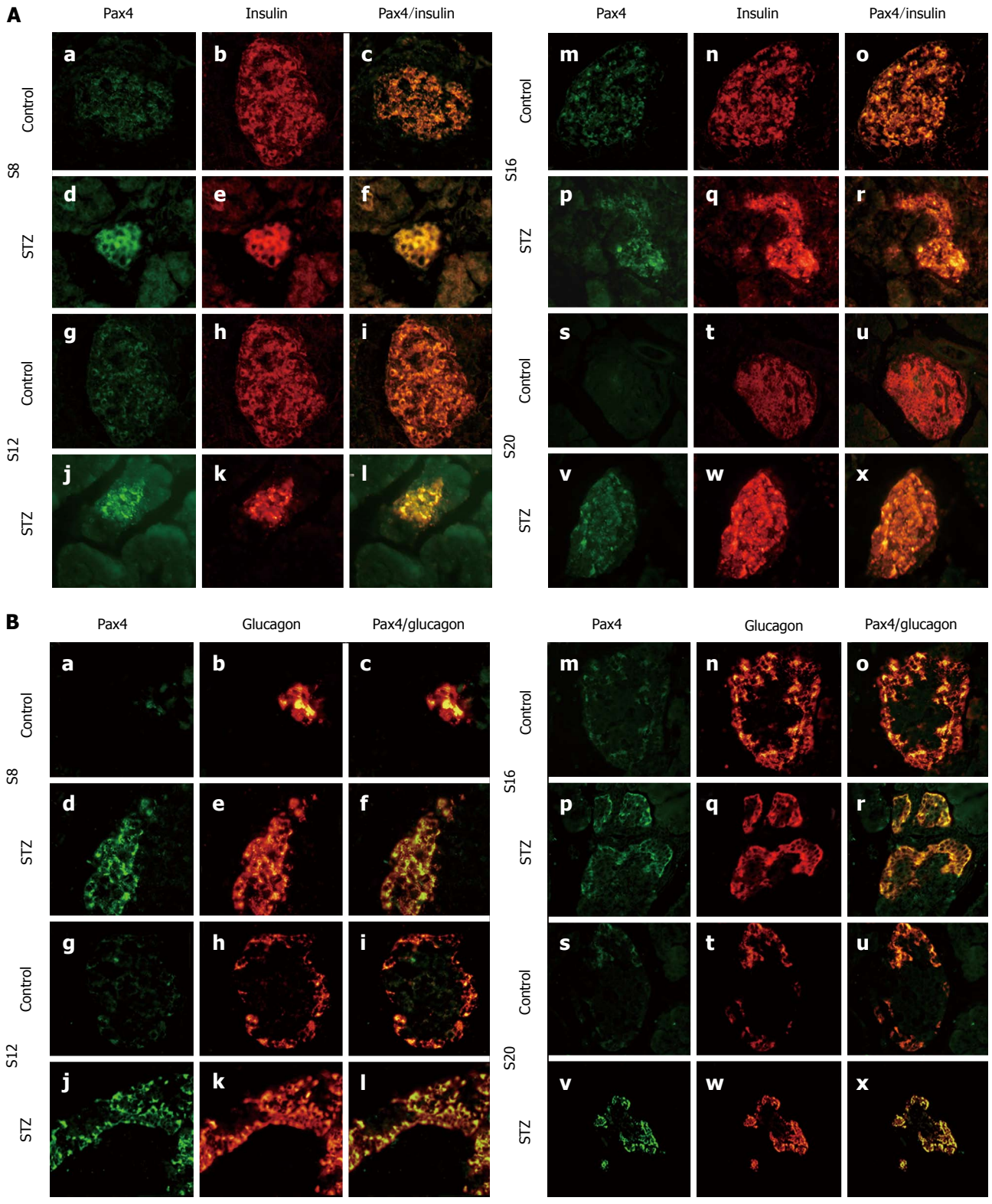

Figure 4 Expression and location of Pax4. A: Immunofluorescent colocalization of Pax4 and insulin in streptozotocin (STZ)-treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-Pax4 antibody (green) and anti-insulin antibody (red); B: Immunofluorescent colocalization of Pax4 and glucagon in STZ-treated rats (d-f, j-l, p-r and v-x) and control rats (a-c, g-i, m-o and s-u) on d 8 (a-f), 12 (g-l), 16 (m-r) and 20 (s-x) after STZ treatment. Pancreatic sections were immunostained with anti-Pax4 antibody (green) and anti-glucagon antibody (red). Original magnification, × 400.

cells into endocrine cells[21]. We observed Ngn3 expression in the α-cells of treated rats, which reached a peak on day 12 after STZ treatment. It has been reported that Ngn3 is activated through partial duct ligation in the adult mouse pancreas[22]. However, Ngn3 is absent in the β-cells of mice that underwent partial pancreatectomy[23]. The dif-

ferent results among these three models indicate that the mechanism of β-cell regeneration in different pathological situations can be varied. Interestingly, we observed abundant expression of Ngn3 in STZ-treated rats but not in control rats. The expression of Ngn3 in α-cells after STZ treatment indicated that α-cells dedifferentiated into precursor cells and may be candidates for β-cell formation. It has been suggested that high Ngn3 expression at an inappropriately early time in the developing mouse pancreas may result in a pancreas entirely consisting of small clusters of glucagon-positive cells[21,24]. Furthermore, by overexpression of Ngn3 in human[25] or mouse[26,27] pancreatic duct cells, the pancreatic duct cells could become endocrine cells. Apparently, the expression of Ngn3 is necessary for the transdifferentiation of α-cells to β-cells.

Next to Ngn3 induction, a complex network of transcription factors, including Pax4, progressively and differentially promotes the particular endocrine fates[28,29]. The expression of Pax4 is first observed around E9.5 in dorsal pancreatic buds of mouse embryos and vanishes shortly after birth[29,30]. Pax4 specifies the β-cell lineage into β and δ precursor cells[31]. In mice lacking Pax4, mature pancreatic and δ-cells were absent[29]. Conversely, ectopic expression of Pax4 in the mouse pancreas converted α-cells into β-cells. Moreover, the transgenic adult mice could survive after STZ-induced hyperglycemia[32]. Activation of Pax4 in endocrine progenitor cells may be mediated by Ngn3 since it binds to the Pax4 regulatory region and is necessary for Pax4 expression[33]. Ngn3 is required for ectopic Pax4 expressing α-cells to acquire a β-cell phenotype[34]. Interestingly, we observed expression of Pax4 in α-cells of both control and STZ-treated rats. In the STZ-treated rats, the expression of Pax4 reached a peak on day 16 after the treatment. The expression of Pax4 in the control animals suggests that Pax4 expression is characteristic of the pancreatic remodeling phase. Both cell differentiation and maturation occur during remodeling of the pancreas, which explains the presence of transcription factors in islet cells. After STZ treatment, the expression of Pax4 increased and a Pax4 signal could still be observed in α-cells 20 d after the treatment. This suggests that STZ treatment exaggerates and extends the period of remodeling.

Another transcript factor which lies downstream of Ngn3 is Nkx6.1, and it is associated with the development and maturation of β-cells. Nkx6.1 appears to be a marker for multipotent pancreatic progenitor cells[35]. At later developmental stages and in the adult pancreas, Nkx6.1 becomes completely restricted to insulin-expressing cells. Consistent with previous researches, we observed coexpression of Nkx6.1 and insulin in both STZ-treated and control rats. However, we did not find any Nkx6.1-positive α-cells. Immunoblotting revealed that the expression of Nkx6.1 decreased 4 d after STZ treatment, and reached normal levels on day 12 after treatment. Although Nkx6.1 is critical for the development of β-cells, it does not affect the generation of β-cells from α-cells.

It has been established that regeneration of neonatal rat β-cells after subtotal destruction by STZ occurs by two mechanisms of equal significance. The first mechanism is the replication of surviving β-cells in the islet compartment, the second mechanism is the replication of cells from a β-cell pool outside the islet compartment. In this article, we have demonstrated that α-cells may also be a source for β-cell regeneration. Mature α-cells converted to β-cells after partial duct ligation plus alloxan treatment, and the contribution of α-cells to the emergence of new β-cells was proportional to the degree of β-cell ablation[36]. However, α-cells could only convert to β-cells when the proportion of β-cell loss reached 99%[37].

In conclusion, during the period of pancreatic remodeling, the islets are not completely matured and the dedifferentiation of α-cells into endocrine precursor cells contributes to the recovery of β-cell mass after impairment by STZ.

COMMENTS

Background

Streptozotocin-induced β-cell loss in neonatal rat pancreas can trigger transient hyperglycemia. β-cell mass recovers 20 d after treatment. It is unknown whether Ngn3, Pax4 and Nkx6.1 participate in the regeneration of β-cells after streptozotocin (STZ) treatment during the remodeling phase of the pancreas.

Research frontiers

It has been shown that mature α-cells converted to β-cells after partial duct ligation plus alloxan treatment, and that the contribution of α-cells to the emergence of new β-cells was proportional to the degree of β-cell ablation.

Innovations and breakthroughs

There is little information on the differentiation factors that are involved in the remodeling of the rat pancreas. This study was designed to determine the expression and location of these transcription factors in the STZ-treated neonatal rat pancreas. The authors for the first time found the expression of Ngn3 and Pax4 in α-cells during remodeling of rat pancreas after STZ treatment.

Applications

Insulin deficiency caused by a reduced pancreatic islet β-cell number underlies the progression of both type 1 and type 2 diabetes, prompting efforts to develop β-cell replacement therapies. This study demonstrated the dedifferentiation of α-cells into endocrine precursor cells may contributes to the recovery of β-cell mass after impairment by STZ and this may provide a alternative way for β-cell replacement therapies.

Terminology

After birth, many major developmental changes occur, including β-cell apoptosis, replication, and exogenesis. This stage is referred to as the remodeling of pancreas.

Peer review

The study is well conducted and the results are interesting. This paper puts up the fact that α-cells dedifferentiate to endocrine precursor cells.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2821

World J Gastroenterol 2011 June 21; 17(23): 2821-2828
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Pathological and MR-DWI study of the acute hepatic injury model after stem cell transplantation

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Supported by The National Natural Science Foundation of China, No. 30070235, No. 30470508 and No. 30870695; The Natural Science Foundation of Hunan Province, No. 06JJ2008, 07JJ6040
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Received: December 30, 2010 Revised: March 17, 2011
Accepted: March 24, 2011
Published online: June 21, 2011

Abstract

AIM: To investigate apparent diffusion coefficient (ADC) values as an indication of reconditioning of acute hepatic injury (AHI) after allogeneic mononuclear bone marrow cell (MBMC) transplantation.

METHODS: Three groups were used in our study: a cell transplantation group (n = 21), transplantation control group (n = 21) and normal control group (n = 10). AHI model rabbits in the cell transplantation group were injected with 5 mL of MBMC suspension at multiple sites in the liver and the transplantation controls were injected with 5 mL D-Hanks solution. At the end of the 1st, 2nd and 4th wk, 7 rabbits were randomly selected from the cell transplantation group and transplantation control group for magnetic resonance diffusion-weighted imaging (MR-DWI) and measurement of the mean ADC values of injured livers. After MR-DWI examination, the rabbits were sacrificed and the livers subjected to pathological examination. Ten healthy rabbits from the normal control group were used for MR-DWI examination and measurement of the mean ADC value of normal liver.

RESULTS: At all time points, the liver pathological scores from the cell transplantation group were significantly lower than those in the transplantation control group (27.14 ± 1.46 *vs* 69.29 ± 6.16, 22.29 ± 2.29 *vs* 57.00 ± 1.53, 19.00 ± 2.31 *vs* 51.86 ± 6.04, P = 0.000). The mean ADC values of the cell transplantation group were significantly higher than the transplantation control group ((1.07 ± 0.07) × 10^{-3} mm^2/s *vs* (0.69 ± 0.05) × 10^{-3} mm^2/s, (1.41 ± 0.04) × 10^{-3} mm^2/s *vs* (0.84 ± 0.06) × 10^{-3} mm^2/s, (1.68 ± 0.04) × 10^{-3} mm^2/s *vs* (0.86 ± 0.04) × 10^{-3} mm^2/s, P = 0.000). The pathological scores of the cell transplantation group and transplantation control group gradually decreased. However, their mean ADC values gradually increased to near that of the normal control. At the end of the 1st wk, the mean ADC values of the cell transplantation group and transplantation control group were significantly lower than those of the normal control group [(1.07 ± 0.07) × 10^{-3} mm^2/s *vs* (1.76 ± 0.03) × 10^{-3} mm^2/s, (0.69 ± 0.05) × 10^{-3} mm^2/s *vs* (1.76 ± 0.03) × 10^{-3} mm^2/s, P = 0.000]. At any 2 time points, the pathological scores and the mean ADC values of the cell transplantation group were significantly different (P = 0.000). At the end of the 1st wk, the pathological scores and the mean ADC values of the transplantation control group were significantly different from those at the end of the 2nd and 4th wk (P = 0.000). However, there was no significant difference between the 2nd and 4th wk (P = 0.073 and 0.473, respectively). The coefficient of correlation between the pathological score and the mean ADC value in the cell transplantation group was -0.883 (P = 0.000) and -0.762 (P = 0.000) in the transplantation control group.

CONCLUSION: Tracking the longitudinally dynamic

change in the mean ADC value of the AHI liver may reflect hepatic injury reconditioning after allogeneic MBMC transplantation.

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Key words: Stem cells; Transplantation; Hepatic injury; Magnetic resonance imaging; Diffusion weighted imaging

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Shang QL, Xiao EH, Zhou QC, Luo JG, Wu HJ. Pathological and MR-DWI study of the acute hepatic injury model after stem cell transplantation. *World J Gastroenterol* 2011; 17(23): 2821-2828 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2821.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2821

INTRODUCTION

Mononuclear bone marrow cells (MBMCs) are adult stem cells that have multi-potential differentiation capabilities and low immunogenicity. Direct allogeneic MBMC transplantation can repair various organ injuries such as hepatic injury without obvious immune rejection[1]. There are many studies evaluating the therapeutic effect of MBMC transplantation therapy for hepatic injury. However, few studies have been undertaken to determine the feasibility of magnetic resonance diffusion-weighted imaging (MR-DWI) to evaluate the therapeutic effect of MBMC transplantation therapy on models of acute hepatic injury (AHI).

MR-DWI is an atraumatic and functional imaging technique that is very sensitive to molecular diffusion from the random and microscopic translational motion of molecules known as Brownian motion[2]. MR-DWI can image the difference in microscopic diffusion movements of water molecules in various tissues. The most noticeable merit of MR-DWI is providing an apparent diffusion coefficient (ADC) value that can distinguish the microscopic diffusion movement of water molecules in different tissues *in vivo* by assigning numerical values[3-7]. When a tissue has a pathological change, the microscopic diffusion movement of water molecules changes and the mean ADC value should also change. It has been generally accepted that MR-DWI is valuable in qualitatively and quantitatively diagnosing cerebral ischemia in the hyper-inchoate period[8]. During recent years, many studies of hepatic pathological changes using MR-DWI have been reported[3-7]. These showed that MR-DWI of the liver seems promising for the characterization of many diseases (especially focal liver lesions) by calculating ADC values.

Similarly, after MBMC transplantation therapy, there should be a dynamic change in the microscopic diffusion movement of water molecules in hepatic tissue during the repair process of AHI. Thus, the aim of our study was to evaluate the contribution of the mean ADC value in reflecting the repair process of AHI after MBMC transplantation therapy by comparison with the pathological change. The pathological mechanisms behind the dynamic change of the mean ADC value from injured hepatic tissue will be discussed in further detail.

MATERIALS AND METHODS

Material and instruments

Experiments were performed using 57 healthy, male New Zealand White rabbits weighing ~2.5 kg with an average age of ~2 mo. All animal work was conducted in accordance with the guidelines provided by the Institutional Animal Control and Utilization Committee. Five rabbits were randomly selected and used to isolate MBMCs. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco (New York, USA). Mononuclear cell separation medium was purchased from Tianjin Haoyang Company (Tianjin, China). D-Hanks solution, an electronic balance, 3% pentobarbital sodium, sterile surgical instruments, an optical microscope, cell separation tools and 2% trypan blue were supplied by the Second Xiangya Hospital. D-galactosamine (D-GaIN) was purchased from Jiangsu Nantong Tonglu Co. Ltd. (Nantong, China). Imaging was performed using a 1.5-Tesla Signa Twinspeed MR scanner (General Electron Medical Systems, USA) with a small diameter cylindrical brain radiofrequency coil.

Study groups and the establishment of AHI models

Acute hepatic injury was induced by D-galactosamine (D-GalN). D-GalN was dissolved in sterile 0.9% NaCl at a concentration of 10 g/100 mL (w/v). Forty-two rabbits were randomly selected to establish the AHI models. According to the weight of each rabbit, D-GalN solution was injected into the upper abdomen at a dosage of 1.0 g/kg. This amount was determined by preliminary experiments. The rabbits' weight, drug dosage and detailed administration times were recorded. The 42AHI rabbits were randomly and equally divided into 2 groups: a cell transplantation group and a transplantation control group. Liver function assays were performed 24 h after drug administration and pathological examinations of liver sections taken during cell transplantation were performed to verify the establishment of the AHI model. The remaining 10 healthy rabbits were assigned to the normal control group, and only MR-DWI examination was performed for measurement of the normal liver mean ADC value.

Isolation and transplantation of MBMCs

After being sacrificed by air injection into the ear vein, the bodies of 5 healthy male rabbits were sterilized by incubation in 75% ethanol. Their limb bones were then isolated and bone marrow was repetitively flushed using D-Hanks solution containing heparin. Soft tissue clumps were removed with 100 pore filters. Recovered cell suspensions were aliquoted into multiple centrifuge tubes. MBMCs were then obtained through density gradient centrifugation. Cell number was counted and the viabil-

Table 1 Criteria for the acute injured liver pathological score

Score	Cellular necrosis and liver hyperplasia	Inflammatory cell infiltration in the area of the header and lobule	Injury of vascular endothelium and thrombus
0	Without	Without	Without
1	Spotty liver cell degeneration, necrosis without change of hepatic sinusoid and lobule shape	Infiltration area < 1/3 lobule or low inflammatory cell infiltration in the area of the header	Hyperemia of hepatic sinusoid or thrombus
2	Scattering severe liver cell degeneration, necrosis of whole lobule or unobvious liver cell hyperplasia	Infiltration area: 1/3-2/3 of lobule or comparatively wide-bound inflammatory cell infiltration	Injury of vascular endothelium or inflammatory cell infiltration under vascular endothelium
3	Large sheet liver cell degeneration and necrosis involving multiple lobules	Infiltration area: > 2/3 of lobule or inflammatory cells surround header	Extensive injury of vascular endothelium and thrombus

Obvious liver cell hyperplasia score: -2; Not obvious liver cell hyperplasia score: +2.

ity examined using trypan blue. The percentage of live cells had to be > 95%. The MBMCs were resuspended in D-Hanks solution at a density of 4×10^6/mL. Five milliliters of cell suspension was aliquoted into 10 mL glass syringes and kept in an incubator at 37℃ with 5% CO_2 for a short time before transplantation.

MBMC transplantations were performed between 24 and 48 h after establishing the AHI models. Each AHI rabbit from the cell transplantation group was properly anesthetized and immobilized on an operating table. The skin of the upper abdomen was prepared and sterilized, then the liver was exposed with a 2 cm cut beneath the xiphoid process. After slowly injecting 5 mL of the MBMC suspension at multiple sites in the liver, the needle was withdrawn and the wound sutured to stop bleeding. The wound was treated with penicillin and covered with a sterile dressing. After transplantation, each model rabbit was given intramuscular injections of penicillin in the buttocks over a 3-d period with normal feeding. In addition to 5 mL of D-Hanks solution substituted for the MBMC suspension, manipulations of the rabbits in the transplantation control group were the same as those in the cell transplantation group.

Pathological management of the liver

At the end of the 1st, 2nd and 4th wk after transplantation, 7 rabbits were randomly selected for MR-DWI examination of the liver at each time point in the cell transplantation group and transplantation control group. Then they were sacrificed for histological examination. Rabbit liver tissue blocks were fixed in 4% paraformaldehyde and were processed for paraffin embedding. Microsections were prepared and stained with hematoxylin and eosin, then examined under an optical microscope. The *criteria* for the liver pathological scores were established according to the characteristic of this study, histology activity index and previous studies[9] (Table 1). Six pathological microsections from each model rabbit were randomly selected to count the pathological scores by 2 experienced pathologists using a double-blind method. The sum of the pathological scores from 10 random high power fields (400 ×) from each microsection was regarded as the pathological score of that microsection.

MR-DWI protocol

Rabbits in the normal control group were subjected only to liver MR-DWI examination. At the end of the 1st, 2nd and 4th wk after transplantation, 7 rabbits from the cell transplantation group and from the transplantation control group were randomly selected at each time point for liver MR-DWI examination.

Rabbits were anesthetized and immobilized, then MR-DWI (axial) was carried out with a 1.5-Tesla Signa Twinspeed MR scanner equipped with a small diameter cylindrical brain radiofrequency coil. The scanning parameters of the MR-DWI included a spin echo echoplanar imaging series, b value 0 and 400 s/mm^2, repetition time 6000 ms, echo time 45 ms, all diffusion directions, frequency coding direction R/L, field of view 20 cm × 15 cm, number of excitations 8, thickness layer 3 mm, 0.5 mm space, and matrix 128 × 128.

ADC values were obtained using Function Software on a GE workstation. Three different regions of interest (ROIs) (~50 mm^2 each) were chosen in the liver parenchyma in every clear axial slice of each liver and their ADC values were measured. The mean value of the above was considered to be the ADC value of each liver.

Statistical analysis

Based on the mean ADC values from ROIs and the pathological scores, the differences between the various groups and time points, and the correlation between the mean ADC value and pathological score were assessed. The statistical significance was calculated by an independent sample *t*-test, analysis of variance and linear correlation using SPSS 11.0 software. P values < 0.05 were considered to indicate statistical significance.

RESULTS

Mean ADC values from each group and analysis

The mean ADC value of the normal control group was $(1.76 \pm 0.03) \times 10^{-3}$ mm^2/s. At all time points after transplantation, the mean liver ADC values from the cell transplantation group were significantly higher than those of the transplantation control group ($P = 0.000$) (Table 2, Figure 1).

The mean liver ADC values from the cell transplantation group and transplantation control group gradually increased to near those of the normal control group over time. At the end of the 1st wk after transplantation,

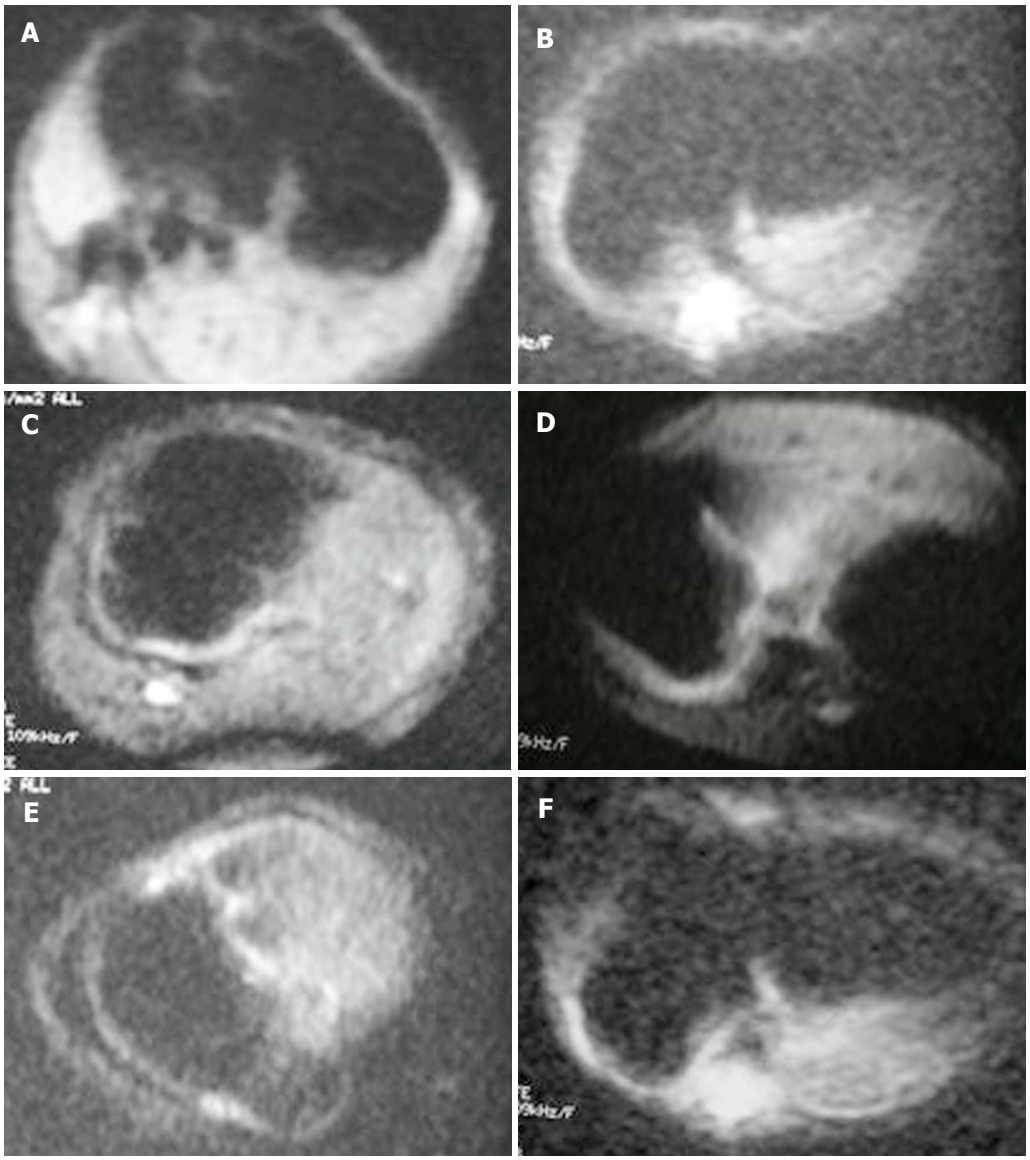

Figure 1 Diffusion-weighted imaging of hepatic injury at different time point. A: At the end of the 1st wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the transplantation control was (0.69 ± 0.05) × 10^{-3} mm^2/s; B: At the end of the 1st wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the cell transplantation group was (1.07 ± 0.07) × 10^{-3} mm^2/s; C: At the end of the 2nd wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the transplantation control group was (0.84 ± 0.06) × 10^{-3} mm^2/s; D: At the end of the 2nd wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the cell transplantation group was (1.41 ± 0.04) × 10^{-3} mm^2/s; E: At the end of the 4th wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the transplantation control group was (0.86 ± 0.04) × 10^{-3} mm^2/s; F: At the end of the 4th wk after transplantation, the mean apparent diffusion coefficient value of the acute hepatic injury liver in the cell transplantation group was (1.68 ± 0.04) × 10^{-3} mm^2/s.

Table 2 Mean apparent diffusion coefficient values from the cell transplantation group and transplantation control group at each time point (mean ± SD) × 10^{-3} mm^2/s

Group	1st wk	2nd wk	4th wk
Cell transplantation group	1.07 ± 0.07	1.41 ± 0.04	1.68 ± 0.04
Transplantation control	0.69 ± 0.05	0.84 ± 0.06	0.86 ± 0.04
t	11.452	21.735	37.876
P	0.000	0.000	0.000

they were both significantly lower than those of the normal control group (t = 23.612, P = 0.000; t = 52.416, P = 0.000).

Between any 2 time points, the differences in the mean liver ADC values from the cell transplantation group were statistically significant (P = 0.000). At the end of the 1st wk after transplantation, the mean ADC values from the transplantation control group were significantly lower than those at the end of the 2nd and 4th wk (P = 0.000). However, there was no significant difference between the end of the 2nd and 4th wk (P = 0.473).

Pathological scores and analysis

The livers' pathological scores from the cell transplantation group and transplantation control group gradually decreased over time (Table 3, Figure 2). At all time

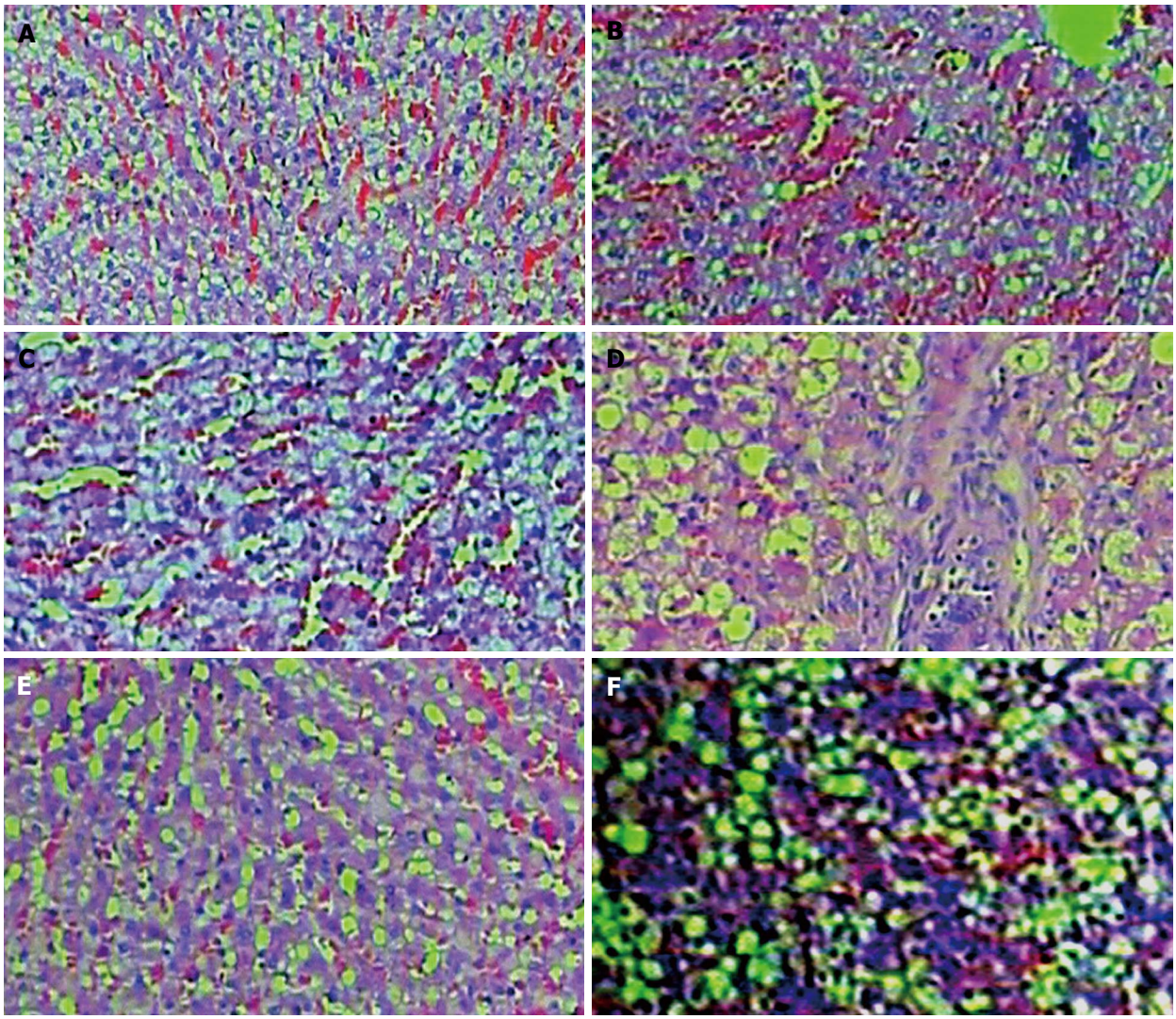

Figure 2 Pathological change of hepatic injury at different time point (Hematoxylin and eosin × 100). A: At the end of the 1st wk after transplantation, the pathological sections of the cell transplantation group showed extensive hepatic cell degenerations, few binucleate cells, abnormal hepatic cords and local inflammatory cell infiltration; B: At the end of the 1st wk after transplantation, the pathological sections of the transplantation control group showed obvious and extensive edema of hepatic cells, spotty hepatolysis, abnormal hepatic cords, local inflammatory cell infiltration and injured vascular endothelium; C: At the end of the 2nd wk after transplantation, the pathological sections of the cell transplantation group showed extensive edema of hepatic cells, abnormal hepatic cords, imperceptible inflammatory cell infiltration and few scattered binucleate cells. (Hematoxylin and eosin ×100); D: At the end of the 2nd wk after transplantation, the pathological sections of the transplantation control group showed obvious and extensive edema of hepatic cells, an increased number of binucleate cells and obvious inflammatory cell infiltration around the header; E: At the end of the 4th wk after transplantation, the pathological sections of the cell transplantation group showed scattering edema of the hepatic cells, hepatic cords, sinusoid near normal and hyperemia in local sinusoid; F: At the end of the 4th wk after transplantation, the pathological sections of the transplantation control group showed scattering and sheet edema of the hepatic cells, hyperemia in local sinusoids and an increased number of binucleate cells.

Table 3 Pathological scores from the cell transplantation group and transplantation control at each time point (mean ± SD)

Group	1st wk	2nd wk	4th wk
Cell transplantation group	27.14 ± 1.46	22.29 ± 2.29	19.00 ± 2.31
Transplantation control	69.29 ± 6.16	57.00 ± 1.53	51.86 ± 6.04
t	-17.619	-33.379	-13.444
P	0.000	0.000	0.000

points after transplantation, the pathological scores of the livers from the cell transplantation group were significantly lower than those from the transplantation control group ($P = 0.000$) (Table 3, Figure 2).

Between any 2 time points, the differences in pathological scores from the cell transplantation group were statistically significant ($P = 0.000$). At the end of the 1st wk after transplantation, the pathological scores from the transplantation control group were significantly higher than those at the end of the 2nd and 4th wk after transplantation ($P = 0.000$). However, there was no significant difference between the end of 2nd and 4th wk ($P = 0.073$).

Correlation analysis

When the *b* value was 400 s/mm^2, there was a significant negative correlation between the pathological score and

mean ADC value in the cell transplantation group and transplantation control group (r = -0.883, P = 0.000; r = -0.762, P = 0.000).

DISCUSSION

The efficacy of allogeneic MBMC transplantation therapy for AHI has already been proven[10,11]. Our results showed that the pathological changes such as cellular edema and inflammatory cell infiltration in the hepatic tissue of the transplantation control group were more obvious than those in the cell transplantation group at any similar time point. The pathological scores from the cell transplantation group were significantly lower than those of the transplantation control group at all time points. These results imply that allogeneic MBMC transplantation into an acute injury of the liver could improve hepatic injury reconditioning. This trend is similar to that found in other studies[10,11].

Because of the high resolution and sensitivity, recent studies have focused on *in vivo* real-time tracking and detecting the fate of transplanted stem cells with MRI[11,12]. However, there are few studies using MR-DWI to evaluate the therapeutic efficacy of MBMC transplantation for acute hepatic injuries.

When the b value is more than 300 s/mm^2, physiological factors such as perfusion have little influence on the mean ADC value of hepatic tissue[13-15]. Therefore, in our study we performed the MR-DWI examination of rabbit liver with 400 s/mm^2 for the b value, as determined by preliminary experiments.

Our study showed that at the end of the 1st wk after transplantation, the mean liver ADC values from the cell transplantation group and transplantation control group were much lower than the normal control group and both gradually increased to near the normal control over time. The correlation between the pathological scores and mean ADC values in the cell transplantation group or transplantation control group was significantly negative. This suggests the possibility of determining the reconditioning of an injured liver after allogeneic MBMC transplantation by tracking the longitudinally dynamic change of the mean ADC value from the AHI liver tissue.

By observing the pathological sections from the cell transplantation group and transplantation control group, we found the main pathological change was varying degrees of cytotoxic edema of the hepatocytes in all pathological sections. Therefore, we hypothesized that the pathological mechanism behind the change of the mean ADC value in hepatic tissue was mainly connected with cytotoxic edema of the hepatocytes in our study. Because the D-GalN injected into the peritoneal cavity was absorbed to injure the hepatic tissue causing Na-K pumps to be dysfunctional, the concentration of intracellular electrolytes increases. This causes water molecules inside cells to increase significantly, while extracellular water molecules decrease significantly. The cytotoxic edema of hepatocytes causes a decrease in gaps between hepatocytes, so that the space where extracellular water molecules can randomly move decreases. Therefore, the mean ADC value of hepatic tissue in the AHI model started to decrease[13-18] and was lower than that of the normal hepatic tissue.

In our study, the mean ADC values from the cell transplantation group and transplantation control group gradually increased over time and their pathological scores gradually decreased. The pathological sections showed their hepatic tissue injury gradually healed. At any similar time point, the mean ADC values from the cell transplantation group were significantly higher than those from the transplantation control group, and the pathological scores of the cell transplantation group were significantly better than those of the transplantation control group. According to previous studies[19-22] and our pathological sections, we hypothesized that there were 2 main hepatic reconditioning mechanisms causing these results. Firstly, the transplanted MBMCs promoted hepatic tissue reconditioning. Secondly, the hepatic injury caused the autologous hepatic reconditioning mechanism to activate. Because of the hepatic reconditioning, the hepatic cells' cytotoxic edema gradually decreased, and the number of extracellular water molecules and the space where extracellular water molecules could move randomly both gradually recovered. Thus, the macroscopic mean ADC values from the cell transplantation group and transplantation control group gradually increased[23,24]. Because of the 2 mentioned hepatic reconditioning mechanisms, the reconditioning in the cell transplantation group was faster than the transplantation control group without transplanted MBMCs. Therefore, at any time point, the mean ADC values from the cell transplantation group were significantly higher than those from the transplantation control group.

Of course, if there were extensive hepatolysis and necrosis, the number of extracellular water molecules and the space where extracellular water molecules randomly move in the AHI model liver would greatly increase. This would cause the mean ADC value of the hepatic tissue in the AHI model to exceed that of the normal hepatic tissue[25,26]. However, we did not observe this phenomenon in our study. Through observing pathological sections from all time points, we found sporadic scattering but no extensive hepatolysis and necrosis. The dominant pathological change in the hepatic tissue was varying degrees of hepatocellular cytotoxic edema. Perhaps the reason was that the dosage of D-GalN used was not high enough to cause extensive hepatolysis in our study. Considering the influence of hepatolysis on the ADC value of hepatic tissue, we chose many ROIs in all clear images from each AHI model's liver to measure ADC values. The mean ADC value was calculated from all ROIs and ADC values from each AHI liver. Thus, we could increase the accuracy of the ADC value of each AHI model's liver to the utmost extent.

In conclusion, when the b value is equal to 400 s/mm^2, tracking the longitudinally dynamic change of the mean ADC value of the AHI liver could determine injured he-

patic tissue reconditioning after allogeneic MBMC transplantation.

COMMENTS

Background

Many studies have proven that allogeneic mononuclear bone marrow cell (MBMC) transplantation therapy is an effective way to repair liver injury. The most notable merit of magnetic resonance diffusion-weighted imaging (MR-DWI) is the provision of an apparent diffusion coefficient (ADC) value that can distinguish the microscopic diffusion movement of water molecules in various tissues in vivo by assigning a numerical value. After MBMC transplantation therapy, there should be a dynamic change in the microscopic diffusion movement of water molecules in the hepatic tissue during the repair process of acute hepatic injury. Thus, the authors attempted to use MR-DWI to evaluate the contribution of the acute injured liver's mean ADC value in reflecting the repair process after MBMC transplantation therapy.

Research frontiers

Allogeneic MBMC transplantation therapy can accelerate the repair of liver injury. Many studies are focusing on the mechanisms behind the transplanted MBMCs repair of the liver injury. Recently, some researchers have been attempting to track and detect the fate of transplanted MBMCs with magnetic resonance imaging.

Innovations and breakthroughs

It has been proven that allogeneic MBMC transplantation can repair liver injury. There are many studies on evaluating the therapeutic effect of MBMC transplantation therapy for hepatic injury, but few studies have determined the feasibility of MR-DWI to evaluate the therapeutic effect of MBMC transplantation therapy on models of acute hepatic injury.

Applications

The results of this study suggest that the dynamic change of the mean ADC value of the acute hepatic injury model's liver can determine the injured liver's reconditioning after allogeneic MBMCs transplantation.

Terminology

MR-DWI is a functional imaging technique that can image the difference in microscopic diffusion movements of water molecules in various tissues. This technique only requires the examinee to lie still on the examining table while the examination is carried out. It is atraumatic and very safe.

Peer review

This article investigates the role of MR diffusion-weighted images for detection of acute liver injury following allogeneic bone marrow cell transplantation in a rabbit model. The conclusion reached confirmed the value and usefulness of this modality. The method is non-traumatic and easily performed. The methodology and design of the study was sound. This study is of significance in the field of bone marrow transplantation.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2829

World J Gastroenterol 2011 June 21; 17(23): 2829-2837
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

NOD2 and *ATG16L1* polymorphisms affect monocyte responses in Crohn's disease

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Supported by Broad Medical Research Program of The Broad Foundation, Inflammatory Bowel Disease Grant IBD-0236. RLR is the recipient of a Sir Charles Hercus Health Research Fellowship from the Health Research Council of New Zealand
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Received: July 1, 2010 Revised: September 30, 2010
Accepted: October 7, 2010
Published online: June 21, 2011

Abstract

AIM: To assess whether polymorphisms in *NOD2* and *ATG16L1* affect cytokine responses and mycobacterium avium subspecies paratuberculosis (MAP) survival in monocytes from Crohn's disease (CD) patients.

METHODS: Monocytes were isolated from peripheral blood of CD patients of known genotype for common single nucleotide polymorphisms of *NOD2* and *ATG16L1*. Monocytes were challenged with MAP and bacterial persistence assessed at subsequent time-points. Cytokine responses were assayed using a Milliplex multi-analyte profiling assay for 13 cytokines.

RESULTS: Monocytes heterozygous for a *NOD2* polymorphism (R702W, P268S, or 1007fs) were more permissive for growth of MAP (P = 0.045) than those without. There was no effect of *NOD2* genotype on subsequent cytokine expression. The T300A polymorphism of *ATG16L1* did not affect growth of MAP in our model (P = 0.175), but did increase expression of cytokines interleukin (IL)-10 (P = 0.047) and IL-6 (P = 0.019).

CONCLUSION: CD-associated polymorphisms affected the elimination of MAP from *ex vivo* monocytes (*NOD2*), or expression of certain cytokines (*ATG16L1*), implying independent but contributory roles in the pathogenesis of CD.

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Key words: Inflammatory bowel disease; Mycobacterium avium subspecies paratuberculosis; Cytokine; CARD15; Autophagy

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Glubb DM, Gearry RB, Barclay ML, Roberts RL, Pearson J, Keenan JI, McKenzie J, Bentley RW. *NOD2* and *ATG16L1* polymorphisms affect monocyte responses in Crohn's disease. *World J Gastroenterol* 2011; 17(23): 2829-2837 Available from: URL:

http://www.wjgnet.com/1007-9327/full/v17/i23/2829.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2829

INTRODUCTION

Crohn's disease (CD) has been proposed as being the product of chronic inflammation caused by a dysfunctional interaction between the intestinal immune system and commensal gut microbiota[1]. The inflammation seen in CD is characterized by pronounced Th1 and Th17/23 responses involving the cytokines interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, IFN-γ[1-3], IL-23 and IL-17[4-6].

Although the commensal intestinal microflora appear to play an important role in the etiology of CD, certain bacterial species have also been implicated as putative causal agents of CD. These include Pseudomonas maltophilia[7], Chlamydia trachomatis[8], Bacteroides fragilis[9] Yersinia species[10], adherent invasive *Escherichia coli* (*E. coli*)[11] and Mycobacterium avium subspecies paratuberculosis (MAP)[12]. The role of MAP in the etiology of CD is unclear. However, MAP causes chronic intestinal inflammation in ruminants (Johne's disease) with similar pathophysiology to CD[13] and it has been reported as a causative agent of regional enteritis with similarities to CD in a number of monogastrics, including two primate species[14,15]. MAP has also been cultured from both resected tissue[16] and peripheral blood of CD patients[17], and has been visualized inside macrophages from CD patients[18]. Meta-analyses of epidemiological studies confirm an association of MAP with CD[19,20], although it remains unknown whether MAP is pathogenic in humans, or whether this association reflects a defective host immune system permissive for the survival of MAP.

Twin and family studies have demonstrated a significant genetic component to the development and progression of CD[21-23]. Linkage analysis, candidate gene approaches and, most recently, genome-wide association studies (GWAS) have identified over 30 risk genes for CD[24-26], many of which are involved with bacterial recognition (e.g. *NOD2*) or processing and elimination of bacteria through the autophagy pathway (e.g. *IRGM*[27] and *ATG16L1*[25]).

In this study, we developed an *ex vivo* monocyte model to assess the impact of the CD-associated single nucleotide polymorphisms (SNPs) in *NOD2* (rs2066842, P268S; rs2066844, R702W; and 1007fs, rs2066847) and *ATG16L1* (rs2241880, T300A) on cytokine responses to the putative pathogen MAP. MAP can survive and replicate within phagocytic cells, and consequently we also evaluated the impact of these polymorphisms on the intracellular persistence of MAP. The use of *ex vivo* monocytes allows functional evaluation of SNPs associated with CD, and may provide a more realistic insight into the impact that genotype has on CD compared to studies which involve abrogation of protein expression or whole gene deletion.

MATERIALS AND METHODS

Patient recruitment

Patients for the current study were selected from a New Zealand population-based Caucasian inflammatory bowel disease (IBD) cohort recruited to investigate genetic and environmental factors that contribute to IBD etiology[28-32]. Inclusion criteria for the current study were a confirmed diagnosis of CD and negative MAP status as ascertained by IS900 PCR in peripheral blood[28].

The genotype combinations and patient phenotype information are detailed in Table 1. Briefly, the potential impact of *ATG16L1* and *NOD2* polymorphisms were assessed separately. For analysis of *ATG16L1*, monocytes were collected from CD patients who had a wild-type *NOD2* genotype and were homozygous for either the major (G) allele (n = 6) or minor (A) allele (n = 6) of *ATG16L1* 1138G > A (rs2241880). Conversely, for experiments evaluating the effect of *NOD2* genotype, monocytes were collected from patients who were *ATG16L1* 1138G homozygotes and were heterozygous for one of the three *NOD2* SNPs previously associated with CD; 2104C > T (R702W, rs2066844), 2722G > C (G908R, rs2066845), or 3020insC (1007fs, rs2066847), and were heterozygous for the background variant 802C > T (P268S, rs2066842). A total of 12 patients were recruited, 6 carried the polymorphisms described, and 6 were homozygous at these *NOD2* loci (Table 1). None of the patients included in this study had the SNPs rs13361189 and rs4958847. These SNPs are in complete linkage disequilibrium with a 20 kb insertion/deletion polymorphism which has been shown to alter expression of the autophagy gene *IRGM*[33].

Preparation of monocytes from peripheral blood

Blood (40 mL) was drawn into heparin tubes (Sigma-Aldrich), divided into 20 mL aliquots, and 15 mL of Phosphate Buffered Saline (PBS) was added to each. Ficoll-Paque™ PREMIUM (10 mL) (GE Healthcare Bio-Sciences Uppsala, Sweden) was layered under each aliquot and the samples centrifuged (1000 × *g*, 20 min). Mononuclear cells collected from the interface were added to 30 mL of PBS, centrifuged (350 × *g*, 10 min) and resuspended in 15 mL of PBS. In order to standardize the number of monocytes used in experiments, mononuclear cells were enumerated using a hemocytometer and a 200 μL aliquot was analyzed on a Beckman Coulter FC500 MPL flow cytometer to determine the percentage of monocytes based on forward and side scatter characteristics. After centrifugation (350 × *g*, 10 min), cells were resuspended in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) to a concentration of 4 × 10^5 monocytes/mL. Then 500 μL of this preparation were plated per well in a 24-well tissue culture plate (Nunc, Roskilde, Denmark). Monocytes were left to adhere for 1 h in a humidified incubator at 37℃ with 5% CO_2 and non-adherent cells were removed by washing three times with 1 mL of room temperature (RT) PBS.

Table 1 ***IRGM*, *ATG16L1*, and *NOD2* genotypes of Crohn's disease patients from whom monocytes were collected for *ex vivo* experiments**

	IRGM		*ATG16L1*	*NOD2*				Clinical characteristics			
Patient	rs13361189[1]	rs4958847[1]	T300A 1138G > A rs2241880	R702W 2104C > T rs2066844	P268S 802C > T rs2066842	G908R 2722G > C rs2066845	1007fs 3020insC rs2066847	Gender	Time since diagnosis (yr)	Disease location	Harvey bradshaw index[2]
1	TT	GG	AA	CC	CC	GG	00	M	53	Ileal	0
2	TT	GG	AA	CC	CC	GG	00	M	5	Ileo-colonic	0
3	TT	GG	AA	CC	CC	GG	00	F	5	Colonic	0
4	TT	GG	GG	CC	CC	GG	00	M	25	Ileo-colonic	2
5	TT	GG	AA	CC	CC	GG	00	F	6	Ileo-colonic	1
6	TT	GG	AA	CC	CC	GG	00	F	9	Ileal	3
7	TT	GG	AA	CC	CC	GG	00	M	1	Ileal	0
8	TT	GG	GG	CC	CC	GG	00	M	6	Colonic	2
9	TT	GG	GG	CC	CC	GG	00	F	10	Colonic	5
10	TT	GG	GG	CC	CC	GG	00	M	24	Ileo-colonic	1
11	TT	GG	GG	CC	CC	GG	00	F	11	Colonic	0
12	TT	GG	GG	CC	CC	GG	00	F	7	Ileo-colonic	4
13	TT	GG	GG	CT	CT	GG	00	F	5	Colonic	4
14	TT	GG	GG	CT	CT	GG	00	F	9	Ileo-colonic	0
15	TT	GG	GG	CT	CT	GG	00	M	17	Ileal	0
16	TT	GG	GG	CT	CT	GG	00	F	18	Colonic	4
17	TT	GG	GG	CC	CT	GG	0C	M	7	Ileal	1
18	TT	GG	GG	CT	CT	GG	00	F	16	Ileo-colonic	4

[1]SNPs located upstream (5') to *IRGM*; [2]Non-invasive clinical index used to assess disease activity in patients with Crohn's disease. A score of ⩾ 7 indicates active disease.

Adherent monocytes were incubated overnight in 500 μL of RPMI 1640/10% FBS.

Culture of MAP

MAP strain Dominic (ATCC 43545) was inoculated into 10 mL volumes of Difco™ Middlebrook 7H9 broth (BD Biosciences, Sparks, MD, USA) supplemented with 10% v/v BBL™ Middlebrook OADC Enrichment (BD Biosciences), 0.05% v/v Tween 80 (Sigma-Aldrich, St Louis, MO, USA) and 2 mg/mL Mycobactin J (Allied Monitor, Fayette, MO, USA) and grown at 37℃. A standard growth curve of MAP was obtained by measuring the optical density at 600 nm (OD_{600}) of an aliquot of bacterial suspension during the log phase, which had been passed repeatedly through a 25-gauge needle, and enumerating the bacteria by plating on the same medium supplemented with 1.5% agar (Invitrogen, Carlsbad, CA, USA).

Monocyte challenge experiments

Bacteria were grown to mid log phase (approximately 1×10^8 cells/mL, with reference to previously obtained growth curve data) and harvested by centrifugation (13000 × *g*, 5 min). Bacteria were resuspended in 500 μL of PBS and passed ten times through a 25-gauge needle to break up clumps of cells. One 500 μL aliquot of MAP was heat-inactivated at 90℃ for 5 min and then both aliquots were diluted to 4×10^6 cells/mL in RPMI 1640/10% FBS. The optimal temperature and incubation time for heat-inactivation had been previously confirmed by plating heat-treated MAP cells onto agar. Growth medium was removed from the monocytes and 500 μL of the MAP suspension was added to each well. Challenge experiments were performed in triplicate for each time-point/genotype combination. After incubation for 4 h at 37℃, 5% CO_2, the supernatant was removed and stored at -80℃ as a zero time-point sample for cytokine assays. Monolayers were washed three times with 1 mL of PBS to remove extracellular bacteria. A zero time-point lysate to assess bacterial uptake and persistence was removed by incubating 500 μL of a 0.1% sodium deoxycholate (Sigma-Aldrich, St Louis, MO, USA) solution with the monolayer for 5 min at RT. Lysates were centrifuged (13000 × *g*, 5 min) and resuspended in 200 μL of PBS. Serial dilutions of the lysates were made with PBS and 50 μL were spread on to a Middlebrook agar plate. The agar plates were left to incubate for four weeks at 37℃ before counting MAP colonies. Subsequent samples at time-points of 24, 48, 72 and 96 h were similarly processed.

Confirmation of genotypes of study participants

ATG16L1 genotypes were confirmed at recruitment by direct DNA sequencing of PCR products. Briefly, genomic DNA was extracted from fresh peripheral blood using GenElute™ (Sigma-Aldrich, St Louis, MO, USA) spin columns according to manufacturer's protocols. A 480 bp fragment containing the *ATG16L1* 1138G > A SNP was amplified for DNA sequencing from patient genomic DNA using the following primers: 5'-CCACAGGTTAGTGTGCAGGA-3' (forward primer) and 5'-CACAGCTGACAGAGCCAAAA-3' (reverse primer). PCR was carried out in a 20 μL volume containing 1 μL of genomic DNA, 0.3 μmol/L of each primer, 200 μmol/L dNTPs, 0.75 mmol/L $MgCl_2$, 1 × TAQ-Ti reaction buffer (Thermo Fisher Scientific, Pittsburgh, PA, USA) and 0.25 U of TAQ-Ti DNA polymerase (Thermo Fisher Scientific). After an initial denaturation step of 94℃ for 2 min, 35 cycles were performed at 94℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s. Five microlitres of each PCR product was checked on 1% agarose. Another 5 μL aliquot was

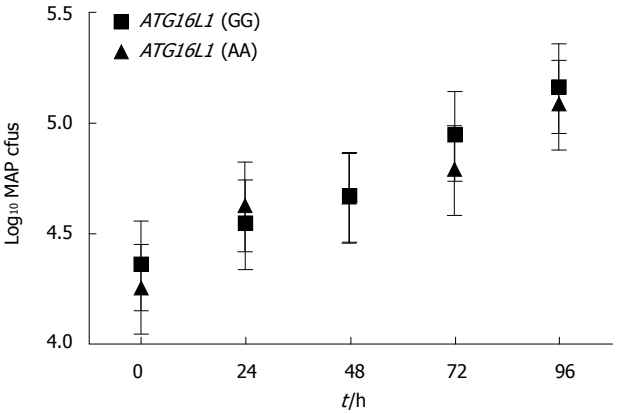

Figure 1 Effect of *ATG16L1* T300A variant on mycobacterium avium subspecies paratuberculosis numbers. Time course of mycobacterium avium subspecies paratuberculosis (MAP) growth in the context of the *ATG16L1* 1138 G > A variant. Monocytes were derived from subjects homozygous for either *ATG16L1* allele and carrying *NOD2* wild-type alleles (n = 6 for each group). MAP growth is expressed as colony forming units (cfu).

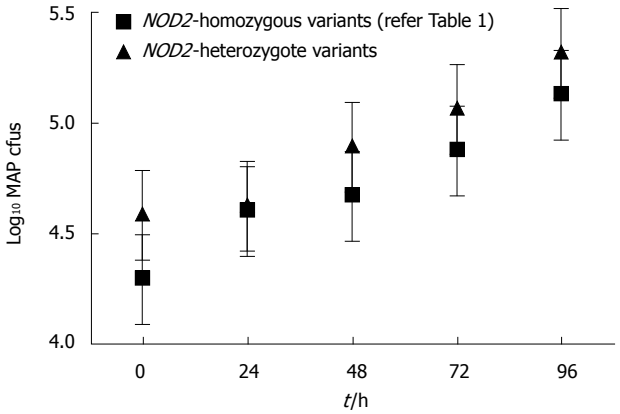

Figure 2 Effect of *NOD2* variants on mycobacterium avium subspecies paratuberculosis numbers. Time course of mycobacterium avium subspecies paratuberculosis (MAP) growth in the context of *NOD2* genetic variation. Monocytes were derived from subjects homozygous for the *ATG16L1* 1138G allele and with/without *NOD2* genetic variants (n = 6 for each group). MAP growth is expressed as colony forming units (cfu).

purified with Exo-SAP-IT (USB Corporation, Ohio, USA) and sequenced using BigDye chemistry (Applied Biosystems, California, USA) on an ABI 3730 Genetic Analyzer (Foster City, California, USA). The *NOD2* and *IRGM* genotypes of study participants were established as previously described using allele-specific PCR and pre-designed TaqMan SNP genotyping assays, respectively[34,35].

Multiplex cytokine analysis

Cytokine analysis was performed using a 13-plex MILLIPLEX™ MAP human cytokine kit according to manufacturer's recommendations (Millipore) for the following: IFNγ, IL-10, IL-12p40, IL-12p70, IL-17, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, TNFα, TNFβ.

Statistical analysis

Data from the five time-points were analyzed by repeated measures ANOVA, with fixed effects for time (as categorical variable) and random effects for subject i.e. genotype. For cytokine analyses, where readings were below the threshold for detection (3.2 pg/mL) they were included as 3.2 pg/mL. Results were considered significant at $P \leqslant 0.05$.

Ethical considerations

Informed written consent was obtained from all participants in this study and ethical approval for this work was granted by the Upper South B Regional Ethics Committee of New Zealand.

RESULTS

Bacterial persistence ATG16L1

Numbers of MAP increased from a mean $\log_{10}$ colony forming units (cfu) of 4.24 at 0 h to 5.08 at 96 h for the AA genotype, and from log10 cfu of 4.47 to 5.29 for the GG genotype (Figure 1). There was no evidence (T-testing) for the effect of the *ATG16L1* T300A polymorphism on MAP numbers at any individual time-point, or overall (pooled time-points ANOVA, $P = 0.175$) (Figure 1).

NOD2

MAP also grew in the monocytes with different *NOD2* genotypes, from a mean $\log_{10}$cfu of 4.30 at 0 h to 5.12 for the cells homozygous for the major alleles of the *NOD2* variants, and from $\log_{10}$cfu of 4.58 to 5.42 for cells heterozygous for any *NOD2* variant (Figure 2). There was no significant effect (*T*-testing) of *NOD2* genotype on MAP numbers at any individual time-point. However, analysis of all time-points indicated that monocytes heterozygous for a *NOD2* polymorphism were more permissive for growth of MAP (ANOVA, $P = 0.045$) (Figure 2).

Multiplex cytokine panels

Thirteen cytokines were evaluated from *ex vivo* monocyte supernatants using a MILLIPLEX™ MAP human cytokine kit. Each time-point/genotype combination was assayed in triplicate. All assays passed quality controls and r^2 values for the standard curves were ⩾ 0.99.

Of the thirteen cytokines analyzed in the multiplex format, only four, IL-10, IL-6, IL-8 and TNFα, had measurable responses above the detection threshold (> 3.2 pg/mL) on the multiplex ELISA platform used. Where samples had values of < 3.2 pg/mL, they were considered to be 3.2 pg/mL for statistical purposes.

Effect of ATG16L1 genotype

Cytokine expression results are shown in Figure 3. The AA genotype of *ATG16L1* was associated with greater expression of cytokines IL-10 and IL-6 in response to challenge with MAP ($P = 0.047$ and $P = 0.019$, respectively). No significant difference was seen between *ATG16L1* genotypes AA and GG for expression of either IL-8 or TNFα ($P = 0.758$ and $P = 0.289$, respectively) (Figure 3).

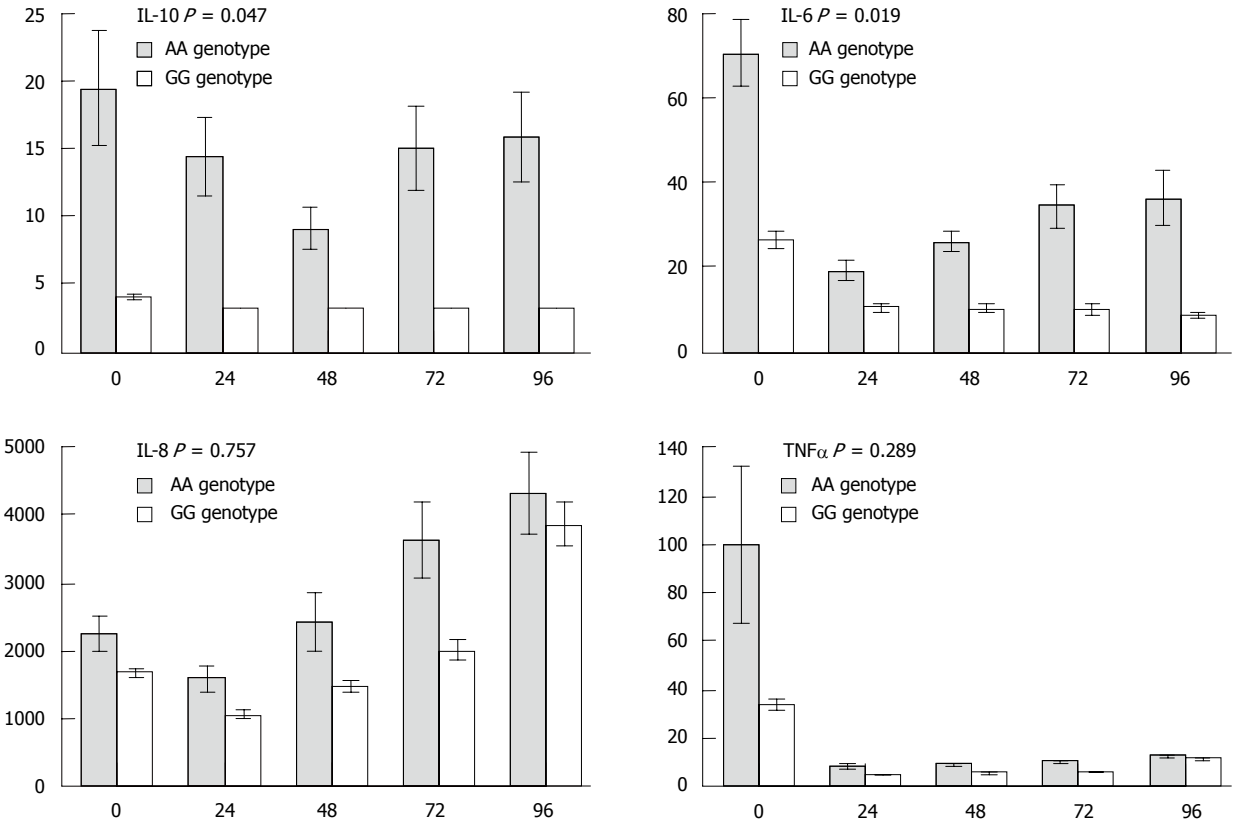

Figure 3 Effect of the *ATG16L1* T300A polymorphism on expression of cytokines following challenge with mycobacterium avium subspecies paratuberculosis. Cytokine concentrations are in pg/mL. Results are the means (and standard error of the mean) of triplicate samples from patients in Table 1. *P*-values are derived from repeated measures ANOVA testing for the effect of genotype on cytokine expression. Fixed effects for time (categorical variable) and *ATG16L1* genotype, random effect for subject. IL: Interleukin; TNF: Tumor necrosis factor.

Effect of NOD2 genotype

Cytokine expression changes for IL-10, IL-6, IL-8 and TNFα by cells of different *NOD2* genotypes are shown in Figure 4. In general, the presence of a *NOD2* polymorphism resulted in a trend of lower expression of these four cytokines throughout the time-course, compared to monocytes without *NOD2* variants.

However, this effect was not significant, with P-values of 0.56, 0.32, 0.41 and 0.97 for cytokines IL-10, IL-6, IL-8 and TNFα, respectively (Figure 4).

DISCUSSION

Numerous genetic association studies have indicated a strong role for *NOD2* and *ATG16L1* in the etiology of CD. However, functional studies have yet to fully elucidate whether, and to what extent, polymorphic variation in these genes affects bacterial clearance and inflammation in CD. Models of gene/environment interaction have mostly used epithelial or monocyte cell lines in vitro with bacterial pathogens such as Salmonella typhimurium that are not generally associated with CD. Also, the use of gene silencing or ablation techniques in these models may assist our understanding of the function of these genes, but may not be representative of the effects of SNPs which have been associated with CD. In this study we developed an *ex vivo* cellular model using monocytes from CD patients and a putative CD pathogen, MAP, in order to assess the effect of SNPs in *NOD2* and *ATG16L1* on bacterial survival and subsequent inflammatory response.

NOD2 is located in the cytoplasm, and plays an important role in cellular responses to bacterial infection[36] through recognition of muramyl dipeptide (MDP), a subunit of bacterial peptidoglycan (PGN)[37]. Furthermore, the R702W, G908R and 1007fs *NOD2* variants, which confer susceptibility to CD, have been shown to impair responses to the bacterial antigen lipopolysaccharide[38].

We found the effect of *NOD2* polymorphisms appeared to be primarily on bacterial persistence/growth, with heterozygosity at R702W or 1007fs making monocytes from CD patients significantly more permissive to growth of MAP. Indeed, a recent study has observed that these variants are associated with an impairment of monocyte phagocytosis and the development of bacteremia in intensive care unit patients[39]. Despite the increased bacterial load, no differences were seen in subsequent cytokine responses for the different host *NOD2* genotypes. Whilst there has been no prior published research with respect to MAP in this type of model of CD, the functional effects of *NOD2* SNPs have been examined for adherent invasive *E. coli* (AIEC). No differences were found in the persistence or growth of AIEC in monocytes from patients who were heterozygous or homozygous for the minor allele of *NOD2* variants R702W, G908R and 1007fs compared to

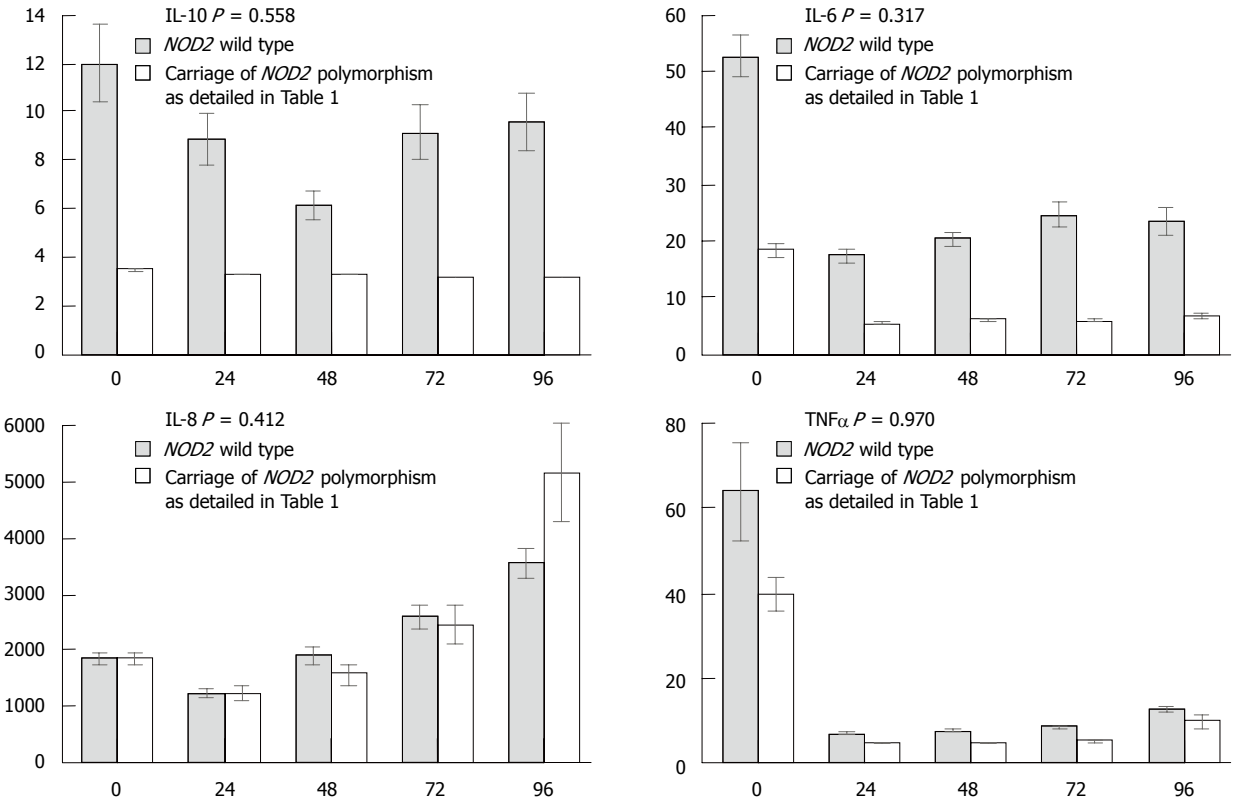

Figure 4 Effect of *NOD2* polymorphisms on expression of cytokines following challenge with mycobacterium avium subspecies paratuberculosis. Cytokine concentrations are in pg/mL. Results are the means (and standard error of the mean) of triplicate samples from patients in Table 1. *P*-values are derived from repeated measures ANOVA testing for the effect of genotype on cytokine expression. Fixed effects for time (categorical variable) and *NOD2* genotype, random effect for subject. IL: Interleukin; TNF: Tumor necrosis factor.

NOD2 major allele homozygotes[40]. It has also been shown that in peripheral blood mononuclear cells from CD patients, homozygosity for R702W does not affect the cytokine response to Gram-negative (*Helicobacter pylori*) bacterial peptidoglycan[41], although homozygosity for the 1007 fs mutation did. In our study we recruited no 1007fs homozygotes and only a single 1007 fs heterozygote so were unable to make any direct comparisons with this previous study. Comparative studies for a broader range of bacterial species in *ex vivo* cells from patients who are homozygous for these *NOD2* SNPs will help clarify the role of this genotype in disease pathogenesis.

Both MAP and AIEC are capable of intracellular survival and growth in monocytes and macrophages, and it appears that the presence of *NOD2* polymorphisms may influence their respective intracellular survival and growth in different ways[42].

Autophagy has been identified as a mechanism for clearing intracellular pathogens, and two autophagy genes, *ATG16L1* and *IRGM*, have been associated with CD[25,27]. In our study, we investigated the effect of the *ATG16L1* T300A polymorphism, and controlled for genotypic variation in *IRGM* (Table 1). *ATG16L1* T300A (rs2241880; 1138G > A) is a common non-synonymous SNP where the G major allele confers greater disease risk and results in a threonine-to-alanine substitution at amino acid position 300 of the *ATG16L1* protein (T300A). This SNP appears to account for all of the disease risk conferred by this locus[43], and functionally, this polymorphism has been proposed to contribute to defective macrophage killing of bacteria[44]. This assertion is supported by the results of two in vitro studies. Kuballa *et al*[45] found that the T300A variant impaired handling and autophagy of Salmonella within human epithelial cells, and Lapaquette *et al*[46] showed that siRNA knockdown of *ATG16L1* led to loss of autophagy of intracellular AIEC bacteria by HeLa cells. Transfection of affected HeLa cells with wild-type *ATG16L1* restored autophagic function, whereas transfection with the T300A polymorphic form did not. In contrast, *ATG16L1* T300A had no effect on the survival of either S. typhimurium or group A Streptococcus in mouse embryonic fibroblasts[47]. Although direct comparison of different model systems is complex and potentially misleading, our results with MAP in *ex vivo* monocytes generate the question as to whether the T300A polymorphism of *ATG16L1* affects autophagic clearance of certain intracellular bacteria as profoundly as indicated by knockdown or silencing models of gene function.

In our study, the T300A polymorphism was associated with significant changes in production of the cytokines IL-6 and IL-10 in response to bacterial challenge with MAP. These two cytokines are components of the Th1 (pro-inflammatory) and Th2 (modulatory) pathways of inflammation, respectively, and it is likely that CD results from an imbalance between these two pathways. It is tempting to speculate that the relative levels of the two

cytokines that were induced are indicative of an imbalance, but extrapolating from a very specific model to describe a complex disease state would be misleading and clearly further comparative work is required in this area in disease-relevant models.

Our study is the first to investigate the effect of *NOD2* and *ATG16L1* genotype on the response of *ex vivo* human monocytes to the putative CD pathogen MAP. Although our results are preliminary and need to be replicated in a larger sample, they provide novel insights into the effect of disease-associated SNPs in innate immunity genes on detection, handling, and elimination of bacteria, and ultimately CD pathogenesis. Our observations indicate that *NOD2* SNPs R702W, P268S, or 1007 fs impair the elimination of MAP yet do not impact on cytokine production. They may, therefore, increase susceptibility to prolonged intracellular bacterial infection. Conversely, the *ATG16L1* T300A polymorphism significantly alters the expression of certain Th1 and Th2 cytokines after MAP challenge, but does not seem to affect the autophagic clearance of this putative CD pathogen.

ACKNOWLEDGMENTS

We thank the people of Canterbury with IBD who generously gave of their time to take part in the study. We also thank Rhondda Brown and Judy Hoar for their assistance in coordinating the recruitment of patients to the Canterbury IBD cohort.

COMMENTS

Background

Polymorphisms of the genes *NOD2* and *ATG16L1* have been associated with susceptibility to Crohn's disease (CD). These genes are important for an effective innate immune response against potential bacterial pathogens [such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP)] which may trigger or exacerbate inflammation. Monocytes from CD patients of known genotype were used to determine whether polymorphisms in *NOD2* and *ATG16L1* alter cytokine responses and bacterial survival following challenge with MAP.

Research frontiers

Previous research has investigated the role of polymorphisms in *NOD2* and *ATG16L1* in various model systems. In general, these have used gene silencing strategies that may not realistically reflect the biological consequences of single nucleotide changes in these genes, or model bacterial pathogens that have little relevance to CD. None have reported the functional consequences of the naturally occurring single nucleotide polymorphisms using patient-derived cells and bacteria such as MAP that have been implicated in CD etiology.

Innovations and breakthroughs

The authors findings indicate that monocytes heterozygous for a *NOD2* polymorphism were more permissive for the intracellular growth of MAP than those without. However, these polymorphisms did not affect subsequent cytokine expression. The T300A polymorphism of *ATG16L1* did not affect growth of MAP in our monocyte model but did result in increased expression of certain cytokines - interleukin (IL)-10 and IL-6.

Applications

By understanding how naturally occurring disease-related polymorphisms of *NOD2* and *ATG16L1* influence bacterial survival and also the production of inflammatory mediators, the authors may gain insight into the contribution of these genetic changes to the function of the host innate immune system. Development of this model system that utilizes patient cells with known single nucleotide changes in key CD-susceptibility genes will provide another research tool to assist better understanding of disease pathogenesis related to bacterial handling.

Terminology

NOD2 (CARD15) - nucleotide oligomerization domain 2 - is a cytosolic pattern recognition receptor that recognizes muramyl dipeptide, a component of bacterial peptidoglycan. Polymorphisms in *NOD2* have been associated with altered susceptibility to CD in many genetic studies. *ATG16L1* - autophagy-related 16-like 1 - is a key component of the autophagic apparatus that is involved with uptake and digestion of intracellular bacteria. The T300A polymorphism of *ATG16L1* has also been associated with altered susceptibility to CD. MAP is an intracellular bacterium that has been cited in several studies as a putative causal agent of CD.

Peer review

This is a very well-designed and well-written study, with interesting and important scientific merit. Not just simple polymorphism descriptions, but their effect on human monocyte cytokine production and intracellular pathogen survival were examined with a very functional methodology. Their *ex vivo* model is much closer to the real pathogenesis of CD than any earlier one. Using the author's concept, more descriptive polymorphism analysis of CD and other diseases may be placed into functional analysis.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2838

World J Gastroenterol 2011 June 21; 17(23): 2838-2843
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Is the schatzki ring a unique esophageal entity?

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Received: October 22, 2010 Revised: November 25, 2010
Accepted: December 2, 2010
Published online: June 21, 2011

Abstract

AIM: To study, whether the association of Schatzki rings with other esophageal disorders support one of the theories about its etiology.

METHODS: From 1987 until 2007, all patients with newly diagnosed symptomatic Schatzki rings (SRs) were prospectively registered and followed. All of them underwent structured interviews with regards to clinical symptoms, as well as endoscopic and/or radiographic examinations. Endoscopic and radiographic studies determined the presence of an SR and additional morphological abnormalities.

RESULTS: One hundred and sixty-seven patients (125 male, 42 female) with a mean age of 57.1 ± 14.6 years were studied. All patients complained of intermittent dysphagia for solid food and 113 (79.6%) patients had a history of food impaction. Patients experienced symptoms for a mean of 4.7 ± 5.2 years before diagnosis. Only in 23.4% of the 64 patients who had endoscopic and/or radiological examinations before their first presentation to our clinic, was the SR previously diagnosed. At presentation, the mean ring diameter was 13.9 ± 4.97 mm. One hundred and sixty-two (97%) patients showed a sliding hiatal hernia. Erosive reflux esophagitis was found in 47 (28.1%) patients. Twenty-six (15.6%) of 167 patients showed single or multiple esophageal webs; five (3.0%) patients exhibited eosinophilic esophagitis; and four (2.4%) had esophageal diverticula. Four (7%) of 57 patients undergoing esophageal manometry had non-specific esophageal motility disorders.

CONCLUSION: Schatzki rings are frequently associated with additional esophageal disorders, which support the assumption of a multifactorial etiology. Despite typical symptoms, SRs might be overlooked.

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Key words: Schatzki ring; Dysphagia; Food impaction; Gastroesophageal reflux disease; Esophageal web

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Müller M, Gockel I, Hedwig P, Eckardt AJ, Kuhr K, König J, Eckardt VF. Is the schatzki ring a unique esophageal entity? *World J Gastroenterol* 2011; 17(23): 2838-2843 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2838.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2838

INTRODUCTION

Lower esophageal (Schatzki) rings are found in 6%-14% of routine barium radiographs[1-4]. Even though most Schatzki rings (SRs) are asymptomatic, they are considered to be the most common cause of episodic dysphagia for solids and food impaction in adults[5,6]. Since their first

description in 1944[7], the etiology and pathogenesis of the SRs has remained obscure and little is known about their association with other structural and functional abnormalities of the esophagus. Theories about their origin include congenital, anatomical, and inflammatory factors as the most likely events that lead to a circular constriction of the esophagogastric junction[8-12].

Therefore, the aims of this study were: (1) to investigate whether the lower esophageal (Schatzki) ring is associated with other esophageal disorders; (2) to determine whether dysphagia is due to the presence of SRs or additional esophageal disorders; and (3) to determine whether one of the pathogenic theories could be supported.

MATERIALS AND METHODS

From 1987 until 2007, all patients with newly diagnosed symptomatic SRs were prospectively registered and followed. The diagnosis of SRs was based on the results of radiographic and/or endoscopic studies. In 119 patients, radiographic and endoscopic studies showed an SR. Fourteen patients had only radiographic and 34 only endoscopic studies. All patients underwent structured interviews to assess clinical symptoms.

Evaluation of symptoms

At the initial investigation and at each follow-up visit, structured interviews were performed. Questions concentrated on clinical symptoms such as the occurrence of food impaction, and frequency of dysphagia, heartburn and regurgitation (less than once a week, weekly, daily, or several times a day).

Radiographic studies

Radiographic studies (n = 133) were performed by senior staff radiologists using the prone-oblique, full-column technique. To distend the lower esophagus maximally, patients were asked to take a deep breath and to perform a Valsalva maneuver during the course of swallowing. Diagnosis of SR (Figure 1A) was based on the presence of a fixed, symmetric, thin (< 4 mm) structure, which intersected the esophagus perpendicular to its long axis, at the squamocolumnar junction[6,13]. The diameter of the lower esophageal ring was measured directly from the radiographic picture in the area of the narrowing. An esophageal web is defined as a thin (< 2 mm) eccentric membrane that can occur anywhere in the esophagus[14]. A sliding hiatal hernia was diagnosed when a pouch was visible between the tubular esophagus and the diaphragmatic narrowing (length of the pouch ⩽ 3 cm = small hernia, > 3 cm = large hernia)[15]. Diagnosis of diverticulum was based on the presence of a pouch in the esophagus (Zenker's diverticulum: pouch in the pharyngoesophageal area; midesophageal diverticulum: pouch in the mid esophagus; epiphrenic diverticulum: pouch just proximal to diaphragm)[14].

Endoscopic procedures

Upper gastrointestinal endoscopy (n = 153) was performed by senior staff gastroenterologists using a variety of upper gastrointestinal endoscopes (Olympus, Hamburg, Germany), which varied in caliber from 8.5 to 9.5 mm. The endoscopic mucosal appearance determined the presence or absence of esophagitis as well as further morphological abnormalities of the upper gastrointestinal tract. SR (Figure 1B) was defined as a thin, symmetric, mucosal structure located at the esophagogastric junction[3]. A sliding hiatal hernia was diagnosed when gastric mucosa extended for > 1.5 cm above the diaphragm[16]. An esophageal web was defined as a thin (no more than 1.5 mm), eccentric membrane, located above the esophagogastric junction[14]. The degree of esophagitis was classified into four stages according to Savary and Miller[17]. If there were mucosal alterations in addition to reflux esophagitis, biopsies were taken from the esophagus. The presence of ⩾ 20 eosinophils per high-power field in the histopathological examination was used as the criterion to diagnose eosinophilic esophagitis[18].

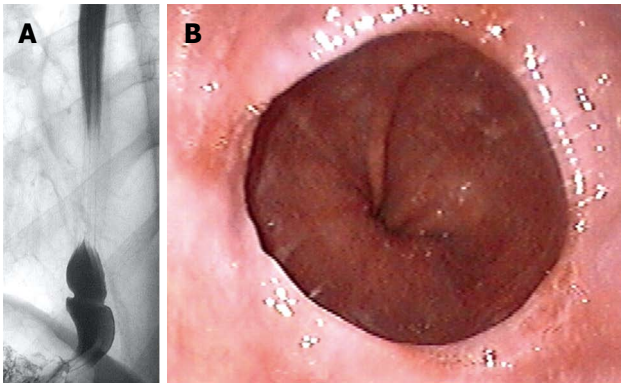

Figure 1 Radiographic (A) and endoscopic (B) image of the lower esophageal (Schatzki) ring.

Esophageal manometry

To exclude an esophageal motility disorder, 57 patients underwent esophageal manometry. Stationary esophageal manometry was performed with the use of a low-compliance capillary perfusion system (Mui Scientific, Mississauga, ON, Canada), using an eight-channel multilumen catheter with a 4.5-mm diameter. The four distal openings were 1 cm apart and the four proximal openings were 5 cm apart. Both sets were radially oriented. The manometric tracings were recorded by a computer polygraph system (Standard Instruments, Karlsruhe, Germany). Manometry was performed using a stationary pull-through method with the catheter introduced transnasally into the stomach. Manometry was carried out and interpreted according to the recommendations of the American Society of Gastroenterology[19]. Non-specific motor disturbance was defined as contractile abnormalities that are insufficient to establish a diagnosis of achalasia, diffuse esophageal spasm or typical scleroderma-like esophageal dysfunction[19,20].

Statistical analysis

Numerical variables are expressed as mean ± SD, counts

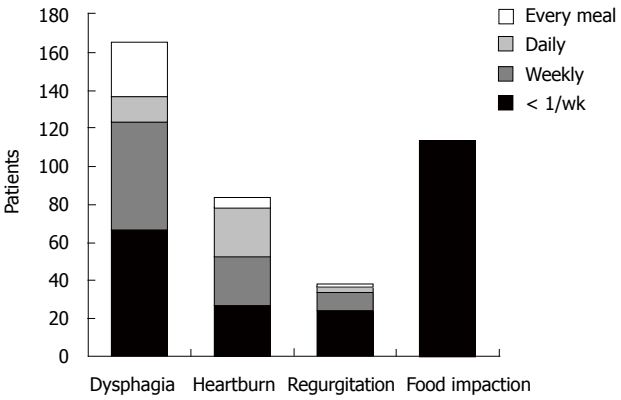

Figure 2 Clinical symptoms of 167 patients with Schatzki rings.

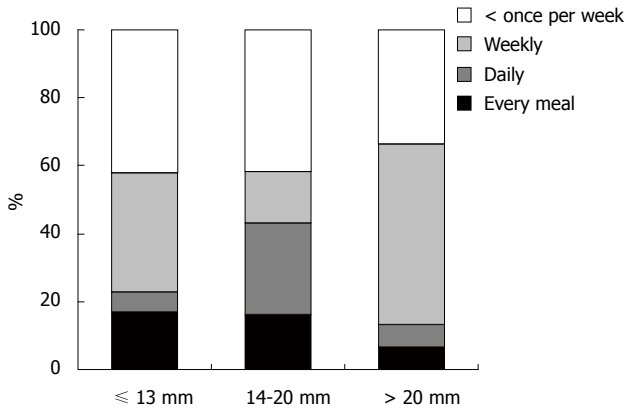

Figure 3 Frequency of dysphagia in relation to the ring size.

and ranges. Categorical variables are described using frequencies and percentages. Statistical significance of the differences between groups was assessed by Mann-Whitney *U* test for metric variables, by the Mantel-Haenszel χ^2 test for trends for ordered categorical variables and by Pearson χ^2 test for binary variables. A two-tailed *P* value < 0.05 was considered statistically significant. The analysis was performed using SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA).

RESULTS

Demographic data and esophageal imaging at initial investigation

One hundred and sixty-seven patients (124 male, 43 female) with a mean age of 57.1 ± 14.6 years were studied. Patients experienced symptoms for a mean of 4.7± 5.2 years before diagnosis. Sixty-four (38.3%) of the 167 patients had endoscopic and/or radiological examinations before their first presentation to our clinic, but only in 15 (23.4%) of these patients was SR was previously diagnosed. In 35 (87.5%) of the 40 patients who received an endoscopic examination, the SR had not been diagnosed, and in 14 (70%) of the 20 patients who underwent radiological examinations, the diagnosis could not be determined.

One hundred and twelve patients received radiological and endoscopic examinations at their initial presentation to our hospital. Endoscopy or a barium swallow accurately diagnosed the SR in all 112 patients in whom both methods were used. In the 34 patients who were only examined by endoscopy, the correct diagnosis was made in all cases, whereas the diagnosis was only made in 14 (66.7%) of the 21 patients who underwent radiological examinations first. In the seven patients without an initial radiological diagnosis, the SR was identified on subsequent endoscopy.

Clinical findings at initial investigation

Clinical symptoms at initial presentation are shown in Figure 2. All patients complained of intermittent dysphagia for solid food: 66 patients (39.5%) less than once per week, 58 (34.7%) weekly, 14 (8.4%) daily, and 29 (16.4%) with every meal. One hundred and thirteen (79.6%) of the 167 patients had a history of food impaction. Forty (23.9%) patients described regurgitation and 87 (52.1%) complained of occasional heartburn.

Table 1 Demographic data, ring size and clinical symptoms of all patients with Schatzki rings, Schatzki rings and additional erosive esophagitis, and with Schatzki rings and additional esophageal webs (mean ± SD)

Variables	SR (*n* = 167)	SR with erosive esophagitis (*n* = 47[1])	SR with esophageal webs (*n* = 26[1])
Age (yr)	57.1 ± 14.6	58.4 ± 13.7	54.4 ± 18.9
Sex (*n*)			
Male	124	33	18
Female	43	14	8
Ring size (mm)	13.9 ± 4.97	14.1 ± 5.3	12.2 ± 3.8
Dysphagia, *n* (%)			
Every meal	29 (17.4)	7 (14.9)	8 (30.8)
Daily	14 (8.4)	3 (6.4)	3 (11.5)
Weekly	58 (34.7)	17 (36.2)	6 (23.1)
< 1 x/wk	66 (39.5)	20 (42.6)	9 (34.6)
Food impaction, *n* (%)	113 (79.6)	34 (72.3)	18 (69.2)
Heartburn, *n* (%)	86 (57.1)	29 (61.7)	12 (46.1)
Regurgitation, *n* (%)	40 (23.9)	11 (23.4)	6 (23.1)

[1]In two patients, erosive esophagitis as well as esophageal webs could be diagnosed. SR: Schatzki ring.

The diameter of the lower esophageal ring was evaluated in 126 patients undergoing radiographic studies, and in 27 patients, endoscopic estimation was used with open biopsy forceps (7 mm) being the reference standard. In 14 patients, no measurement was performed. At initial presentation, the mean ring diameter was 13.9 ± 4.97 mm (range: 5-25 mm). Eighty-three (54.2%) of 153 patients had a ring size ⩽ 13 mm, and 15 (9.8%) had a ring size > 20 mm. There was no correlation between ring size and frequency of dysphagia (*P* = 0.29, Figure 3). Also, sex (*P* = 1.0) and age ⩽ 40 or > 40 years (*P* = 0.93) had no influence on the frequency of dysphagia. Demographic data, ring size and clinical findings of all patients with Schatzki rings are shown in Table 1.

Additional findings and their influence on symptoms

A sliding hiatal hernia was found in 162 (97%) of 167

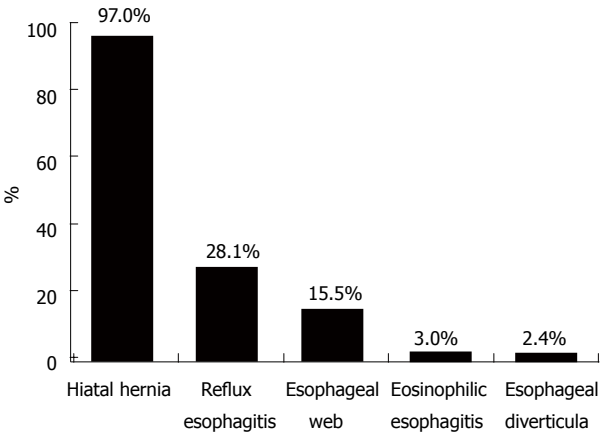

Figure 4 Additional structural abnormalities of the esophagus.

patients (n = 28 radiographic examinations, n = 29 endoscopic examinations, n = 105 radiographic and endoscopic examinations). One hundred and nineteen patients exhibited a small hernia and 43 had a large one. data, ring size and clinical findings of patients with Schatzki rings and additional erosive esophagitis, and /or esophageal webs are demonstrated in Table 1.

Further structural and functional abnormalities of the esophagus were diagnosed in 87 (52%) of 167 patients (Figure 4). The most frequent additional endoscopic finding was erosive reflux esophagitis, which was found in 47 (28.1%) patients; 40 (85.1%) of whom had stage Ⅰ esophagitis, whereas seven (14.9%) presented with stage Ⅱ and Ⅲ, and none with stage Ⅳ. All but one patient with reflux esophagitis showed a hiatal hernia. Twenty-six (15.6%) of 167 patients showed single (n = 15) or multiple ⩾ 2 (n = 11) esophageal webs, and four (2.4%) patients had esophageal diverticula. Two of the four patients with esophageal diverticula showed a Zenker's diverticulum, and in two patients, midesophageal diverticula were diagnosed. Four patients exhibited erosive reflux esophagitis in addition to esophageal webs. Five (16.6%) of the 30 patients in whom biopsies of the esophagus were taken exhibited histopathological signs of eosinophilic esophagitis. All but one patient complained of food impaction, whereas the frequency of dysphagia varied in this subgroup of patients (two patients, every meal; one patient, daily; and two patients, less than once per week).

Four (7%) of 57 patients undergoing esophageal manometry showed pathological results (two with non-specific motor disturbance, one with low contraction amplitudes, and one with diffuse esophageal spasm). Patients with an additional motility disorder of the esophagus showed a higher frequency of dysphagia than patients without (P = 0.03), although there was no difference in ring diameter (patients with motility disorders, 13.53 ± 3.6 mm; patients without, 13.7 ± 4.1 mm; P = 0.92).

The mean ring diameter in patients with additional esophageal webs (12.3 ± 3.8 mm) was smaller than in patients without webs (14.2 ± 5.1 mm) (P = 0.057). However, there were no differences in the frequency of dysphagia in patients with further structural abnormalities of the esophagus (sliding hernia, P = 0.1; erosive esophagitis, P = 0.48; and esophageal webs, P = 0.15) in comparison to the patients with an SR alone.

DISCUSSION

The lower esophageal SR is a common clinical finding and the most common cause of intermittent dysphagia, especially after consumption of solid food[6,21]. However, little is known about its etiology, its pathogenesis, or its association with other esophageal disorders. In addition, the clinical importance of associated disorders has not been described.

In our study, we were able to show that symptomatic SRs cannot always be diagnosed with a single imaging technique, and that the SR is not a unique entity. It is frequently associated with other esophageal disorders, such as hiatal hernias, reflux esophagitis and esophageal webs. In addition, ring size and most other structural abnormalities do not predict symptoms. In contrast, dysphagia was more common in patients with an additional motility disorder, and food impaction was the most common presentation in patients with eosinophilic esophagitis. Whether the frequent association with hiatal hernias and inflammatory esophagitis plays a pathogenic role remains unclear, but a multifactorial etiology is suspected.

In the present study, we confirmed previous observations that patients with symptomatic SRs complain about episodic dysphagia for solid food (mean duration 4.7 years prior to diagnosis), and more than two-thirds develop food impaction. Despite the typical clinical presentation, there was a significant diagnostic delay. Prior to presentation to our hospital, diagnosis of an SR was made in less than half of symptomatic patients; a surprisingly low number of patients. One of the difficulties in detecting lower esophageal rings might be related to the fact that the radiographic and endoscopic visualization depends on proper distension of the esophagogastric region beyond the caliber of the ring, which is often not accomplished[22]. This is especially true for wider rings with a luminal diameter > 13 mm[23]. In such instances, the radiographic examination has been shown to be superior to endoscopy in detecting lower esophageal rings[24]. In contrast, the present study could show a better diagnostic yield of endoscopy as compared with a barium swallow. This is most likely related to our special attention to membranous structures. Our findings suggest that a second imaging study should be performed in patients with typical clinical presentation if the first study fails to make the diagnosis of SR.

The current investigation showed that SRs are not a unique entity, but associated with additional esophageal disorders in 57% of symptomatic patients. Besides the nearly unanimous association with a hiatal hernia[2,24], we found a common association with reflux esophagitis and esophageal webs. In addition, esophageal diverticula were occasionally diagnosed. However the presence of additional structural abnormalities of the esophagus did not change the clinical presentation. Dysphagia was not more common in these patients. Even in patients with addition-

al esophageal webs, whose ring diameters were generally smaller, dysphagia was not more common. These findings suggest that the ring diameter alone may not be responsible for the observed symptoms.

In contrast, dysphagia was more frequently observed in patients with non-specific motility disorders, regardless of ring size, which suggests that the motility disorder may be responsible for the symptoms. Therefore, manometry should be considered in patients with wide ring diameters and symptoms of dysphagia. In addition, we found that intermittent bolus obstructions were a very frequent finding in those patients with an additional diagnosis of eosinophilic esophagitis, and clinicians should keep this clinical entity in mind. Therefore, the presence of an SR should not deter the endoscopist from taking esophageal biopsies in a patient in whom eosinophilic esophagitis remains a possibility. In fact, ring-like structures are common in eosinophilic esophagitis, and we became aware of this entity only a decade ago. Although an association of eosinophilic esophagitis and SRs has been previously suggested, it is not known if this is caused by shared clinical and endoscopic findings, or rather a shared pathogenesis[25,26]. Common clinical features might also explain the frequent coexistence of esophageal webs and eosinophilic esophagitis in our study. However, it is not clear if the additional esophageal disorders occur by chance, or if there is a common pathogenesis. The etiology of SRs remains obscure, and several theories about their etiology and pathogenesis exist. One of these is the developmental theory that holds that the presence of a congenital mucosal ridge at the esophagogastric junction is a rather frequent anatomical phenomenon that could fold in a valve-like fashion to create the ring[3,8]. Arguing against this, is the fact that the majority of symptomatic individuals were over the age of 40 years in the present and previous studies[27]. In the so called plication theory, Stiennon has postulated that longitudinal shortening in the presence of a hiatal hernia may lead to folding of redundant esophageal mucosa[11]. Consistent with this theory is the fact that most of the patients in the present study had a sliding hiatal hernia. However, it still remains unclear why some patients with a hernia develop an SR and others do not. Therefore, the plication theory is unlikely to be the only cause for the development of SRs. Currently, the inflammation theory with gastroesophageal reflux as the main cause of inflammation, is the most popular theory[28], and consequently, some authors have recommended an antireflux regimen to prevent symptomatic recurrence[29,30]. Others have pointed out that, if less than two-thirds of patients are found to have pathological gastroesophageal reflux[31,32], it might not be the main pathogenic factor. Similar to the latter findings, even if reflux esophagitis were one of the most frequent associated esophageal disorders in the present study, less than one-third of all investigated patients showed endoscopic signs of erosive esophagitis, and only half complained of occasional heartburn, which suggests that gastroesophageal reflux is not the only cause for the development and narrowing of the SR. Therefore, we assume that the etiology of SR is multifactorial.

In conclusion, SRs might be overlooked in endoscopic and/or radiological examinations. Therefore, in patients with a typical clinical presentation, a second diagnostic imaging should be considered. Other esophageal disorders are frequently observed and should be kept in mind; most of which do not alter clinical presentation. Non-specific motility disorders and eosinophilic esophagitis should be considered, especially when frequent dysphagia or food impaction is present. With regard to the etiology of SRs, the present findings suggest a multifactorial genesis, which supports the inflammation theory as well as the plication theory.

COMMENTS

Background

The Schatzki ring (SR) is the most common cause of episodic dysphagia to solid food. Nevertheless its etiology and pathogenesis remains unknown and little is known about its association with other structural and functional abnormalities of the esophagus. Theories regarding its origin include inflammatory, developmental, and congenital factors as the most likely events leading to a circular constriction of the esophagogastric junction. In addition, the clinical importance of associated disorders has not been described.

Research frontiers

Currently, the 'inflammation theory' with gastroesophageal reflux disease (GERD) as the main cause of inflammation, is the most popular theory. However, prospective studies have documented an association with GERD in less than two thirds of patients, suggesting that additional pathogenic factors might be responsible for the development of the SR.

Innovations and breakthroughs

It is known that SRs are associated with hiatal hernias and reflux esophagitis, whereas this is the first study that could show the frequent association of SRs with additional esophageal disorders, most of which do not alter clinical presentation. Nonspecific motility disorders and eosinophilic esophagitis should be considered, especially when frequent dysphagia or food impactions are present, respectively. In regards to the etiology of SRs the present findings suggest a multifactorial pathogenesis. Furthermore, despite the typical clinical presentation SRs might be overlooked in endoscopic and /or radiological examinations.

Applications

SRs are frequently associated with other esophageal disorders. These should be sought, especially when frequent dysphagia or food impaction is the presenting symptom. SRs might be overlooked on radiographic or endoscopic examinations, therefore, a second diagnostic modality should be used when suspicion remains high.

Terminology

"Stiennon's plication theory", postulated that longitudinal shortening in the presence of an hiatal hernia may lead to folding of a redundant esophageal mucosa, creating the SR.

Peer review

The manuscript reads very well and flows nicely. The conclusions are supported by the data and the limitations are well addressed. In addition, the study is novel and the topic chosen is original.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2844

World J Gastroenterol 2011 June 21; 17(23): 2844-2847
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Factors influencing lower esophageal sphincter relaxation after deglutition

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Received: December 1, 2010 Revised: March 1, 2011
Accepted: March 8, 2011
Published online: June 21, 2011

Abstract

AIM: To study the relationship between upper esophageal sphincter (UES) relaxation, peristaltic pressure and lower esophageal sphincter (LES) relaxation following deglutition in non-dysphagic subjects.

METHODS: Ten non-dysphagic adult subjects had a high-resolution manometry probe passed transnasally and positioned to cover the UES, the esophageal body and the LES. Ten water swallows in each subject were analyzed for time lag between UES relaxation and LES relaxation, LES pressure at time of UES relaxation, duration of LES relaxation, the distance between the transition level (TL) and the LES, time in seconds that the peristaltic wave was before (negative value) or after the TL when the LES became relaxed, and the maximal peristaltic pressure in the body of the esophagus.

RESULTS: Relaxation of the LES occurred on average 3.5 s after the bolus had passed the UES and in most cases when the peristaltic wave front had reached the TL. The LES remained relaxed until the peristaltic wave faded away above the LES.

CONCLUSION: LES relaxation seemed to be caused by the peristaltic wave pushing the bolus from behind against the LES gate.

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Key words: Deglutition; Lower esophageal sphincter; Peristalsis; Relaxation; Upper esophageal sphincter

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Tibbling L, Gezelius P, Franzén T. Factors influencing lower esophageal sphincter relaxation after deglutition. *World J Gastroenterol* 2011; 17(23): 2844-2847 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2844.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2844

INTRODUCTION

Swallowing dysfunction with esophageal food retention is a common problem in an adult population with hiatus hernia. Most articles reporting lower esophageal sphincter (LES) studies depict LES relaxation caused by neurogenic mechanisms[1] in gastroesophageal reflux (GER). The ability of the LES to allow esophagogastric transit after deglutition has received little attention. Efforts have been made to prove that LES relaxation is triggered by a neurogenic reflex following deglutition[2], that the LES and the upper esophageal sphincter (UES) relaxations are simultaneous events and that the cervical portion of the vagus nerve mediates inhibitory and excitatory changes in LES pressure[3]. In a recent study, a biodynamic approach was proposed for LES opening when GER takes place in hiatal hernia and the gastric wall tension pulls open the LES[4]. As long ago as 1978[5], LES was suggested to be a biodynamic gate which is forced to open when a bolus is propelled by esophageal peristalsis. We decided to further

challenge the idea of LES as an esophagogastric pressure zone dependent on complex neuro-humoral factors.

The recent development of high-resolution solid-state manometry (HRM) systems with closely spaced circumferential pressure sensors has made it possible to display simultaneous recordings along the entire esophagus in color-coded pressure plots (Figure 1) and has dramatically improved the diagnostic assessment of esophageal function and disease. With conventional esophageal manometry, simultaneous events in the UES and LES have been difficult to display especially since deglutition often will displace the pressure sensors due to shortening of the esophagus. The HRM technique enables, therefore, a unique possibility to study UES and LES pressure during the entire deglutition period and independent of any sphincter dislocation. In order to find out if there is an interplay between pulling forces and LES opening, the aim was therefore to study the relationship between UES relaxation, peristaltic pressure and LES relaxation following deglutition using the HRM technique.

MATERIALS AND METHODS

The HRM system (ManoScan 360 A-100, ManoView analysis software ver. 2.0.1 from Sierra Scientific Instruments Inc., Los Angeles, CA) uses a solid state catheter (Ø 4.2 mm) with closely spaced circumferential pressure sensors with 1 cm intervals over 36 cm. The HRM catheter was passed transnasally and positioned to be recording simultaneously from the hypopharynx, through the body of the esophagus, to the stomach. The catheter was calibrated outside the patient before and immediately after the investigation using the thermal compensation option in the software.

We performed a prospective HRM study in 10 adult patients (median age 45 years, range 38-63 years; 7 women, 3 men) who were admitted to an esophageal laboratory for suspected dyspepsia. It can be claimed that our patients with dyspepsia are not representative for studies of normal LES and UES function. Dyspepsia is a diagnosis without organic lesion and with diffuse symptoms predominantly in the gastric region. The patients did not have any symptoms of dysphagia and reflux, they were free of medication, and the presence of hiatus hernia was excluded at gastroscopy and HRM. It is therefore regarded that confounding factors have been excluded in the study material.

The investigation comprised ten swallows of a 10 mL water bolus in a supine position. The characteristics analyzed were: (1) the time lag between UES relaxation and LES relaxation; (2) the LES pressure at time of UES relaxation; (3) the duration of LES relaxation; (4) the length of the esophagus between UES and LES; (5) the distance between the transition level (TL) and the LES; (6) time in seconds that the peristaltic front wave was before (negative value) the TL or after the TL when the LES pressure dropped to nadir; and (7) the maximal peristaltic pressure in the esophagus. The eight best readable swallow recordings in each subject (a total of 78 swallows) were calculated upon, and the mean values of each individual item are given.

TL is defined as an esophageal zone with striated-to-smooth muscle fiber transition[6] showing as a short loss of peristaltic pressure (Figure 1).

RESULTS

Individual values and the mean of all values are presented in Table 1. The LES relaxed on average 3.5 s after the UES had opened. The mean LES pressure was 26 mmHg at time of UES opening. The mean duration of LES remaining relaxed was 6.7 s. The mean length of the esophagus between UES and LES was 26 cm. The mean distance between the TL and the LES was 18 cm, corresponding to on average 72% of the total length. The mean time that the peristaltic wave was before (negative value) the TL or after the TL when the LES opened was -0.5 s. The mean maximum pressure of the peristaltic wave was 138 mmHg. The time lag between UES and LES relaxation was never over 6.4 s. The individual LES pressure, as well as the maximum peristaltic pressure, varied remarkably from swallow to swallow (Table 1).

DISCUSSION

This study shows quite clearly that the LES becomes relaxed several seconds (on average 3.5 s) after the water bolus has passed the UES. This corresponds with findings by Nguyen *et al*[7] when impedance and manometry techniques were used. They found a mean latency between bolus entry into the esophagus and LES relaxation of 3.6 s. This is also in agreement with the esophageal transit time (3.8 s) as assessed with the biomagnetic technique[8]. The LES pressure did not change in our study at time of UES opening. Therefore, it seems unlikely that LES relaxation is triggered by the start of deglutition.

It seems as if opening of the LES coincides with the peristaltic wave front reaching close to the TL, which is an esophageal level with striated-to-smooth muscle fiber transition. Reasonably, this depends on the premise that the distal end of the water bolus has reached the LES, that the water bolus fills up the esophageal lumen between TL and LES, and that the peristaltic wave pushes the water bolus from behind against the LES gate. Only the combination of manometry and impedance measurement or the combination of HRM and radiography can find out whether LES relaxation and opening take place at the moment when the bolus arrives at the LES. In an impedance study of LES opening and bolus transit, it was found that LES relaxation seemed to be modulated by the bolus transit and occurred predominantly upon arrival of the bolus in the distal esophagus[7]. This discovery clashes with the findings by Pandolfino *et al*[9] who used a combined impedance/manometry technique. They showed that LES relaxation did not necessary coincide with bolus passage or LES opening. The hydrostatic pressure in the esophageal body necessary to open the LES in an upright position, as shown in a combined manometric and radiographic study from 1978[5], was approximately the same as the LES pressure before opening. If no deglutition activ-

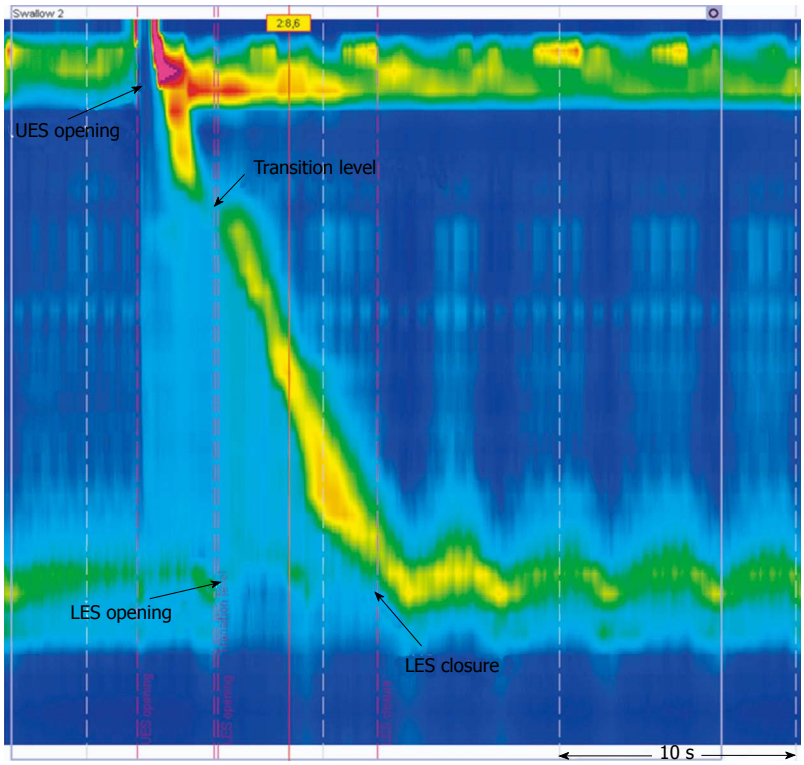

Figure 1 An high-resolution solid-state manometry recorded swallowing event. Transition level = TL, LES relaxation = LES opening to LES closure. Red ⩾ 60 mmHg. Blue ⩽ 0 mmHg. LES fluctuations represent respiration, upwards expiration, downwards inspiration. UES: Upper esophageal sphincter; LES: Lower esophageal sphincter.

Table 1 Different esophageal events in ten subjects

Event	F1	F2	M3	F4	F5	F6	M7	M8	F9	F10	All 10 subjects; mean: ranges
(A) s	2.6	5.2	5.0	3.6	3.8	3.2	3.6	1.7	3.0	3.5	3.5: 1.7-5.2
(B) mmHg	21	19	20	32	13	24	23	52	29	29	26: 13-52
(C) s	5.1	4.2	4.2	6.7	5.5	7.7	10.3	8.5	7.7	7.5	6.7: 4.2-10.3
(D) cm	25	26	28	25	29	23	26	26	22	26	26: 22-29
(E) cm	16	17	20	18	20	17	19	20	16	18	18: 16-20
(F) s	-1.4	0.7	0.9	0.3	0.5	-0.2	-1.4	-2.0	-1.6	-0.3	-0.5: -2.0-0.9
(G) mmHg	109	121	123	71	102	232	187	169	152	116	138: 71-232

Different esophageal events, mean values of ten recordings in ten subjects, and mean values of all ten subjects. A: Time lag between relaxing of UES and LES; B: LES pressure when UES opened; C: Duration of LES remaining relaxed; D: Esophagus length; E: Distance between TL and LES; F: Time in seconds that the peristaltic wave was before (minus value) or after the TL when the LES relaxed; G: maximum pressure of the peristaltic wave. F: Female; M: Male. UES: Upper esophageal sphincter; LES: Lower esophageal sphincter; TL: Transition level defined as the zone when the striated muscle layer transitions into the smooth muscle layer.

ity took place and a contrast medium was instilled into the esophagus, the LES opened when the hydrostatic pressure exceeded the resting LES pressure and closed again when the hydrostatic pressure in an upright position fell short of the LES pressure. Deglutition caused the LES to relax and open when the peristaltic wave reached the upper level of the infused contrast medium. These different findings indicate that the LES is a barrier which is forced to open by the peristaltic pressure. In the clinic, this would explain why, for instance, patients with achalasia cardia or with lack of esophageal peristalsis do not have any LES relaxation after deglutition.

If we look upon the LES as a gate that will be pulled open, it is of interest to compare the pressure of the LES and the maximum pressure of the peristaltic wave in the distal esophagus. In this study, the peristaltic pressure was five times stronger than the LES pressure. HRM and conventional manometry are claimed to be the same in their measurement of LES resting pressure[10]. It has been shown that the longitudinal esophageal muscle is contracted during the peristaltic activity[11] which will decrease esophageal wall compliance. Certainly, the peristaltic force displays an interaction between the longitudinal and circular esophageal muscles[12], and a compliance decrease of the esophageal wall will facilitate bolus transit. The reason for the deglutition-induced pressure overload in the esophageal body is therefore of importance, in order to overcome the stiffness of the esophageal wall. In the study by Babaei *et al*[12], it was

even proposed that the longitudinal esophageal smooth muscle has an important role in the relaxation of LES. In our study, the LES remained relaxed as long as the peristaltic wave was present in the esophagus. This indicates that there is a close interplay between LES function and peristaltic activity. It is reasonable to believe that the LES will be pulled open when the pulling direction is either from the esophagus or from the stomach[12].

In conclusion, LES opening seems to be caused by the peristaltic wave pushing the bolus from behind against the LES gate. Therapeutic attention in patients with dysphagia of non-stricture origin should therefore be focused on esophageal motility function rather than on drugs affecting LES pressure.

COMMENTS

Background

Swallowing problems with food retention in the gullet are present in at least 8% of an adult population. For accurate treatment, it is of importance to know whether transit from the esophagus to the stomach can be treated with drugs aimed at opening the lower esophageal sphincter (LES) or whether transit is due to dynamic properties of gullet muscles. So far, it has been claimed that LES is triggered to open by a neurogenic reflex from the upper esophageal sphincter (UES).

Research frontiers

The newly developed high-resolution manometry (HRM) system is a technical innovation and breakthrough for the understanding of dynamic esophageal events, meaning the interplay between esophageal motility and esophageal sphincter relaxations.

Innovations and breakthroughs

The esophageal HRM probe was used in ten non-dysphagic patients with dyspepsia in order to study the time relationship between UES and LES relaxation and to study where the peristaltic wave front was located when the LES relaxed after deglutition of a 10 mL water bolus in the supine position. These simultaneous activities have previously not been possible to study with conventional esophageal manometry.

Applications

The LES was shown to relax 3.5 s later than the UES after deglutition which is the average time it takes a water bolus front to reach the LES. The LES remained relaxed on average 6.8 s; that is until the propulsive force had faded away. The peristaltic front wave reached a level close to the transition level (TL), either 2 s before or 1 s after the TL.

Terminology

Esophagus-gullet. Dyspepsia-a diagnosis without any specified organic lesion and with diffuse symptoms from the gastric region. Dysphagia-swallowing difficulties. HRM-a pressure catheter with a 36 cm long segment of sensors spaced 1 cm apart giving simultaneous pressure information of sphincter and muscular activity from a total of 432 locations from UES to LES. The pressure can be displayed as a color plot offering visual information of the esophagus and its sphincters very similar to an anatomic picture. LES-lower esophageal sphincter. Manometry-pressure measurement. Smooth muscle-a muscle under autonomic, non-volitional control. Striated muscle-a muscle under volitional control. TL, transition level-the level in the esophagus between the upper striated muscle level and the lower smooth muscle level which is located about 7 cm distal of the UES.

Peer review

More emphasis on why HRM is a great technique and the knowledge it provides compared to conventional esophageal manometry has now been given in the Methods section. The reason why we believe that dyspepsia patients can be regarded as normal people with regard to esophageal transit studies is addressed in the Materials section. The previous conclusion, regarding older theories of a neurogenic reflex causing LES relaxation, has been omitted and basic data for this are given in the discussion.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2848

World J Gastroenterol 2011 June 21; 17(23): 2848-2854
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Effects of sargentgloryvine stem extracts on HepG-2 cells *in vitro* and *in vivo*

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Supported by National Science and Technology Key Project for the Development of New Drugs in China, No. 2009ZX09103-422
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Received: January 24, 2011 Revised: February 24, 2011
Accepted: March 3, 2011
Published online: June 21, 2011

Abstract

AIM: To observe the effects of sargentgloryvine stem extracts (SSE) on the hepatoma cell line HepG-2 *in vitro* and *in vivo* and determine its mechanisms of action.

METHODS: Cultured HepG-2 cells treated with SSE were analysed by 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium bromide and clone formation assay. The cell cycle and apoptosis analysis were conducted by flow cytometric, TdT-Mediated dUTP Nick End Labeling and acridine orange/ethidium bromide staining methods, and protein expression was examined by both reverse transcriptase-polymerase chain reaction and Western blotting. The pathological changes of the tumor cells were observed by haematoxylin and eosin staining. Tumor growth inhibition and side effects were determined in a xenograft mouse model.

RESULTS: SSE treatment could not only inhibit HepG-2 cell proliferation in a dose- and time-dependent manner but also induce apoptosis and cell cycle arrest at the S phase. The number of colonies formed by SSE-treated tumor cells was fewer than that of the controls ($P < 0.05$). SSE induced caspase-dependent apoptosis accompanied by a significant decrease in Bcl-xl and Mcl-1 and elevation of Bak expression ($P < 0.05$). Tumor necrosis factor α in the xenograft tumor tissue and the liver functions of SSE-treated mice showed no significant changes at week 8 compared with the control group ($P > 0.05$). Systemic administration of SSE could inhibit the HepG-2 xenograft tumor growth with no obvious toxic side effects on normal tissues.

CONCLUSION: SSE can induce apoptosis of HepG-2 cells in vitro and in vivo through decreasing expression of Bcl-xl and Mcl-1 and increasing expression of Bax.

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Key words: Sargentgloryvine stem extract; Apoptosis; Human hepatocellular carcinoma; HepG-2; *Bcl-2* family

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Wang MH, Long M, Zhu BY, Yang SH, Ren JH, Zhang HZ. Effects of sargentgloryvine stem extracts on HepG-2 cells *in vitro* and *in vivo*. *World J Gastroenterol* 2011; 17(23): 2848-2854 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2848.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2848

INTRODUCTION

Although significant progress has been made over the past decades in cancer prevention and treatment, the development of effective treatment regimens remains one of the greatest challenges in the area of cancer che-

motherapy. Recently, plant-derived natural products are becoming important as anti-cancer derivatives, including vincristine, vinblastine, paclitaxel and camptothecin, which are invaluable contributions of nature to modern medicine[1-4]. However, the quest to find novel therapeutic compounds for cancer treatment is a never-ending venture, and diverse plant species are being studied to identify prospective anti-cancer agents[5,6]. Sargentgloryvine stem of *Sargentodoxa cunneata* (Oliv.) has been widely used as an ingredient in Chinese medicine according to the Chinese herbal medicine principles for thousands of years in the treatment of several kinds of diseases, such as chronic pelvic cavity inflammation, rheumatism and appendicitis. Sargentgloryvine stem extract (SSE) as a chemotherapeutic adjuvant can enhance the efficacy and ameliorate the side effects of cancer chemo- or radio-therapy. Because SSE has been used in Chinese herbal medicine as a bioactive constituent in a complex herbal mixture, an important question is whether its biological activity can be largely or exclusively ascribed to one or more individual compounds present in this herb. To address this question, we prepared SSE and studied its effects. Our previous research showed that SSE possesses potent anticancer activities[7]. However, the molecular mechanisms underlying the anticancer effects of SSE are poorly understood and need to be elucidated. To identify potential anticancer mechanisms of SSE in human hepatocellular carcinoma (HCC), the molecular effects of SSE on HepG-2 cells were examined. Down-regulation of the two anti-apoptotic Bcl-2 family proteins Bcl-xl and Mcl-1 may be responsible for antiproliferative and cell apoptotic effects of SSE on HepG-2 cells. Meanwhile, HepG-2 xenograft nude mice treated systemically with SSE were also monitored in tumor growth inhibition and toxicity *in vivo*. The purpose of this study was to observe the effects of SSE on the hepatoma cell line HepG-2 *in vitro* and *in vivo*, and preliminarily analyse its mechanisms of action.

MATERIALS AND METHODS

Materials

SSE was extracted from 10 g dried powder of *Sargentgloryvine* stem in a rotary shaker with 200 mL of 50 mL/L ethanol at 60℃ for 24 h. The extract was then filtered, concentrated using a rotary evaporator to remove the solvent, and finally lyophilised in a freeze-dryer to obtain crude freeze-dried powder. The same batch of SSE was used in all studies in order to keep the results reliable. The powder was dissolved in DMEM culture medium at a stock solution of 5 g/L for further use. The hepatoma cell line HepG-2 was preserved in our laboratory. Specific pathogen-free male athymic BALB/c nude mice of 6-7 wk of age with a body mass of 20-30 g were purchased from the Animal Experimental Centre of the Fourth Military Medical University. HepG-2 cells were cultured in DMEM supplemented with 100 mL/L foetal calf serum, 100 kU/L penicillin, 0.1 g/L streptomycin and 250 μg/L amphotericin B, incubated at 37℃ in a humified atmosphere of 50 mL/L carbon dioxide. The primers for PT-PCR detection of transcript are described in Table 1.

Methods

Cell viability and clone formation assay: HepG-2 and ECV304 cells (control cells) were cultured in 96-well plates at a density of 3×10^8 cells/L with 100 μL per well in DMEM. Cells were treated with 1.5, 15, 45, 60 and 90 mg/L SSE for 6, 12, 24, 36 and 48 h or medium only as a control (DMEM-treated) group. After incubation, cell proliferation was detected by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and cytotoxicity was studied with a commercial assay kit (Beyotime Biotechnology, China) following the manufacturer's instructions. Absorbance was measured at 570 nm using the Bio-Rad 550 microplate reader. The protracted cell growth curves and the inhibition of cell growth were calculated based on the absorbance (*A*) value as follows: inhibitory rate = $(1-A_{\text{treated}}/A_{\text{control group}}) \times 100\%$. For the colony formation assay, a total of 3×10^2 HepG-2 and control cells, and ECV304 tumor cells were plated in 75 mm culture dishes and treated with SSE at a concentration of 30 mg/L. After incubation for an additional 10-14 d, the cells were fixed with methanol and stained with 1 g/L crystal violet (Sigma, USA), and colonies of > 50 cells were manually counted. All experiments were performed in independent triplicates.

Cell apoptosis and cycle analysis: For apoptosis, HepG-2 cells and ECV304 cells were treated with SSE at concentrations of 15, 30 or 60 mg/L, or no drug as a control for 24 h. The cells were harvested and washed twice with cold PBS and resuspended in binding buffer. FITC-labelled annexin V and propidium iodide (PI) were added and incubated for 10 min at room temperature, and the cell suspensions were immediately analysed by flow cytometry. For cell cycle detection, the cells treated with 45 mg/L SSE for 24 h were fixed with 700 mL/L cold ethanol and resuspended in phosphate-buffered saline containing 20 mg/L PI and then analysed for PI fluorescence intensity by flow cytometry to measure the cellular DNA content. The HepG-2 cells were suspended in 75 mm plates and treated with SSE at a concentration of 45 mg/L for 24 h or an equal volume of culture medium as the control. The total volume of each well was one mL. The cells were collected, and a TdT-Mediated dUTP Nick End Labeling assay (Keygen Biotech Co., Ltd., Nanjing, China) was performed as suggested by the manufacturer to detect the incorporation of labelled nucleotides into DNA. At least 300 cells were counted under a light microscope, and apoptotic cells were identified. All experiments were performed in triplicate. The negative control cells were set up with no TdT enzyme added, and positive control cells were pretreated with DNase during the staining process.

Apoptosis quantification and $\Delta\psi$m detection: HepG-2 cells were cultured in 6-well plates and treated with SSE at a concentration of 45 mg/L for 24 h. Acridine orange/ethidium bromide staining was performed following the

Table 1 The primers for detection of transcript

Gene	Sense sequence 5'-3'	Antisense sequence 5'-3'
β-actin	GACTTAGTTGCGTTACACCTTTC	TGCTGTCACCTTCACCGTTC
Bax	ATGGACGGGTCCGGGGAG	TCAGCCCATCTTCTTCCAGAT
Bak	ATGGCTTCGGGGCAAGGC	TCATGATTTGAAGAATCTTCGTACC
Bal-2	ATGGCGCACGCTGGGAGAACG	GTACTCAGTCATCCACAGGGC
Bcl-xl	ATGTCTCAGAGCAACCGGGAGCT	TCATTTCCGACTGAAGAGTGAGC
Mcl-1	TGCCGCTGCTGGAGTTGGT	TTACAGTAAGGCTATCTTATTAGAT
Bcl-w	CTCTGGTGGCAGACTTTGTAG	CCGTCCCCGTATAGAGCTGTGA
Bcl-b	ATGGCCGACTCGCAGGACCCA	TTATAAACGTTTCCATATAAAA
Blf-1	ATGAGTGATCCAGAAACCAG	TTAATCCTCTTCTGAACTTTCA

manufacturer's instructions (Keygen Biotech Co., Ltd., Nanjing, China). Acridine orange is a vital dye and can stain both live and dead cells. Ethidium bromide can only stain the cells that have lost membrane integrity. Live cells appear uniformly green, while early apoptotic cells stain green but contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporate more ethidium bromides and therefore stain orange and show condensed and often fragmented nuclei. At least 300 cells were counted under a fluorescence microscope to quantify apoptosis. All experiments were performed independently in triplicate. Additionally, HepG-2 cells were harvested following treatment with SSE at 45 mg/L for 12 and 24 h. After washing twice with PBS, 1×10^6 cells were incubated with 10 mg/L of Rh123 (Sigma, USA), a cationic lipophilic fluorochrome that is taken up by mitochondria in proportion to the $\Delta\psi m$, for 30 min at 37℃. Fluorescence intensities were determined by flow cytometry (Becton Dickinson Inc., USA).

Gene expression study: Total RNA from 1×10^6 SSE-treated (45 mg/L for 24 h) HepG-2 cells and control cells were extracted by Trizol™ reagent (Invitrogen, USA), and 1 μg total RNA was used to synthesise cDNA with the superscript first-strand synthesis kit (Takara BioTechnology, Dalian, China) following the manufacturer's instructions. One microliter cDNA was used to amplify the specific genes by reverse transcriptase-polymerase chain reaction (RT-PCR). To normalize cDNA loading, the *β-actin* gene was also amplified from each sample. RT-PCR was performed with the primers listed above. HepG-2 cells treated with SSE (45 mg/L for 24 h) and control cells were harvested by suspension in lysis buffer. The cell extracts were clarified by centrifugation, and the protein concentrations were determined using the Bio-Rad protein assay kit. Each protein extract (25 μg) was electrophoresed on a 100 g/L SDS-polyacrylamide gel, transferred to a membrane and blocked in 50 g/L skimmed milk in tris buffered saline-Tween 20 for 1 h at room temperature. Membranes were probed with anti-Bax, -Bak, -Bcl-xl, -Mcl-1, -caspase-8, -caspase-9, -cytochrome C and -β-actin antibodies overnight at 4℃. Primary antibodies were removed, and the blots were extensively washed with TBS-T three times. Blots were then incubated for 1 h at 37℃ with the secondary antibodies in 10 g/L skimmed milk dissolved in TBS-T. After removal of the secondary antibody, blots were extensively washed and developed using the enhanced chemiluminescence kit in the dark (Santa Cruz Biotechnology). The primary antibodies used in this experiment were monoclonal mouse anti-human Bax, Bcl-xl, Mcl-1, cytochrome C, caspase-8 and-9 (latter two from Abcam, UK) and β-actin and polyclonal goat anti-human Bak (Santa Cruz). Goat anti-mouse and rabbit anti-goat IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies for detection of protein expression.

Solid tumor growth assay: Athymic BALB/c nude mice were housed in laminar flow cabinets under specific pathogen-free conditions. All animal studies were performed in compliance with the Institutional Guidelines of the Fourth Military Medical University. Aliquots of 1.0×10^6 HepG-2 cells were suspended 1:1 in PBS and subcutaneously inoculated into the right flank of each mouse. When 300 mm^3 tumors were observed, the mice were randomly assigned to two groups ($n = 5$). The mice of the treatment group received 18 mg/kg SSE suspended in 50 μL DMEM, and the mice of the control group were treated with an equal volume of DMEM *via* vena caudalis injection every 2 d for 14 d. The tumor volume was measured each time before SSE administration with callipers using a standard formula as follows: $width^2 \times length \times 0.5$. An average tumor volume per mouse was used to calculate the group mean tumor volume ± SD ($n = 5$). Mice were sacrificed 24 h after the last administration of SSE to harvest the tissues of tumors and the heart, liver, spleen, kidneys and lungs. All tissues were then fixed in 40 g/L paraformaldehyde overnight and sectioned, and haematoxylin and eosin staining was performed to identify the toxicity of SSE *in vivo*. The supernatants of nude mice xenografts were preserved at -70℃. After protein concentration determination, tumor necrosis factor α (TNFα) levels were detected by ELISA in tumor tissues from nude mice according to the manufacturer's instructions. Liver function (aspartate aminotransferase and alanine aminotransferase) and kidney function (creatinine) were also detected.

Statistical analysis

All statistical analyses were performed using SPSS13.0. Data were expressed as mean ± SD. Comparisons among all groups were performed with the one-way ANOVA analysis of variance test. Differences were considered significant at $P < 0.05$.

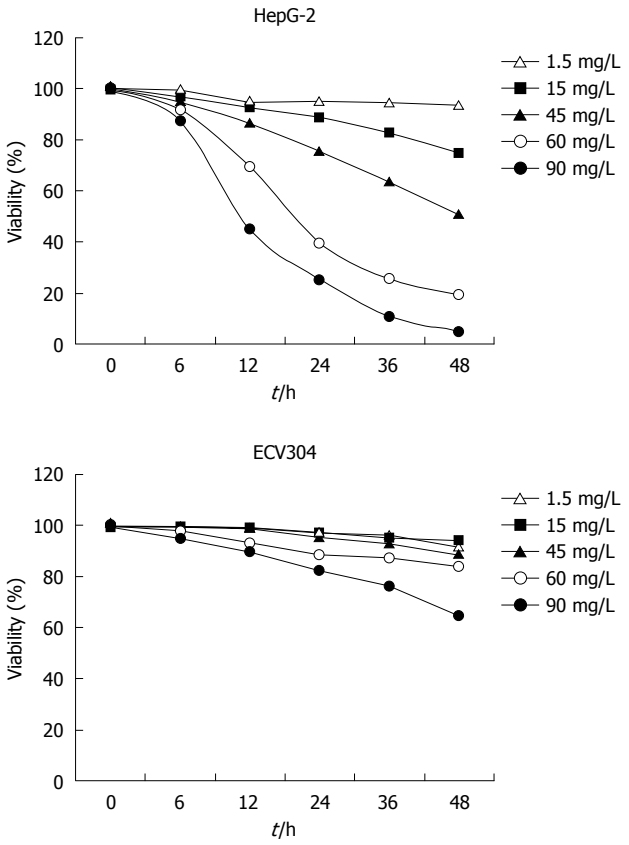

Figure 1 Effects of sargentgloryvine stem extract on HepG-2 viability *in vitro* (MTT assay). HepG-2 and ECV304 cells were treated with sargentgloryvine stem extract (SSE) at five concentrations in five different time points. MTT assay showed that cell proliferation inhibition rates were enhanced with increased SSE concentration and treatment time in HepG-2 cells but not in ECV304 cells. Experiments were repeated in triplicate.

RESULTS

Proliferation and colony formation

SSE-treatment at concentrations of 1.5 and 15 mg/L showed no significant growth inhibition of HepG-2 cells, but higher SSE concentrations of 30, 60 and 90 mg/L significantly inhibited the proliferation of the HepG-2 cells (Figure 1). At an SSE concentration of 30 mg/L, the inhibition rate (mean ± SD) increased with treatment time; the rates were 5.0 ± 1.4, 13.7 ± 2.7, 23.3 ± 6.0, 34.2 ± 5.3 h and 53.7 ± 3 at 6, 12, 24, 36 and 48 h, respectively ($P < 0.05$). All concentrations showed no obvious growth inhibition on ECV304 cells. Additionally, the number of colonies of SSE treated HepG-2 cells was lower than that of the control cells (261 ± 16 *vs* 492 ± 21, Student's *t* test, $P < 0.05$), but no difference was observed on the plates of ECV304 cells. Finally, SSE treatment at 45 mg/L for 24 h significantly increased the proportion of S phase HepG-2 cells from 17.8 ± 1.9 to 63.3 ± 3.3 ($P < 0.05$), but decreased the proportion of G_0/G_1 phase and G_2/M phase cells.

SSE-induced apoptosis

SSE treatment of HepG-2 cells increased the apoptosis rates in a dose-dependent manner (0.2%, 4.7%, 9.5%, 28.7%, as analysed by flow cytometry) (Figure 2). Western blotting analysis showed that SSE treatment (45 mg/L) for 24 h significantly increased caspase-9 but not caspase-8 cleavage (Figure 3), indicating that SSE induces apoptosis through the intrinsic pathway. Additionally, SSE-treated HepG-2 cells lost $\Delta\psi m$, as indicated by a decrease in Rh-123 fluorescence (Figure 4), which was significantly weaker than that of the control cells. Consistent with the results of fluorescence microscopy above, Western blotting analysis demonstrated that the expression of cytochrome C in plasmosin was also altered in SSE-treated HepG-2 cells (Figure 3).

Gene expression

In SSE-treated (45 mg/L) HepG-2 cells, the expression of Bak but not Bax was significantly increased, while the expression levels of Bcl-xl and Mcl-1 were significantly down-regulated compared with the control cells (Figure 5A). Western blotting analysis further confirmed the up-regulation of Bax and down-regulation of Bcl-xl and Mcl-1 (Figure 5B).

Xenograft growth in mice

Compared with DMEM-treated mice, i.v. administration of 18 mg/kg SSE every 2 d for 14 d led to an inhibition of HepG-2 cell growth (Figure 6). No lesions were found in the heart, liver, spleen, lungs or kidneys in SSE-treated xenograft mice, and their functions were also normal. Compared with the control group, the TNFα levels in SSE-treated tumor tissue showed no significant difference at week 8 (9.9 ± 6.8 mg/L *vs* 9.1 ± 5.7 mg/L, $P > 0.05$). In addition, the food intake, mental status and activities were similar in the two groups during the treatment.

DISCUSSION

Recently, the discovery of active compounds from natural products has gained considerable attention as a new source of anticancer drugs[6-10]. The quest to find novel therapeutic compounds for cancer treatment is a never-ending venture. Sargentgloryvine stem, a traditional Chinese medicine, has been used for thousands of years in the treatment of several inflammatory diseases. Recently, we have successfully developed a novel extract from sargentgloryvine stem SSE that has a potent anticancer activity on HCC cells[1]. However, the molecular mechanisms underlying the anticancer effects of SSE have not been elucidated.

Our research shows that SSE can induce apoptosis of the hepatoma cell line HepG-2 *in vitro* and *in vivo*, and its mechanism of action may be through decreasing expression of Bcl-xl and Mcl-1 and increasing expression of Bax. Compared with the control group, TNFα in the tumor tissue and liver function in SSE-treated mice had no significant changes at 8 wk ($P > 0.05$). In addition, this study provides evidence that SSE may be a potent therapeutic agent in the treatment of HCC without obvious toxic side effects.

In this study, the inhibitory effect of SSE on HepG-2 cells was tested, and ECV304 cells as the control were also studied. It was demonstrated that SSE profoundly

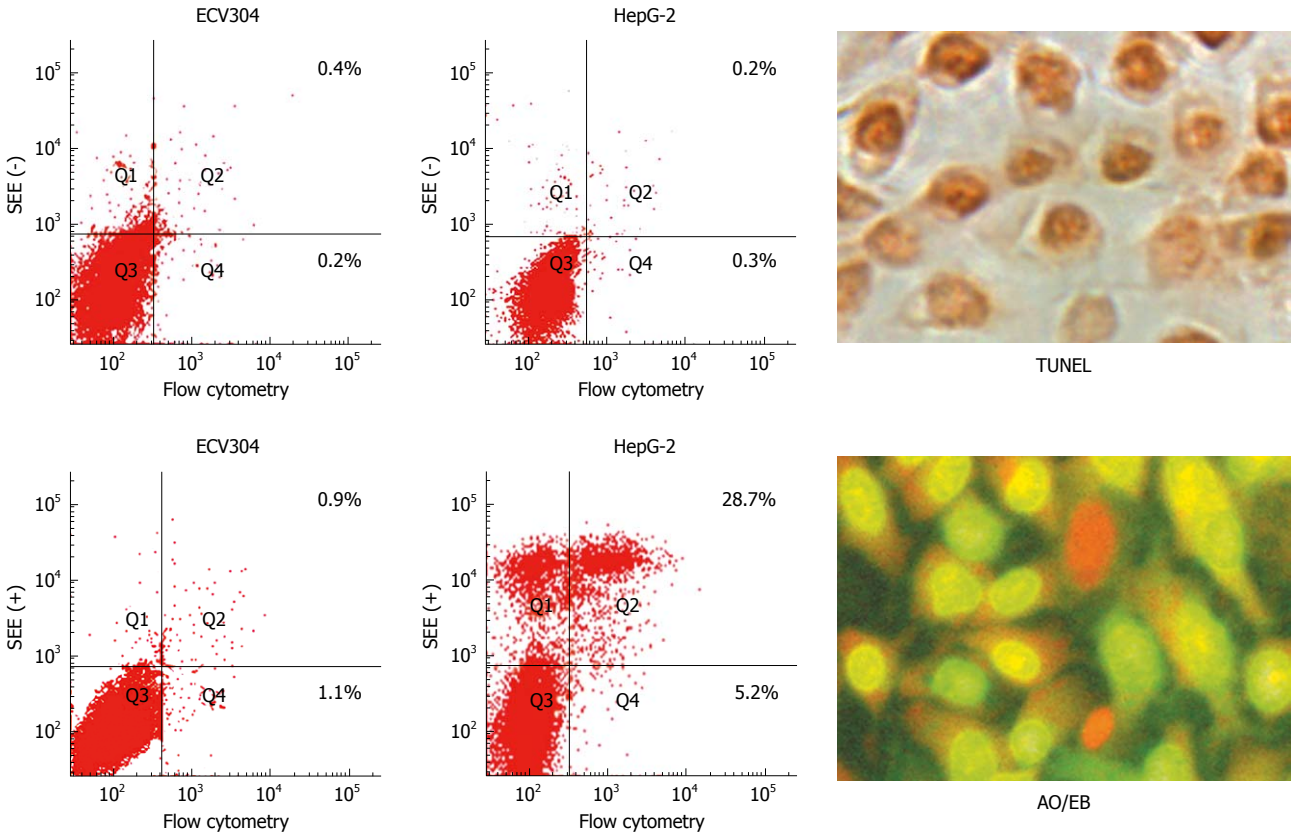

Figure 2 Apoptosis of sargentgloryvine stem extract-treated HepG-2 cells (× 400). HepG-2 and ECV304 cells were treated with sargentgloryvine stem extract (SSE) at a concentration of 45 mg/mL for 24 h. Flow cytometry showed that apoptosis rate was increased obviously compared with the non-treated control cells, $P < 0.05$; while ECV304 cells did not show obvious diversity, $P > 0.05$. Positive signals in nucleus were observed obviously in SSE-treated HepG-2 cells by TdT-Mediated dUTP Nick End Labeling (TUNEL) and acridine orange/ethidium bromide (AO/EB) assays.

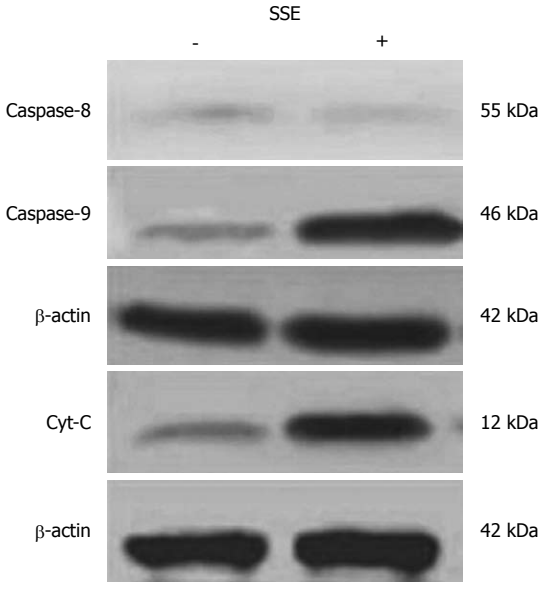

Figure 3 Caspase-8, caspase-9 and cytochrome C in sargentgloryvine stem extract-treated HepG-2 cells. Significantly up-regulated cleavage was found in caspase 9 but not in caspase 8 in HepG-2 cells treated with 45 mg/L sargentgloryvine stem extract (SSE) for 24 h. Cytochrome C in plasmosin was notably increased. $P < 0.05$.

inhibited the growth of HepG-2 cells in a concentration- and time-dependent manner but not in ECV304 cells. MTT and colony formation assays indicate that SSE possesses specific anti-HCC cell activity rather than general cytotoxicity. Additionally, the study suggested that SSE may be safe for normal cells, thus, SSE may have advantages for clinical application.

To reveal the mechanisms of the inhibitory effect of SSE on HepG-2 cells, changes in the cell cycle were analysed. It was demonstrated that the cell cycle of HepG-2 cells was blocked at S phase after 45 mg/L SSE treatment for 24 h, which indicated that the growth inhibition by SSE might be, in part, due to cell cycle arrest. Because cell cycle arrest is always accompanied with cell apoptosis, we analysed apoptosis in the SSE-treated cells. The data showed that SSE treatment induced cell apoptosis, and the apoptosis rates increased with increasing SSE concentrations[11]. More significantly, the induction of apoptosis by SSE in HepG-2 cells was observed at an initial concentration of 30 mg/L within 24 h, further suggesting the safety of SSE for systemic use in the treatment of HCC. It is known that apoptosis is regulated by two main pathways: the extrinsic pathway, which is initiated by the binding of ligands to specific death receptors on the cell surface, and the intrinsic pathway, which is initiated in mitochondria[12,13]. To understand the major *in vivo* pathway through which SSE induces HepG-2 cell growth suppression and apoptosis, the expression of caspase-8 and caspase-9, which play important roles in apoptosis triggered by various proapoptotic signals,

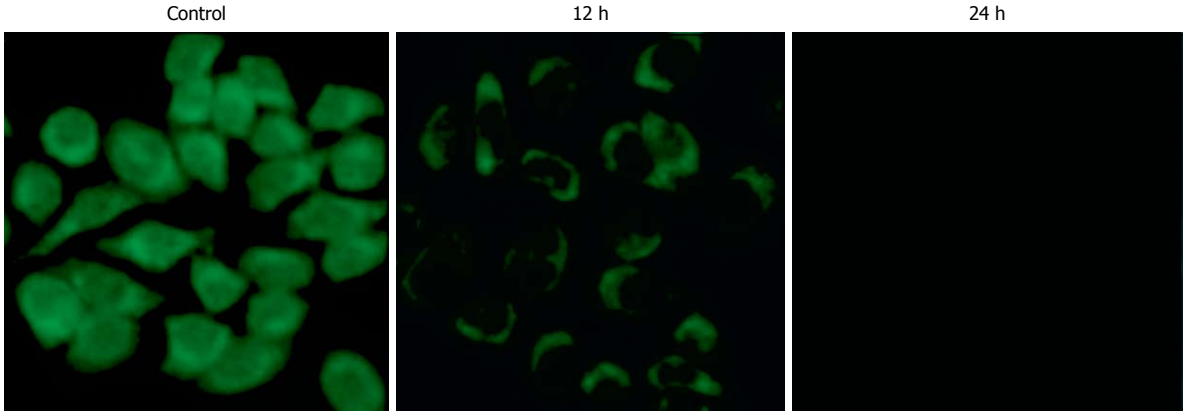

Figure 4 Rh-123 fluorescence in sargentgloryvine stem extract-treated HepG-2 cells (× 400). Flow cytometry showed that fluorescent intensities in sargentgloryvine stem extract (SSE)-treated (45 mg/L) cells for 12 h maintained their $\Delta\psi$m and only displayed minor changes in Rh-123 fluorescence. In contrast, SSE-treated (45 mg/L) cells for 24 h, fluorescent intensities were significantly weaker than control cells.

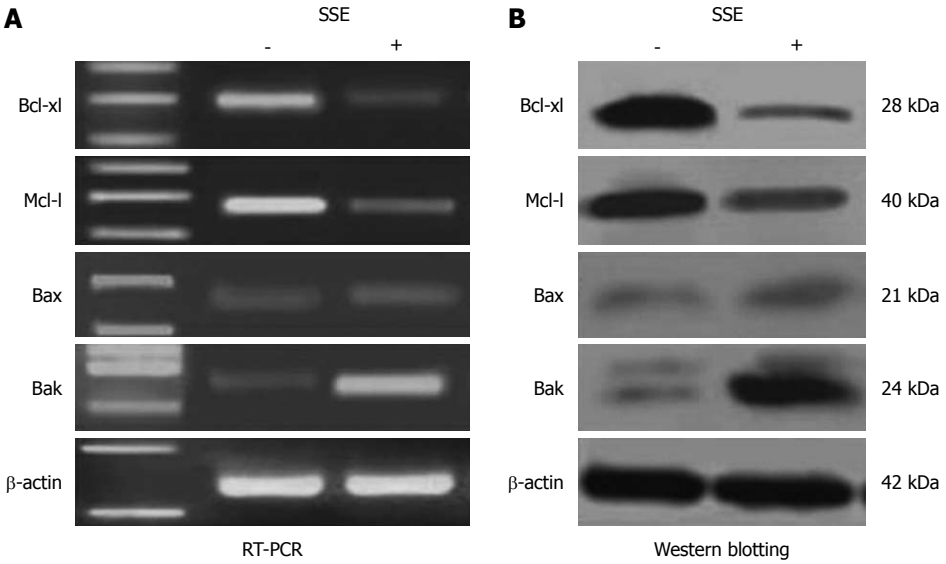

Figure 5 Gene expression in sargentgloryvine stem extract-treated HepG-2 cells. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that the expression of Bak but not Bax was significantly increased in HepG-2 cells treated with 45 mg/L sargentgloryvine stem extract (SSE) for 24 h, and the expression of Bcl-xl and Mcl-1 was significantly down-regulated; B: Western-blotting confirmed the SSE induced gene expression changes with up-regulated Bak and down-regulated Bcl-xl and Mcl-1 in HepG-2 cells.

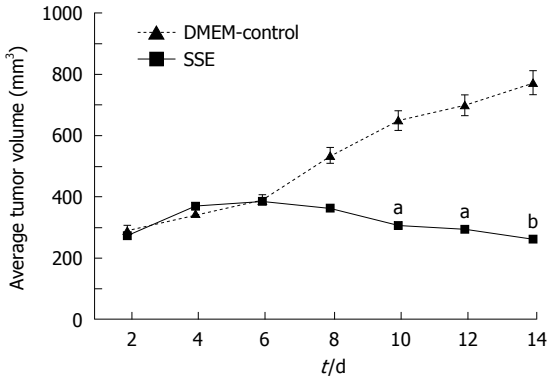

Figure 6 Tumor growth inhibition by systemic sargentgloryvine stem extract-treatment *in vivo*. HepG-2 xenograft tumor (approximately 300 mm^3) growth in Athymic BALB/c nude mice injected with 50 μL of 18 mg/kg sargentgloryvine stem extract (SSE) or DMEM-control every 2 d for 14 d. The tumor size was measured and the tumor volume was calculated as: $width^2$ × length × 0.5. $^aP < 0.05$, $^bP < 0.01$ *vs* DMEM control.

were studied[14]. The results showed that 45 mg/L SSE treatment for 24 h significantly increased caspase-9 but not caspase-8 expression, indicating that SSE-induced apoptosis may be through the intrinsic pathway.

The intrinsic pathway for apoptosis involves several steps including mitochondrial membrane permeabilization and release of cytochrome C, followed by caspase-9 activation[15,16]. Our results showed that treatment with SSE at 45 mg/L for 24 h led to loss of $\Delta\psi$m as indicated by a decrease in Rh-123 fluorescence. SSE also induced cytochrome C release from the mitochondria to cytosol in HepG-2 cells. Taken together, these results further confirmed that SSE may directly trigger the intrinsic pathway to induce the mitochondrial pathway for apoptosis in HCC cells.

Previous studies have shown that in apoptotic cells, anti-apoptotic *bcl-2* members are often inactivated whereas pro-apoptotic members, such as *bax* and *bak*, are activated

and oligomerized in the mitochondria outer membrane. This triggers mitochondrial membrane permeabilization and release of soluble apoptogenic factors such as cytochrome C into the cytosol, which results in caspase activation[17,18]. Further studies revealed that 45 mg/L SSE treatment significantly decreased Bcl-xl and Mcl-1 expression and increased Bak expression and led to mitochondria endomembrane action. Finally, it was confirmed that SSE-induced HepG-2 cell apoptosis is mediated through the *bcl-2* pathway. These results demonstrated that SSE-induced apoptosis is mediated primarily by down-regulated expression of Bcl-xl and Mcl-1, which led to the release of Bak and ultimately activated the intrinsic apoptosis pathway.

In addition to inducing tumor cell apoptosis, systemic injection of SSE into HepG-2 xenografted mice inhibited tumor growth and significantly minimised tumor size but caused no obvious pathological changes in the heart, lungs, liver, spleen or kidneys. The levels of TNFα in the tumor tissue and liver function tests suggest that SEE treatment is relatively safe for the mice.

In conclusion, our research shows that the extract of the Chinese herb sargentgloryvine stem has *in vitro* anticancer effects including inhibition of proliferation and induction of apoptosis in the hepatoma cell line HepG-2 by mechanisms involving expression of Bcl-2 family proteins activating the intrinsic mitochondria apoptosis pathway. Moreover, an *in vivo* solid tumor growth assay further confirmed that systemic administration of the extract could inhibit tumor growth with little cytotoxicity to normal tissues. These *in vitro* and *in vivo* studies provide evidence urging the development of SSE as a novel regimen for human HCC.

ACKNOWLEDGMENTS

We thank our colleagues from the Department of Laboratory Medicine for their technical support.

COMMENTS

Background

Plant-derived natural products have become available such as anticancer derivatives of vincristine, vinblastine, paclitaxel and camptothecin.

Research frontiers

Sargentgloryvine stem extract (SSE) as a chemotherapeutic adjuvant can enhance the efficacy and ameliorate the side effects of cancer chemo- or radiotherapy. However, the effect of SSE on the human hepatocellular carcinoma (HCC) cells remains unknown.

Innovations and breakthroughs

This study showed that SSE treatment was not only able to inhibit the proliferation of human HCC cell HepG-2 cells in a dose and time dependent manner, but also induce apoptosis and cell cycle arrest at S phase.

Applications

SEE is able to inhibit proliferation of human HCC cells and is relatively safe for the mice, and therefore it has a great potential to be a therapeutic agent in the treatment of HCC.

Terminology

SSE: Sargentgloryvine stem is the dried vine stem of *Sargentodoxa cuneata* (Oliv.) and has been widely used as an ingredient in formulated Chinese medicine for thousands of years in the treatment of diseases such as chronic pelvic cavity inflammation, rheumatism and appendicitis.

Peer review

The paper by Wang *et al* addresses an important issue, i.e. novel options for systemic therapy of HCC. In general, the paper is well written, the methods used are sound and the described approach is of potential interest.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2855

World J Gastroenterol 2011 June 21; 17(23): 2855-2859
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Sonographic features of duodenal lipomas in eight clinicopathologically diagnosed patients

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Received: January 23, 2011 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: June 21, 2011

Abstract

AIM: To investigate the sonographic features and diagnostic value of endoscopic ultrasonography (EUS) for duodenal lipomas (DLs).

METHODS: A total of eight consecutive patients with DL diagnosed pathologically were included in the study. One EUS expert reviewed the ultrasonic images for all lesions, including the original layer of the duodenal wall, the echo intensity and the echo homogeneity. The size of the lesions and the perifocal structures were also investigated. The diagnosis by EUS was compared with the histological results.

RESULTS: Using routine endoscopy, only one case was correctly diagnosed as DL. Four cases were classified as submucosal tumors, and three cases were mistaken for stromal tumors. All tumors appeared as round or oval intensive hyperechoic lesions with distinct anterior borders that originated from the submucosal layer on EUS. Tumors ranged from 8 to 36 mm in size, with an average size of 16 mm. Homogeneous echogenicity was seen in all cases except one that had a tubular structure inside the tumor. Echo attenuation was observed only in the area behind the tumors in five cases, and it was observed both inside and behind the tumors in three cases in which the posterior border was obscure or invisible. Seven (87.5%) cases were correctly diagnosed as DL, and one (12.5%) was mistaken as Brunner's gland adenoma by EUS. Pathologically, all tumors originated from the submucosal layer and consisted of mature fat cells without heteromorphism. Among the fat cells, there was a small amount of thick-wall vessels infiltrating the lymphocytes, and abundant fibrous connective tissues.

CONCLUSION: On EUS, DL is featured as an intensive homogeneous hyperechoic submucosal lesion with marked echo attenuation and without involvement of the mucosa.

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Key words: Duodenum; Lipoma; Endoscopic ultrasonography; Hyperecho; Echo attenuation

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Chen HT, Xu GQ, Wang LJ, Chen YP, Li YM. Sonographic features of duodenal lipomas in eight clinicopathologically diagnosed patients. *World J Gastroenterol* 2011; 17(23): 2855-2859 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2855.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2855

INTRODUCTION

Gastrointestinal lipomas are uncommon benign tumors that occur anywhere along the gut. The most common location for these lesions is the colon, followed by the il-

eum and the jejunum[1]. Lipomas found in the duodenum are rare and the literature regarding duodenal lipomas (DLs) is only limited to case reports[2-8], and no systematic study of diagnostic means for DLs has been reported. Since preoperative diagnosis of DLs is difficult, and large DLs can mimic malignant tumors on endoscopy, patients could be subjected to extensive surgical procedures that are sometimes destructive. Endoscopic ultrasonography (EUS) is an optimal method for detecting gastrointestinal submucosal tumors (SMTs) such as stromal tumors and leiomyomas; however, the features and diagnostic value of EUS for examining DLs have not been well established because of the rareness of this disease. In this study, we studied the sonographic features and diagnostic value of EUS for identifying DLs.

MATERIALS AND METHODS

Patients

A total of eight consecutive patients with DL were included. The diagnosis of DL for all patients was pathologically established after surgical excision in five patients and endoscopic resection in three patients during the period from June 2000 to December 2010 in the First Affiliated Hospital, School of Medicine, Zhejiang University, China. The patient group was composed of five males and three females, and aged from 42 to 78 years, with a mean of 60 years. Except for one patient who was asymptomatic, DLs presented as bleeding in four patients, dyspepsia in two patients, and epigastric pain in one patient (Table 1). Laboratory examinations, including liver function tests, serum lipids, and tumor markers (carcinoembryonic antigen, carbohydrate antigen 19-9, carbohydrate antigen 125, and alpha-fetoprotein), showed no obvious abnormalities except for anemia in five patients.

Instrument

The EUS system included Olympus EU-M2000 sonogram processing equipment, an Olympus GIF-2T-240 double-cavity electronic gastroscope, Olympus MAJ drive systems with a high-frequency echo probe, UM-DP12-25R miniature ultrasonic probes with a frequency spectrum of 12-15 MHz, and a Daker WP-800 water pump (Olympus Medical System Corp., Tokyo Japan).

Methods

One EUS expert reviewed the ultrasonic image of all lesions, including the original layer of the duodenal wall, the echo intensity, and the echoic homogeneity. The size of the lesions and the perifocal structures were also investigated. The EUS diagnosis was compared with the histological results.

RESULTS

Endoscopic studies

The tumors were located at the bulb in one case and in the descending part of the duodenum in seven cases. The lesions, with a hemispherical or oval shape, were sessile in six cases and had a stalk in two. The surface of the lesions was intact in three cases (Figure 1A) and had an ulcer (Figure 2A) or an erosion in five cases. Prior to EUS, only one case was correctly diagnosed as DL by routine endoscopy because of the yellowish and soft appearance (Figure 3A). Four cases were classified as SMTs, and three cases were mistaken for stromal tumors.

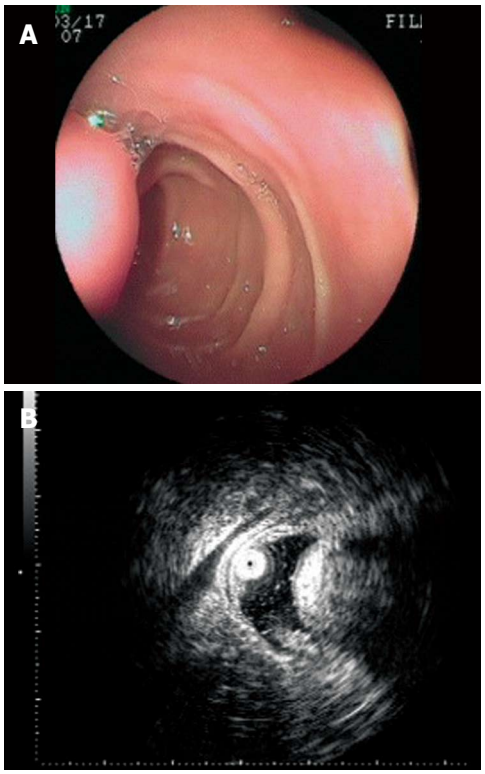

Figure 1 Endoscopic and endoscopic ultrasonography findings in Case 7. A: A submucosal tumors of the descendant duodenum with an intact surface; B: Endoscopic ultrasonography showed a hyperechoic lesion with a distinct anterior border originating from the submucosal layer. There was marked echo attenuation both inside and behind the lesion, and the posterior border was obscure.

Endoscopic ultrasonography

The endoscopic ultrasonography (EUS) findings in all cases are shown in Table 1. All tumors appeared as intensive hyperechoic lesions with a distinct anterior border that originated from the third EUS layer without involvement of the overlying first and second layers (Figures 1B, 2B, 3B and 4). Tumors ranged from 8 to 36 mm in size, with an average size of 16 mm. The margins of the tumors were clear in five cases (Figures 3B and 4), and the posterior borders of three lesions were obscure or invisible because of marked echo attenuation (Figures 1B and 2B). Homogeneous echogenicity was seen in all cases except for one that had a tubular structure inside the tumor (Figure 2B). Echo attenuation was observed only in the area behind the tumors in five cases (Figures 3B and 4) and both inside and behind the tumors in three cases (Figures 1B and 2B). With EUS, seven patients were correctly diagnosed as having DLs, but one

Table 1 Clinical settings and endoscopic ultrasonography observations

Case	Sex	Age	Symptoms	Location	Treatment	EUS features					
						Layer	Size (mm)	Echogenicity	Homogeneity	Border	Echo attenuation
1	M	50	None	Bulb	Endoscopic resection	3rd	12	Hyperecho	Homogenous	Distinct	Behind
2	F	64	Melena	2nd portion	Surgery	3rd	10	Hyperecho	Homogenous	Distinct	Behind
3	M	63	Dyspepsia	2nd portion	Surgery	3rd	25	Hyperecho	Homogenous	Indistinct	Behind and inside
4	F	54	Melena	2nd portion	Surgery	3rd	12	Hyperecho	Homogenous	Distinct	Behind
5	M	67	Dyspepsia	2nd portion	Endoscopic resection	3rd	8	Hyperecho	Homogenous	Distinct	Behind
6	M	78	Melena	2nd portion	Endoscopic resection	3rd	11	Hyperecho	Homogenous	Distinct	Behind
7	F	62	Epigastric pain	2nd portion	Surgery	3rd	15	Hyperecho	Homogenous	Indistinct	Behind and inside
8	M	42	Melena	2nd portion	Surgery	3rd	36	Hyperecho	Heterogeneous	Indistinct	Behind and inside

EUS: Endoscopic ultrasonography; M: Male; F: Female.

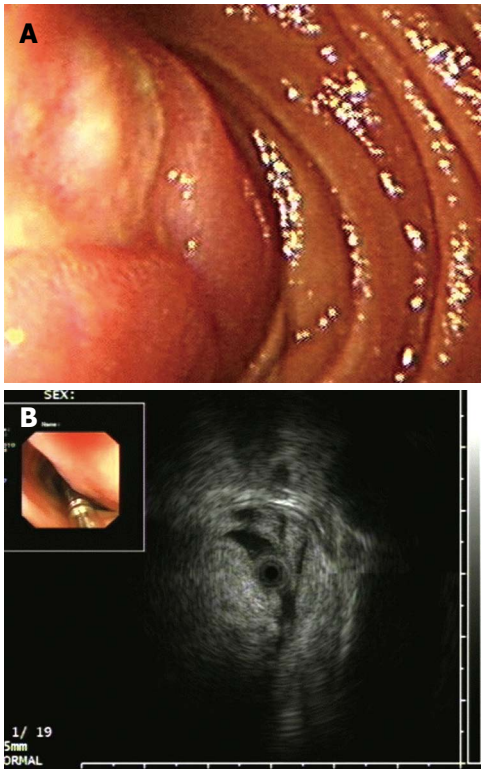

Figure 2 Endoscopic and endoscopic ultrasonography findings in Case 8. A: A lobulated submucosal tumor in the descendant duodenum with an ulcer on its surface; B: Endoscopic ultrasonography showed an intensive hyperechoic lesion with a distinct anterior border originating from the submucosal layer without involvement of the overlying mucosal layer. The posterior border of the tumor was invisible because of the marked echo-attenuation.

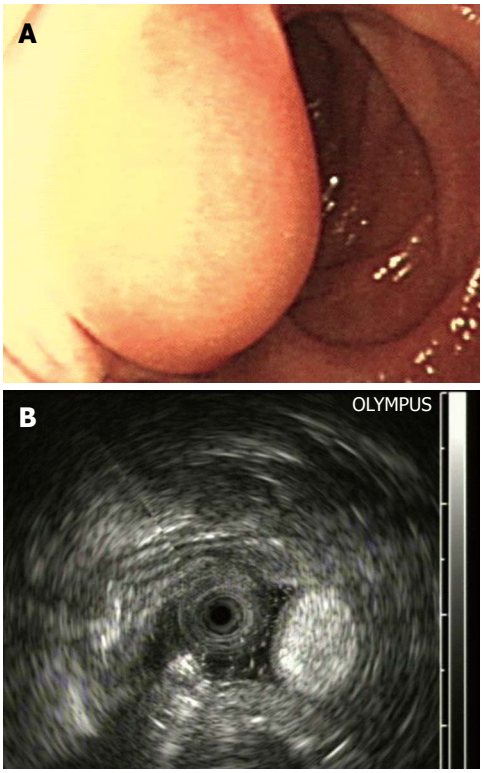

Figure 3 Endoscopic and endoscopic ultrasonography findings in Case 2. A: A submucosal tumor located in the descendant duodenum with a yellowish and soft appearance; B: Endoscopic ultrasonography showed an oval intensive hyperechoic lesion with homogeneous parenchymal echogenicity and a clear margin originating from the third layer without involvement of the overlying mucosal layers. Echo attenuation was seen at the area behind the focus.

patient with DL was mistaken as having Brunner's gland adenoma (BGA) due to the appearance of a tubular structure.

Pathology

The pathological diagnosis after routine endoscopic biopsy was chronic inflammation of the mucosa, whereas the postoperative diagnosis after surgical excision or endoscopic resection was DL. The tumors presented by gross appearance as a node or finger in six cases and as a sub-lobe or cauliflower in two cases. All tumors originated from the submucosal layer, sometimes involving the muscularis propria, with a fully or partially coated fiber peplos. Microscopically, the tumors were composed of mature fat cells without heteromorphism. Among the fat cells, there were small amounts of thick-wall vessels, infiltrating lymphocytes, and abundant fibrous connective tissues (Figures 5-7).

DISCUSSION

Lipomas of the duodenum are rare, with fewer than 230 cases reported in the literature, and most of the described cases are from autopsy records rather than clinical experience[9]. DLs were mostly detected after bleeding or obstruction occurred. Among the patients in

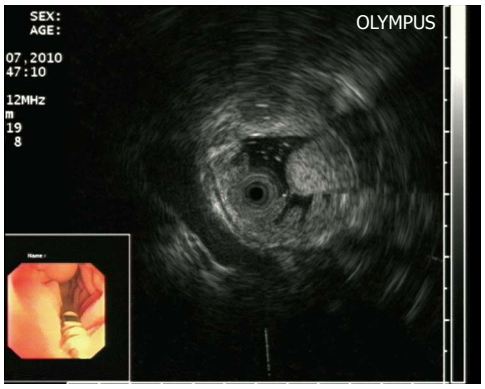

Figure 4 Endoscopic and endoscopic ultrasonography findings in Case 4. A submucosal tumor located in the descendant duodenum. Endoscopic ultrasonography showed a submucosal hyperechoic lesion with marked echo-attenuation behind the focus.

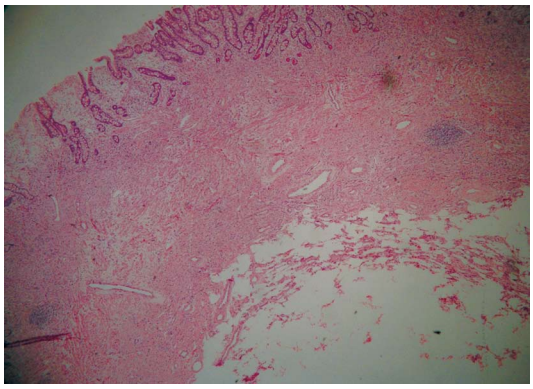

Figure 5 Pathological findings in Case 3. Microscopically, the tumor was located in the submucosa without involvement of the mucosa (HE, × 40).

this study, four (50%) experienced bleeding, two (25%) experienced dyspepsia, one (12.5%) had epigastric pain, and one (12.5%) was asymptomatic. The symptoms of DL are nonspecific, however, and they are not useful for differential diagnosis.

Endoscopy is the preferred means for detecting upper gastrointestinal diseases. However, it can only suggest the presence of submucosal protruding lesions, but cannot provide additional details even though lesions have been detected[10,11]. In this study, four patients were diagnosed as having SMTs by endoscopy, and three patients were mistaken as having stromal tumors. Only one patient was correctly diagnosed with lipoma by routine endoscopy due to the yellowish and soft appearance of the lesion (Figure 2B).

EUS has been reported to be an effective modality for assessing gastrointestinal tumors and evaluating the original layer of the submucosal tumor, the homogeneity of the internal parenchymal echo, and the echogenicity of the lesions[10]. The typical EUS observations of DLs are intensive homogeneous hyperechoic lesions originating from the submucosa, with echo attenuation behind and/or inside the rear area. Thus, the signs of DLs are similar to the ultrasonic features of a fatty liver.

Intensive hyperecho is the most noticeable EUS feature of DLs. A newly-devised echogenicity classification system for SMTs by Okanobu[10] demonstrated the highest echo level of lipomas (levels 5-6). The marked echo-attenuation is another EUS feature of DLs. In our series, there was a more apparent echo decline in the area behind the focus than anywhere else in all cases regardless of the size of the lesions, suggesting a greater attenuation coefficient for DLs than for normal intestinal wall tissues. Furthermore, visible echo decline was seen inside the lesion when the size of the focus was larger than 15 mm. The anterior border was as distinct as the overlying first and second layers of the DLs; however, the appearance of the posterior border depended on the related echo de-

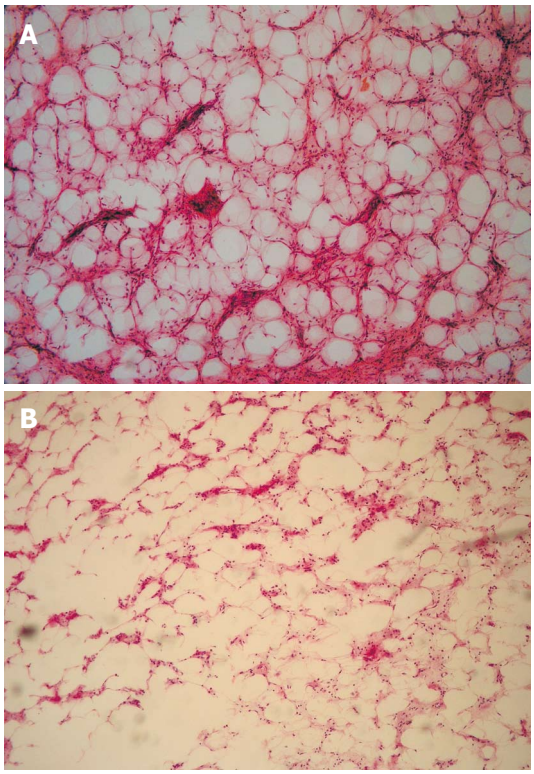

Figure 6 Pathological findings in Cases 2 and 4. A, B: The tumors consisted of mature fat cells. Abundant fibrous connective tissues were found among the fat cells (HE, × 100).

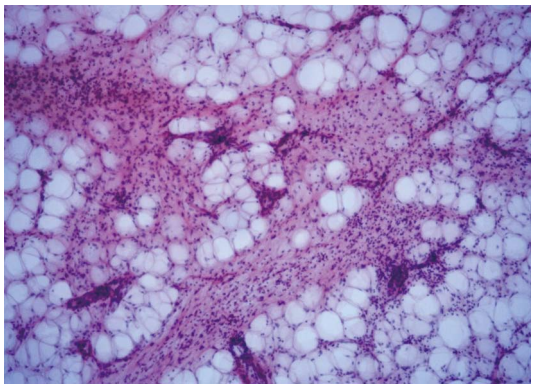

Figure 7 Pathological findings in Case 7. Massive fiber ropes were inside the tumor parenchyma (HE, × 100).

cline. The latter portion and the posterior border of DLs larger than 25 mm are often invisible with the 12-MHz probe. The internal parenchymal echo of DLs is generally homogenous[11]. Occasionally, blood vessels may present as tubular structures inside the focus.

According to two ultrasonic characteristics, we could distinguish DLs from the majority of other SMTs such as leiomyomas, stromal tumors, or cysts. It is noteworthy that Brunner's gland adenomas (BGAs) also show hyperechoic lesions originating from the submucosa, and therefore, DLs may be mistaken for BGAs[11,12]. In our study, one patient was misdiagnosed with duodenal BGA. BGAs appear primarily in the bulb portion or at the junction of the bulb and the descending duodenum, whereas DLs are located primarily in the descending portion of the duodenum. In addition, the echogenicity of BGAs is not as intensive or homogeneous as that of lipomas. Although the second EUS layer often becomes blurry or invisible in BGAs because of involvement of the lamina propria, it is always readable in DLs.

The reason why all gastrointestinal lipomas appeared as hyperechoic is not clear. Interestingly, normal subcutaneous fat tissues appeared as a hypoechoic zone with a small amount of hyperechoic fiber ropes. An earlier report showed that 29% of superficial soft tissue lipomas were hypoechoic, 22% were isoechoic, 29% were hyperechoic, and 20% were of a mixed pattern[13]. Thus, the echo types of lipomas mostly depend on the quantity of the boundary in relation to the mixture of fat and other connective tissues.We found abundant fibrous connective tissues among the fat cells of DLs. The heterogeneous mixture generated innumerable acoustic boundaries that appeared hyperechoic because of the marked acoustic impedance difference between the fat and fibrous tissues.

In summary, EUS has significant value for the differential diagnosis of DLs. The appearance of a round or oval lesion originating from the submucosal layer with intensive homogenous hyperecho and marked echo-attenuation, without involvement of the mucosal layers, suggests a diagnosis of DL. Abundant fibrous connective tissues among the fat cells may be the acoustic basis of this appearance.

COMMENTS

Background

Lipomas located in the duodenum are rare. Because the preoperative diagnosis for duodenal lipomas is difficult to establish and some large lipomas can mimic malignant tumors on endoscopy, patients could be subjected to extensive surgical procedures that sometimes were destructive. Endoscopic ultrasonography (EUS) is the optimal method for the diagnosis of gastrointestinal stromal tumors, but its diagnostic value for duodenal lipomas has not been well established because of its rareness.

Research frontiers

Up to date, less than 230 cases of lipomas have been reported in the literature, but most of them are from autopsy records rather than clinical experience. Clinically, duodenal lipomas are mostly revealed by bleeding or obstruction. Endoscopy can only detect the submucosal lesion but fail to judge its nature. EUS is an effective modality in the assessment of gastrointestinal submucosal tumors and evaluation of the original layer, homogeneity of internal parenchymal echo and echogenicity of the lesions.

Innovations and breakthroughs

EUS is of significant value in the diagnosis and differential diagnosis of duodenal lipomas. The sonographic features were round or oval lesions originated from submucosal layer with intensive homogenous hyperecho and marked echo-attenuation, without involvement of the mucosal layers.

Applications

According to this study, duodenal lipomas can be defined by EUS, thus needless surgery can be avoided. By EUS, they can differentiate duodenal lipomas from other submucosal tumors. The sonographic features of duodenal lipomas can be used for the diagnosis of lipomas in esophagus, stomach and colon.

Peer review

This is an original report on the correlations of the sonographic findings and clinicopathological features of duodenal lipomas. The material is characterized by small case series but the conclusive suggestions are appropriate and interesting. Duodenal lipoma is a rare clinicopathological entity. Therefore, it is interesting to get an eight consecutive duodenal lipoma cases. The EUS is probably the best option to diagnose this pathology.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2860

World J Gastroenterol 2011 June 21; 17(23): 2860-2866
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Association between *ITGA2* C807T polymorphism and gastric cancer risk

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Supported by The National Natural Science Foundation of China, No. 30873099; Nanjing Medical University start-up research fund for Wang XR; the Natural Science Foundation of education Department, Jiangsu Province, No. 08KJB320004
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Received: October 9, 2010 Revised: November 5, 2010
Accepted: November 12, 2010
Published online: June 21, 2011

Abstract

AIM: To evaluate the impact of the *ITGA2* gene polymorphism on gastric cancer risk.

METHODS: A hospital-based case-control study was conducted, including 307 gastric cancer patients and 307 age- and gender-matched control subjects. The genotypes were identified by polymerase chain reaction-restriction fragment length polymorphism assay.

RESULTS: The frequencies of the wild and variant genotypes in cases were significantly different from those of controls (P = 0.019). Compared with individuals with the wild genotype CC, subjects with the variant genotypes (CT + TT) had a significantly higher risk of gastric cancer (adjusted odds ratio = 1.57, 95% CI = 1.13-2.17, P = 0.007). In stratified analyses, the elevated gastric cancer risk was especially evident in older individuals aged > 58 years, nonsmokers and rural subjects. Further analyses revealed that the variant genotypes were associated with poor tumor differentiation and adjacent organ invasion in the sub-analysis of gastric cancer patients.

CONCLUSION: The *ITGA2* gene C807T polymorphism may be associated with an increased risk of gastric cancer, differentiation and invasion of gastric cancer.

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Key words: Gastric cancer; Integrin; *ITGA2*; Polymorphism; Genotype

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Chen J, Liu NN, Li JQ, Yang L, Zeng Y, Zhao XM, Xu LL, Luo X, Wang B, Wang XR. Association between *ITGA2* C807T polymorphism and gastric cancer risk. *World J Gastroenterol* 2011; 17(23): 2860-2866 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2860.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2860

INTRODUCTION

Gastric cancer remains a major public health issue as the fourth most common cancer type and the second leading cause of cancer death worldwide[1,2]. Nearly half of the gastric cancer cases occur in China[3]. Although the

cause of gastric cancer is largely unknown, it has been shown that diet, tobacco smoking, alcohol, gastroesophageal reflux and *Helicobacter pylori* (*H. pylori*) infection are associated with the risk of this cancer[4-7]. As genetic polymorphisms are responsible for the inter-individual variation and diversity, they have been recently considered as the main genetic elements involved in the development of common and complex diseases, including various cancers. Like many malignancies, it is believed that gastric cancer is the result of interactions between environmental factors and genetic factors[8]. Our previous epidemiological studies also provided the evidence that genetic polymorphisms were associated with the risk of gastric cancer[9-12].

Integrins are members of a family of cell-surface heterodimeric proteins that mediate cell-matrix and cell-cell interactions. The 18 α-subunits and 8 β-subunits form together at least 25 different integrins, each pair being specific for a unique set of ligands. It has been demonstrated that integrins may play a crucial role in carcinogenesis, tumor behavior and metastasis[13,14]. Several integrins such as α2β1, α Ⅱ bβ3 and αvβ3 are considered as key factors for cancer development and progression. Integrin α2β1, also known as platelet glycoprotein Ⅰa-Ⅱa, is expressed by epithelial cells, and its level of expression in tumor cells is associated with motility, invasiveness and cellular differentiation[15-17]. Several studies have shown that integrin α2β1 expression is closely associated with invasion and metastasis of gastric cancer[18-21].

The integrin, α2 gene (*ITGA2*) is located on chromosome 5q23-31. A silent change in the coding region at nucleotide 807 (TTT/TTC at codon Phe253) has been identified. The C807T single nucleotide polymorphism (NCBI SNP ID: rs1126643) of the *ITGA2* gene was associated with the integrin α2β1 density. The genotype 807 TT was associated with a higher receptor density and the genotype 807 CC with a lower density, whereas heterozygous individuals expressed intermediate receptor levels[22,23].

Recent studies indicated that the *ITGA2* gene C807T polymorphism was associated with various diseases, including stroke, retinal vein occlusion, acute coronary syndrome, colorectal cancer, and breast cancer[24-29]. To the best of our knowledge, there has been no study that assessed the association between the polymorphism and gastric cancer risk.

Given that the roles of *ITGA2* in the progression of gastric cancer as well as the effect of the polymorphism in *ITGA2* gene on the receptor function, it is plausible that the polymorphism may be associated with the risk of gastric cancer. To test the hypothesis, we performed a hospital-based case-control study in a Chinese population.

MATERIALS AND METHODS

Subjects

This hospital-based case-control study consisted of 307 consecutive inpatients with histologically confirmed gastric cancers without synchronous and/or metachronous secondary malignancy and a population-based and sex- and age-matched 307 cancer-free inpatients as controls. All subjects were recruited between March 2005 and November 2009 from the patients who were admitted to the First Affiliated Hospital of Nanjing Medical University. The most common causes for hospitalization in the control subjects were hernias, appendicitis, hydrocele, cholecystitis and cataract. All subjects were of unrelated Han nationality from Jiangsu Province or its surrounding regions. Information on age, gender, smoking status, residence (urban or rural), body weight and personal medical history was collected by questionnaire. Individuals who formerly or currently smoked ≥ 10 cigarettes per day for at least 2 years were defined as smokers. Depth of tumor invasion and local lymph node status were classified according to the TNM classification criteria of International Union Against Cancer[30]. Differentiation was graded according to World Health Organization classification. The study was approved by the Ethics Committee of Nanjing Medical University First Affiliated Hospital and informed consent was obtained from all the participating subjects.

Genotyping

The protocol for genomic DNA extraction was described in our previous study[9]. The polymerase chain reaction (PCR)-restriction fragment length polymorphism assay was used to identify the *ITGA2* C807T genotypes. The PCR was performed in a total volume of 20 μL reaction mixtures containing 2 μL 10 × PCR buffer (MBI Fermentas), 1.75 mmol/L $MgCl_2$, 0.25 μmol/L each primer (forward 5'-GTGTTTAACTTGAACACATAT-3', reverse 5'-ACCTTGCATATTGAATTGCTT-3'), 0.15 mmol/L dNTP, 1 unit *Taq* polymerase (MBI fermentas) and 150 ng genomic DNA. The amplification protocol is as follows: primary denaturation at 95℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min, then a final elongation at 72℃ for 5 min. The 115 bp PCR products including the polymorphic site were digested at 65℃ for 12 h, using restriction enzyme *Taq* I (MBI Fermentas) and then separated on a 3% ethidium bromide-stained agarose gel. The wild-type homozygotes (CC) produced two bands at 92 and 23 bp, while the variant homozygotes (TT) produced one band at 115 bp, and the heterozygous (CT) produced three bands at 115, 92 and 23 bp (Figure 1). To control the quality of genotyping, all assays were conducted by two researchers separately in a blind fashion. In addition, a 10% masked samples were randomly selected and retested, and the reproducibility was 100%.

Statistical analysis

Statistical analyses were conducted using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed and $P < 0.05$ was considered statistically significant. Quantitative variables departing from the normal distribution including age and weight were summarized as median and analyzed by Mann-Whitney rank

Table 1 Demographic information *n* (%)

Characteristics	Cases (n = 307)	Controls (n = 307)	P value
Gender (male)	231 (75.2)	231 (75.2)	1.000
Age[1] (yr), (range)	59 (50-68)	58 (49-66)	0.145
Weight[1] (kg), (range)	62 (55-70)	65 (57-72.75)	0.001
Hypertension	65 (21.17)	59 (19.22)	0.546
Diabetes	17 (5.54)	24 (7.82)	0.262
Smoking	82 (26.71)	53 (17.26)	0.005
Residence			
Rural	139 (45.28)	139 (45.28)	1.000
Urban	168 (54.72)	168 (54.72)	

[1]Median (25th-75th percentiles).

sum test. Pearson's χ^2 test was used to compare the difference in the distribution of categorical variables and genotype frequencies between cases and controls. The Hardy-Weinberg equilibrium of the *ITGA2* genotypes was estimated for cases and controls by a goodness-of-fit χ^2 test. Odds ratio (OR) and 95% CI were calculated to evaluate the association between the polymorphism and the risk of gastric cancer. Carriers of the wild genotype CC were used as the reference. The crude OR was obtained using the Woolf approximation method and the adjusted OR was calculated by unconditional logistic regression method, with adjustment for age, sex, smoking status, residence, hypertension and diabetes.

RESULTS

Demographic information

A total of 614 subjects (307 cases and 307 controls) were analyzed. Baseline demographic characteristics of the study groups are shown in Table 1. The age distribution and proportion of males were quite similar due to the fact that we selected the age- and gender-matched subjects. The two groups were similar with respect to residence, history of hypertension and diabetes. Nevertheless, compared with controls, gastric cancer patients had a lower body-weight (P = 0.001) and more smokers were found among gastric cancer cases than among the controls (26.71% *vs* 17.26%, P = 0.005).

Distribution of ITGA2 genotype in cases and controls and risk estimates

Table 2 shows the frequency distributions of the genotypes and their association with gastric cancer risk by unadjusted OR, adjusted OR and 95% CI. The genotype distributions in cases and controls were consistent with those from the Hardy-Weinberg equilibrium model (P = 0.988, P = 0.675, respectively). The frequencies of the *ITGA2* genotype were significantly different between gastric cancer cases and controls (P = 0.019). Compared with the control group, T allele frequency was significantly higher in the case group (P = 0.024). With the wild genotype CC as reference, we found that the CT genotype was associated with an increased risk of gastric cancer (adjusted OR = 1.54, 95% CI = 1.10-2.18, P = 0.013).

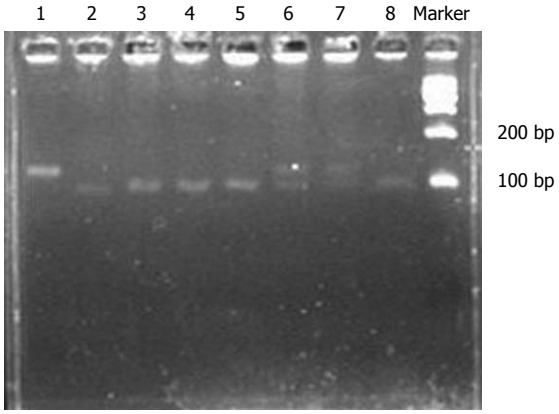

Figure 1 *ITGA2* C807T polymorphism in gastric cancer patients and controls. Amplified polymerase chain reaction products were digested with restriction enzyme *Taq* I and analyzed on a 3% agarose gel. Lane 1: The TT homozygous; Lanes 2-5, 8: The CC homozygous; Lanes 6, 7: The CT homozygous.

Individuals with the variant genotypes (CT + TT) had a 1.57-fold increased risk of developing gastric cancer (adjusted OR = 1.57, 95% CI = 1.13-2.17, P = 0.007).

Stratified analysis of polymorphism and gastric cancer risk

As shown in Table 3, stratified analyses were performed by the median age of controls (58 years), sex, smoking status, and residence. The elevated risk of gastric cancer associated with the variant genotypes was noteworthy in subjects aged > 58 years (adjusted OR = 1.88, 95% CI = 1.17-3.03, P = 0.010), but not in subjects aged ⩽ 58 years. In non-smoking subjects, the variant genotypes were associated with a 51% increased risk of gastric cancer (adjusted OR = 1.51, 95% CI = 1.05-2.18, P = 0.028), whereas the correlation was not statistically significant in smoking subjects. When stratified by residence, the elevated risk was evident in rural subjects (adjusted OR = 2.35, 95% CI = 1.42-3.90, P = 0.001), but not in urban subjects. No statistically significant difference was observed in the association of the polymorphism and susceptibility to gastric cancer between males and females.

Variant genotypes and clinicopathological characteristics of gastric cancer

We also observed the correlations between the *ITGA2* variant genotypes and clinicopathologic features of gastric cancer patients in this study (Table 4). A significantly increased risk was found in individuals with the variant genotypes in both poorly differentiated tumors (adjusted OR = 2.21, 95% CI = 1.12-4.38, P = 0.022) and adjacent invaded organs (adjusted OR = 2.12, 95% CI = 1.10-4.07, P = 0.024) of gastric cancer. However, no significant association was observed between the polymorphism and lymph node metastasis or tumor location.

DISCUSSION

In the present study, we investigated the role of *ITGA2* gene C807T polymorphism in gastric cancer susceptibil-

Table 2 Distributions of *ITGA2* genotype in cases and controls and risk estimates *n* (%)

ITGA2 genotype	Cases[1]	Controls[1]	Crude OR (95% CI)	*P* value	Adjusted OR[2] (95% CI)	*P* value
Overall	307	307				
CC	141 (45.93)	170 (55.37)	1.00		1.00	
CT	135 (43.97)	113 (36.81)	1.44 (1.03-2.01)	0.033	1.54 (1.10-2.18)	0.013
TT	31 (10.10)	24 (7.82)	1.56 (0.87-2.78)	0.133	1.62 (0.90-2.91)	0.112
CT + TT	166 (54.07)	137 (44.63)	1.46 (1.06-2.01)	0.019	1.57 (1.13-2.17)	0.007
C allele	417 (67.92)	453 (73.78)				
T allele	197 (32.08)	161 (26.22)				

[1]Distributions of the *ITGA2* genotype in cases and controls were in Hardy-Weinberg equilibrium ($P = 0.988$, $P = 0.675$, respectively); [2]Adjusted for age, sex, smoking status, residence, hypertension and diabetes. OR: Odds ratio.

Table 3 Stratified analyses for variant *ITGA2* genotypes in cases and controls *n* (%)

Variable	(CT + TT)/CC		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value
	Cases	Controls				
Age (yr), (median)						
≤ 58	81 (26.4)/68 (22.1)	80 (26)/85 (27.7)	1.27 (0.81-1.97)	0.298	1.31 (0.83-2.06)	0.247
> 58	85 (27.7)/73 (23.8)	57 (18.6)/85 (27.7)	1.74 (1.10-2.75)	0.018	1.88 (1.17-3.03)	0.010
Sex						
Females	45 (14.7)/31 (10.1)	38 (12.4)/38 (12.4)	1.45 (0.76-2.76)	0.255	1.52 (0.73-2.93)	0.206
Males	110 (35.8)/121 (39.4)	99 (32.2)/132 (43)	1.21 (0.85-1.73)	0.304	1.29 (0.82-2.01)	0.287
Smoking status						
Smokers	40 (13)/42 (13.7)	19 (6.2)/34 (11.1)	1.70 (0.84-3.46)	0.141	1.87 (0.89-3.94)	0.100
Non-smokers	126 (41)/99 (32.3)	118 (38.4)/136 (44.3)	1.47 (1.02-2.10)	0.037	1.51 (1.05-2.18)	0.028
Residence						
Urban	89 (29)/79 (25.7)	84 (27.4)/84 (27.4)	1.13 (0.73-1.73)	0.585	1.17 (0.76-1.81)	0.479
Rural	77 (25.1)/62 (20.2)	53 (17.2)/86 (28)	2.02 (1.25-3.25)	0.004	2.35 (1.42-3.90)	0.001

[1]Adjusted for age, sex, smoking status, residence, hypertension, and diabetes. OR: Odds ratio.

Table 4 Associations between variant *ITGA2* genotypes and clinicopathological characteristics of gastric cancer[1]

Variable	CT + TT	CC	Crude OR (95% CI)	*P* value	Adjusted OR[2] (95% CI)	*P* value
Tumor differentiation						
Well	42	42	1		1	
Moderate	65	69	0.94 (0.55-1.63)	0.830	0.94 (0.54-1.63)	0.828
Poor	55	27	2.04 (1.09-3.82)	0.027	2.21 (1.12-4.38)	0.022
Depth of tumor infiltration						
T1	24	30	1		1	
T2	21	17	1.54 (0.67-3.56)	0.308	1.76 (0.73-4.25)	0.208
T3	34	40	1.06 (0.53-2.15)	0.866	1.10 (0.52-2.32)	0.797
T4	83	51	2.03 (1.07-3.86)	0.030	2.12 (1.10-4.07)	0.024
Lymph node metastasis						
Negative	59	51	1		1	
Positive	103	87	1.02 (0.64-1.64)	0.923	0.98 (0.61-1.58)	0.942
Localization						
Cardia	38	41	1		1	
Non-cardia	128	100	1.38 (0.83-2.31)	0.217	1.38 (0.81-2.35)	0.231

[1]Data of seven plaintively treated cases were not obtained for the inoperable tumors; [2]Adjusted for age, sex, smoking status, residence, hypertension, and diabetes. OR: Odds ratio.

ity in a Chinese population. We found that the polymorphism may be associated with an increased risk of gastric cancer, differentiation and invasion of gastric cancer.

It has been reported that integrin α2β1 is one of the key factors accelerating tumor progression and metastasis in various types of cancers[15-21,31]. Koike *et al*[20] found that the α2 integrin was expressed in the intestinal-type and diffuse-type gastric carcinoma cells, and invasion through basement membrane and type I collagen gel was inhibited by anti-α2 integrin monoclonal antibody, indicating that the α2 integrin plays an important role in invasion of gastric carcinoma cells. Another study conducted by Lee *et al*[32] elucidated the potential mechanisms underlying the spreading and invasiveness of gastric carcinoma

cells, the integrin transduces signaling directly *via* engagements with extracellular matrix proteins, thereby leading to the regulation of downstream intracellular signaling molecules. It also functions in collaborative (indirect) signaling, in which integrins cosignal with other membrane receptor-mediated signal pathways, e.g. growth factor receptors, G-protein coupled receptors or the transforming growth factor β1 signaling pathway.

The *ITGA2* gene C807T polymorphism is associated with integrin density, but the precise molecular mechanism remains unclear. It is a silent polymorphism in codon 253 (Phe253Phe) and does not cause an altered structure of the integrin molecule, but in linkage disequilibrium with a yet unknown functional polymorphism affecting *ITGA2* expression. Another explanation could be a direct effect on the stability of the *ITGA2* mRNA, which resulted in a change of the amount of integrin protein being expressed.

Limited studies have reported the association between the polymorphism in *ITGA2* gene and cancer risks, although the results remain inconsistent[27-29]. Gerger *et al*[27] found that the *ITGA2* gene C807T polymorphism was associated with reduced colorectal cancer risk (OR = 0.77, 95% CI = 0.64-0.94, P = 0.011). In their another case-control study, they found that carriers of the most common *ITGA2* haplotype (807C_1648G) had a decreased risk for breast cancer (OR = 0.72, 95% CI = 0.53-0.98)[28]. Nevertheless, Ayala *et al*[29] reported that no association was observed between the *ITGA2* gene C807T polymorphism and breast cancer risk.

Based on these studies, we conducted this hospital-based case-control study to investigate the association between the *ITGA2* gene C807T polymorphism and the risk of gastric cancer in a Chinese population. The frequency of the variant T allele in our control group was 26.22%, which was similar to that in another study in a Chinese Taiwanese population (27.1%)[33] and HapMap database (26.7% for Han Chinese). Our results showed that the variant genotypes had a 57% increased risk of developing gastric cancer.

In the subgroup analyses, we found that the polymorphism was associated with the increased risk of gastric cancer in the subgroup of the subjects aged > 58 years, but not in the subjects aged ≤ 58 years. Milne *et al*[34] indicated that carcinogenesis is considered as accumulation of genetic events, and gastric cancer has a steep slope for age-specific increase in incidence. The increased risk observed in older subjects implies that the *ITGA2* genotype effects tend to be age specific. The polymorphism may contribute to elevated integrin α2β1 levels beyond the age of 58, thus representing a significant risk factor in this age group. However, this is just a hypothesis to interpret the results of our study, and further research is warranted to clarify the mechanism underlying the interaction between the polymorphism and age.

Similarly, in statistical analyses stratified by smoking status, a significant association was observed in nonsmokers, but not in smokers. Tobacco smoking has been undoubtedly accepted as a independent risk factor for gastric cancer[3,5,6]. The association between the polymorphism and gastric cancer risk could be masked by the overwhelming accumulated exposure to tobacco carcinogens in smokers so that the association is more evident in nonsmokers.

We also noted that increased risk of gastric cancer associated with the polymorphism was pronounced in rural subjects, but not in urban subjects. It has been suggested that the genetic differences have their strongest effects under conditions of low environmental pollution[9,35]. Our results plausibly agree with the hypothesis that the genetic effects might be more prominent in the better environments of rural areas[9]. However, this result may be found accidentally, further studies are needed to verify it.

In addition, in the stratified analyses by clinicopathological characteristics of gastric cancer, we observed a significant correlation of the variant genotypes with poorly differentiated tumors. Similarly, Langsenlehner *et al*[28] suggested that a histological grade of 3 or 4 was found more often in breast cancer subjects with TT genotype. The result is consistent with our findings. In contrast, Yasoshima *et al*[21] found no correlation between the expression of integrin α2β1 and histopathological features such as the histological grade, stromal type, and infiltrating growth pattern. We also observed the significant association of the variant genotypes with adjacent organ invasions. Several studies have suggested that integrin α2β1 was closely associated with invasion and metastasis in gastric cancer or tumor cells[18-21,31]. These studies might explain the result we observed. However, no correlation between the polymorphism and lymph node metastasis or location of gastric cancer was found in the stratified analyses. Because the number of cases in the subgroups was relatively small and clinicopathological variables were obtained at the time of diagnosis, our findings should be interpreted with caution before being confirmed in further studies. Thus, large-sized studies which prospectively follow up the clinical outcome, especially the survival rate, may be required to elucidate the association between the polymorphism and gastric cancer progression as well as prognosis.

Some limitations may exist in the present study. First, our study is a hospital-based case-control study, so we can not rule out the selection and recall bias. Nevertheless, the T allele frequency in control subjects is quite similar to that reported in HapMap database for Han Chinese in Beijing (0.262 in our study *vs* 0.267 in HapMap database) and the genotype distributions of cases and controls were in Hardy-Weinberg equilibrium. The second limitation is our relatively small sample size, with 307 cases and 307 controls. So gene-environment interactions may have been underpowered in stratified analyses. However, our preliminary data certainly provides some interesting information and valuable guidance for the future studies in this area. Finally, no enough information on *H. pylori* status was available in cases and controls, because of the ethical reasons.

In conclusion, the present study provides evidence that the *ITGA2* gene C807T polymorphism is associated with an increased risk of gastric cancer in a Chinese population. The association is especially evident in older individuals, non-smokers and rural subjects, and the variant genotypes may also play a role in the differentiation and invasion of gastric cancer, indicating that the polymorphism may be a useful diagnostic marker for genetic susceptibility to gastric cancer. Further studies with larger samples and functional studies are needed to elucidate the role of genetic variations in *ITGA2* and the pathogenesis of gastric cancer.

COMMENTS

Background

Integrin α2β1 has been considered as a key factor for cancer development and progression, especially in gastric cancer. Polymorphisms in *ITGA2* gene is responsible for the expression of integrin α2β1. Recent studies indicated that the *ITGA2* gene C807T polymorphism was associated with cancer risk.

Research frontiers

Using polymerase chain reaction-restriction fragment length polymorphism method, this study explored the relationship between *ITGA2* C807T polymorphism and gastric cancer risk.

Innovations and breakthroughs

The results suggest that the polymorphism is associated with the elevated risk of gastric cancer in a Chinese population, especially in older individuals aged > 58 years, nonsmokers, and rural subjects. Further analyses revealed that the polymorphism may play a role in differentiation and invasion of gastric cancer.

Applications

The results of this study could help further understand the genetic determinants of gastric cancer. The polymorphism may be a useful diagnostic marker for genetic susceptibility to gastric cancer.

Terminology

Integrins are members of a family of cell-surface heterodimeric proteins that mediate cell-matrix and cell-cell interactions. Single nucleotide polymorphisms represent a natural genetic variability at a high density in the human genome, which are responsible for the inter-individual variation and diversity. They have been recently considered as the main genetic elements involved in the development of common and complex diseases, including various cancers.

Peer review

The current study was designed, processed and concluded well, deserving publication.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2867

World J Gastroenterol 2011 June 21; 17(23): 2867-2872
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Log-normal censored regression model detecting prognostic factors in gastric cancer: A study of 3018 cases

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Supported by the Gastric Cancer Laboratory and Pathology Department of Chinese Medical University, Shenyang, China; the Science and Technology Program of Shenyang, No. 1081232-1-00
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Received: October 23, 2010 Revised: January 11, 2011
Accepted: January 18, 2011
Published online: June 21, 2011

Abstract

AIM: To investigate the efficiency of Cox proportional hazard model in detecting prognostic factors for gastric cancer.

METHODS: We used the log-normal regression model to evaluate prognostic factors in gastric cancer and compared it with the Cox model. Three thousand and eighteen gastric cancer patients who received a gastrectomy between 1980 and 2004 were retrospectively evaluated. Clinic-pathological factors were included in a log-normal model as well as Cox model. The akaike information criterion (AIC) was employed to compare the efficiency of both models. Univariate analysis indicated that age at diagnosis, past history, cancer location, distant metastasis status, surgical curative degree, combined other organ resection, Borrmann type, Lauren's classification, pT stage, total dissected nodes and pN stage were prognostic factors in both log-normal and Cox models.

RESULTS: In the final multivariate model, age at diagnosis, past history, surgical curative degree, Borrmann type, Lauren's classification, pT stage, and pN stage were significant prognostic factors in both log-normal and Cox models. However, cancer location, distant metastasis status, and histology types were found to be significant prognostic factors in log-normal results alone. According to AIC, the log-normal model performed better than the Cox proportional hazard model (AIC value: 2534.72 *vs* 1693.56).

CONCLUSION: It is suggested that the log-normal regression model can be a useful statistical model to evaluate prognostic factors instead of the Cox proportional hazard model.

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Key words: Gastric cancer; Log normal regression model; Cox proportional hazard model; Prognostic factors

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, Korea

Wang BB, Liu CG, Lu P, Latengbaolide A, Lu Y. Log-normal censored regression model detecting prognostic factors in gastric cancer: A study of 3018 cases. *World J Gastroenterol* 2011; 17(23): 2867-2872 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2867.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2867

INTRODUCTION

The survival of patients with gastric cancer has recently been improved because of early detection, rational lymphadenectomy and several therapeutic modalities[1,2]. However, gastric cancer still remains the second leading cause

of cancer mortality in the world. It is acknowledged that surgery and systemic chemotherapy can clearly improve the survival of patients with gastric cancer[3,4]. However, a sensible treatment option must be fundamentally based on the current evaluation of prognostic factors, so a rational method to evaluate the prognostic factors is very important in establishing therapeutic strategies and evaluate prognosis.

Survival analysis is a branch of statistics which deals with death in biological organisms and failure in mechanical systems. The Cox model is the standard tool for assessing the effect of prognostic factors; however, there may be substantive differences in the estimated prognosis obtained by the Cox model rather than a log-normal model[5]. The Cox model is semiparametric, in that the baseline hazard takes on no particular form[6]. In contrast to Cox, a link to parametric survival models comes through alternative functions for the baseline hazard. In this case, one can let the baseline hazard be a parametric form such as log-normal. It is acknowledged that most of studies used the Cox proportional hazard model to find the relation between survival time and covariates of patients with gastric cancer[7-9]. On the other hand, some studies reported that log-normal regression could estimate the parameter more efficiently than the Cox model[5]. However, the efficiency of log-normal regression was still controversial.

The aim of this retrospective study was to elucidate the factors affecting the survival of patients with GC using log-normal regression, and to compare these results with the Cox model.

MATERIALS AND METHODS

Patients

In this study, three thousand and eighteen cases with gastric cancer were selected on whom an operation was performed at the China Medical University between 1980 and 2004. The selection criteria for inclusion were as follows: (1) an operation was performed; (2) lymph nodes were dissected and then pathologically examined; and (3) the patient medical records were available. All patients were periodically followed up through post letters, and/or telephone interviews with patients and their relatives. Clinical, surgical and pathological findings, and all follow-up information were collected and recorded in a database, and 5-year survival rate was calculated. The study protocol was approved by the Ethics Committee of China Medical University.

Reference standard

Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected lymph nodes was determined by surgeons who reviewed the excised specimens after surgery based on the Japanese Classification of Gastric Carcinoma[10]. Accordingly, lymphadenectomy was classified as D1, dissection of all the Group 1 lymph nodes; D2, dissection of all Group 1 and Group 2 lymph nodes; and D3, dissection of all the Group 1, Group 2 and Group 3 lymph nodes. pN category was defined as pN0 (no metastatic lymph node), pN1 (1-6 metastatic lymph nodes), pN2 (7-15 metastatic lymph nodes) and pN3 (> 15 metastatic lymph nodes), according to the 5th Edition of UICC[11]. The location of tumors was defined as upper, middle and lower third gastric cancer, according to JCGC[10] and the histological grade was defined as poorly differentiated, moderately differentiated and well differentiated, according to the latest World Health Organization (WHO) classification[12]. The Borrmann type was defined as Borrmann Ⅰ, Borrmann Ⅱ, Borrmann Ⅲ and Borrmann Ⅳ, according to JCGC[10]. The histological type was determined according to Lauren's classification.

Statistical analysis

All data were analyzed using STAT statistics software (Version 10.0, Stata Corp LP). Clinic-pathologic factors were entered to a log-normal censored regression, as well as a Cox proportional hazard model in univariate and multivariate analysis in order to find the prognostic factors. The term of relative risk (RR) was used to interpret the risk of death in parametric results and the term of Akaike Information Criterion (AIC) was employed to compare the efficiency of models. Disease-specific survival was analyzed using the Kaplan-Meier method. The log-rank test was used to analyze survival differences. Lower AIC indicates better likelihood. A P value of less than 0.05 was considered statistically significant.

RESULTS

Clinic-pathological characteristics of patients with gastric cancer

The male-to-female ratio among the 3018 patients enrolled was 2.74:1 and the mean age was 57.54 years (range: 19 to 90 years) at operation. 269, 1362 and 608 cases received D1, D2 and more than D2 lymph node dissection respectively. In addition, six hundred and fifty seven cases received palliative surgery. From 3018 cases, a total of 46081 lymph nodes were removed and examined, and the mean number of examined lymph nodes was 15.27. One thousand six hundred and forty three cases were observed lymph node metastasis. Thus, the incidence of lymph node metastasis was 54.44%. The last follow-up was Jan 1, 2009, with a total follow-up rate of 70.68%. More clinic-pathologic factors are shown in Table 1.

Multivariate analysis of prognostic factors in gastric cancer

Univariate analysis indicated that age at diagnosis, past history, cancer location, distant metastasis status, surgical curative degree, combined other organ resection, Borrmann type, Lauren's classification, pT stage, total dissected nodes and pN stage were prognostic factors in both log-normal and Cox models. In the final multivariate model, age at diagnosis, past history, surgical curative degree, Borrmann type, Lauren's classification pT stage, and pN stage were significant prognostic factors in both

Table 1 Clinicopathological characteristics of 3018 gastric cancers included in the study *n* (%)

Variable	Subgroups	Frequency
Gender ratio	Male	2211 (73.26)
	Female	807 (26.74)
Age at diagnosis (mean ± SD; yr)		57.54 ± 11.24
Past history		
	Without	2234 (74.02)
	With	784 (25.98)
Family history	Without	2467 (81.74)
	With	551 (18.26)
Cancer number	Single	2883 (96.65)
	Multiple	100 (3.35)
Cancer location	Lower stomach	1873 (62.64)
	Middle stomach	492 (16.46)
	Upper stomach	355 (11.87)
	Total stomach	270 (9.03)
Distant metastasis status	Without	2540 (85.04)
	With	447 (14.96)
Maximum tumor diameter (mean ± SD, cm)		5.85 ± 3.30
Surgical curative degree	Absolutely radical	1396 (49.73)
	Relatively radical	809 (28.82)
	Palliative	602 (21.45)
Lymph node dissection	More than D2	608 (20.99)
	D2	1362 (47.03)
	D1	269 (9.29)
	Palliative surgery	657 (22.69)
Combined other organ resection	Without	2016 (76.80)
	With	609 (23.20)
Histological type	Well differentiated	755 (27.43)
	Middle differentiated	382 (13.88)
	Poor differentiated	1615 (58.69)
Borrmann classification	Ⅰ	70 (2.98)
	Ⅱ	426 (18.15)
	Ⅲ	1571 (66.94)
	Ⅳ	280 (11.93)
Lauren classification	Intestinal type	1170 (43.89)
	Diffuse type	1496 (56.11)
pT stage	pT1	328 (11.89)
	pT2	1486 (53.88)
	pT3	737 (26.72)
	pT4	207 (7.51)
Total dissected lymph node (mean ± SD)		15.27 ± 13.11
Pathological lymph node status	pN0	1375 (45.56)
	pN1	1039 (34.43)
	pN2	432 (14.31)
	pN3	172 (5.70)

log-normal and Cox models. However, cancer location, distant metastasis status and histology types were found as significant prognostic factors in log-normal results alone (Table 2). According to AIC, the log-normal model performed better than the Cox proportional hazard model (AIC value: 2534.72 *vs* 1693.56) (Table 3).

Survival outcomes

Overall, the 5-year disease-specific survival rate was 29.57%. The survival was observed significantly different in patients with different cancer locations (5-year disease-specific survival rate, L tumor *vs* M tumor *vs* U tumor *vs* T tumor: 33.11% *vs* 30.46% *vs* 25.66% *vs* 7.59%, χ^2 = 190.27, P = 0.000) (Figure 1). In addition, the cases with distant metastasis received a poorer prognosis than those without distant metastasis (5-year disease-specific survival

Table 2 Univariate model of Cox and log normal regression with prognostic factors

	HR (95% CI)	
	Cox	Log normal
Sex		
Male	0.953 (0.843-1.078)	0.925 (0.818-1.046)
Female	1.00	1.00
Age at diagnosis	1.015[1] (1.009-1.020)	1.016[1] (1.011-1.021)
Past history		
Without	1.00	1.00
With	0.715[1] (0.631-0.831)	0.694[1] (0.612-0.787)
Family history		
Without	1.00	1.00
With	0.871 (0.752-1.009)	0.875 (0.754-1.014)
Cancer number		
Single	1.00	1.00
Multiple	0.870 (0.622-1.218)	0.924 (0.661-1.294)
Cancer location		
Lower third	1.00	1.00
Middle third	1.181[1] (1.017-1.373)	1.302[1] (1.233-1.374)
Upper third	1.436[1] (1.212-1.701)	1.695[1] (1.429-1.897)
Total stomach	2.464[1] (2.062-2.944)	2.207[1] (2.011-2.677)
Distant metastasis		
Absent	1.00	1.00
Present	2.554[1] (2.194-2.973)	2.596[1] (2.227-3.027)
Surgical curative degree		
Absolutely radical	1.00	1.00
Relatively radical	1.835[1] (1.593-2.114)	2.159[1] (2.020-2.308)
Palliative	4.236[1] (3.714-4.832)	4.661[1] (4.214-4.759)
Lymph node dissection		
> D2	1.00	1.00
D2	0.989 (0.859-1.138)	1.536[1] (1.458-1.619)
D1	1.056 (0.853-1.307)	2.359[1] (2.121-2.574)
< D1	3.310[1] (2.854-3.839)	3.624[1] (3.231-3.862)
Combined other organ resection		
Without	1.00	1.00
With	1.981[1] (1.749-2.245)	2.070[1] (1.825-2.348)
Histologic types		
Well differentiated	1.00	1.00
Middle differentiated	0.706[1] (0.592-0.843)	0.976 (0.918-1.039)
Poor differentiated	0.918 (0.814-1.036)	0.952 (0.897-1.011)
Borrmann classification (*n* (%))		
Ⅰ	1.00	1.00
Ⅱ	1.005 (0.892-1.340)	0.981 (0.894-1.019)
Ⅲ	1.247[1] (1.173-1.638)	1.176[1] (1.074-1.293)
Ⅳ	2.512[1] (1.842-3.075)	2.610[1] (2.416-3.153)
Lauren classification (*n* (%))		
Intestinal type	1.00	1.00
Diffuse type	1.245[1] (1.082-1.184)	1.171[1] (1.015-1.384)
pT stage		
pT1	1.00	1.00
pT2	2.936[1] (2.299-3.751)	1.787[1] (1.666-1.916)
pT3	4.305[1] (3.357-5.522)	3.193[1] (3.066-3.321)
pT4	7.697[1] (5.759-10.287)	5.707[1] (5.579-5.833)
Total dissected nodes	0.993[1] (0.988-0.998)	0.994[1] (0.988-0.998)
pN stage		
pN0	1.00	1.00
pN1	1.555[1] (1.372-1.764)	1.633[1] (1.533-1.740)
pN2	2.510[1] (2.133-2.953)	2.667[1] (2.561-2.772)
pN3	3.669[1] (2.901-4.640)	4.355[1] (4.249-4.460)

[1]Statistically significant (P < 0.05). HR: Hazard radio; CI: Confidence interval.

Table 3 Multivariate model of Cox and log normal regression with prognostic factors (full model and final model)

	Cox HR (95% CI)		Log normal HR (95% CI)	
	Full model (AIC = 1508.49)	Final model (AIC = 2534.72)	Full model (AIC = 913.34)	Final model (AIC = 1693.56)
Sex				
Male	0.91 (0.801-1.034)		0.886 (0.781-1.005)	
Female	1.00			
Age at diagnosis	1.014[1] (1.009-1.02)	1.011[1] (1.006-1.017)	1.015[1] (1.010-1.021)	1.015[1] (1.009-1.020)
Past history				
Without	1.00	1.00	1.00	1.00
With	0.840[1] (0.738-0.955)	0.858[1] (0.755-0.975)	0.813[1] (0.716-0.914)	0.809[1] (0.713-0.919)
Family history				
Without	1.00		1.00	1.00
With	0.957 (0.8254-1.11)		0.967 (0.833-1.234)	
Cancer number				
Single	1.00		1.00	
Multiple	1.21 (0.861-1.701)		1.312 (1.935-1.840)	
Cancer location				
Lower third	1.00		1.00	1.00
Middle third	1.033 (0.885-1.205)		1.135[1] (1.073-1.199)	1.129[1] (1.069-1.194)
Upper third	1.406[1] (1.173-1.686)		1.288[1] (1.224-1.353)	1.277[1] (1.211-1.338)
Total stomach	1.466[1] (1.214-1.771)		1.462[1] (1.365-1.558)	1.439[1] (1.343-1.535)
Distant metastasis				
Absent	1.00		1.00	1.00
Present	1.21[1] (1.013-1.447)		1.205[1] (1.011-1.437)	1.198[1] (1.009-1.424)
Surgical curative degree				
Absolutely radical	1.00	1.00	1.00	1.00
Relatively radical	1.389[1] (1.197-1.611)	1.383[1] (1.194-1.601)	1.724[1] (1.537-1.934)	1.672[1] (1.538-1.817)
Palliative	3.889[1] (2.583-5.855)	2.687[1] (2.316-3.116)	2.972[1] (2.770-3.174)	2.796[1] (2.653-2.938)
Lymph node dissection				
> D2	1.00		1.00	
D2	0.908 (0.784-1.051)		0.967 (0.892-1.049)	
D1	0.901 (0.712-1.14)		0.935 (0.855-1.015)	
< D1	0.607[1] (0.395-0.935)		0.904 (0.784-1.024)	
Combined other organ resection				
Without	1.00		1.00	
With	1.406[1] (1.227-1.61)		1.447[1] (1.264-1.657)	
Histologic types				
Well differentiated	1.00		1.00	1.00
Middle differentiated	1.056 (0.878-1.271)		1.110[1] (1.042-1.183)	1.120[1] (1.051-1.193)
Poor differentiated	1.179[1] (1.035-1.343)		1.232[1] (1.160-1.304)	1.254[1] (1.182-1.327)
Borrmann classification				
Ⅰ	1.00	1.00	1.00	1.00
Ⅱ	1.142 (0.957-1.319)	1.201 (1.068-1.433)	1.018 (0.943-1.106)	1.015 (0.941-1.102)
Ⅲ	1.315[1] (1.113-1.672)	1.394[1] (1.205-1.741)	1.246[1] (1.052-1.539)	1.241[1] (1.047-1.533)
Ⅳ	2.126[1] (1.758-3.119)	2.253[1] (1.827-3.284)	2.530[1] (2.376-2.713)	2.526[1] (2.372-2.708)
Lauren classification				
Intestinal type	1.00		1.00	1.00
Diffuse type	1.131[1] (1.012-1.358)		1.307[1] (1.154-1.528)	1.302[1] (1.148-1.523)
pT stage				
pT1	1.00	1.00	1.00	1.00
pT2	1.851[1] (1.431-2.394)	1.971[1] (1.528-2.542)	1.195[1] (1.102-1.297)	1.193[1] (1.100-1.294)
pT3	1.981[1] (1.511-2.598)	2.19[1] (1.678-2.858)	1.428[1] (1.328-1.527)	1.423[1] (1.324-1.522)
pT4	2.344[1] (1.699-3.235)	2.501[1] (1.821-3.435)	1.706[1] (1.557-1.855)	1.697[1] (1.549-1.847)
Total dissected nodes	0.987[1] (0.981-0.993)		0.988[1] (0.982-0.993)	
pN stage				
pN0	1.00	1.00	1.00	1.00
pN1	1.281[1] (1.123-1.461)	1.266[1] (1.11-1.444)	1.507[1] (1.393-1.620)	1.500[1] (1.387-1.622)
pN2	2.139[1] (1.783-2.566)	2.095[1] (1.749-2.51)	2.271[1] (2.151-2.391)	2.250[1] (2.130-2.370)
pN3	3.24[1] (2.446-4.292)	3.325[1] (2.52-4.386)	3.422[1] (3.242-3.602)	3.375[1] (3.196-3.554)

[1]Statistically significant (< 0.05); HR: Hazard radio; CI: Confidence interval; AIC: Akaike Information Criterion.

rate, 33.50% *vs* 7.56%, χ^2 = 372.21, P = 0.000) (Figure 2). Furthermore, the cases with different histologic types were investigated with a different prognosis (5-year disease-specific survival rate, well differentiated tumors *vs* middle differentiated tumors *vs* poor differentiated tumors: 39.27% *vs* 29.67% *vs* 25.03%, χ^2 = 12.37, P = 0.002) (Figure 3).

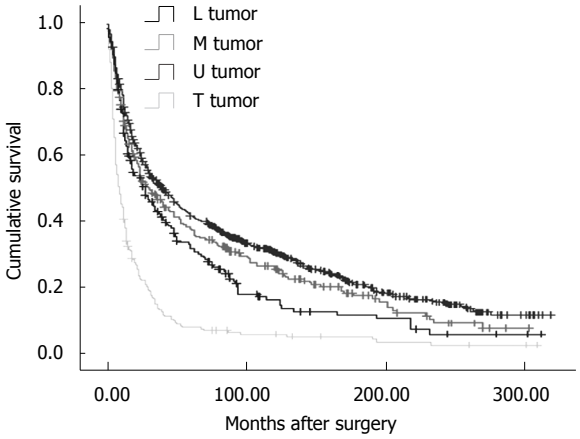

Figure 1 Disease-specific survival analysis according to cancer locations (χ^2 = 190.27, *P* = 0.000, Log Rank test). L tumor: Lower third tumors; M tumor: Middle third tumors; U tumor: Upper third tumors; T tumor: Tumor occupied the total stomach.

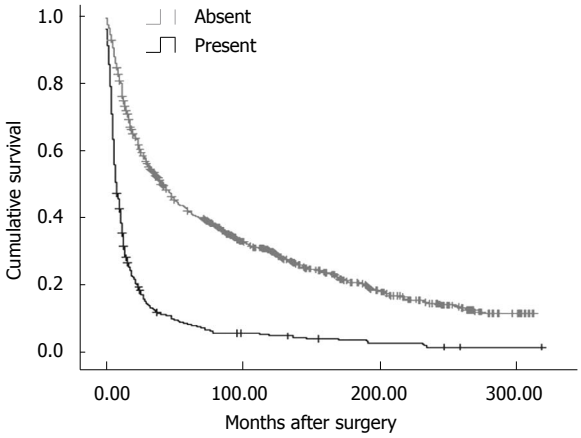

Figure 2 Disease-specific survival analysis according to distant metastasis status (χ^2 = 372.21, *P* = 0.000, Log Rank test).

DISCUSSION

There were several studies that have investigated the factors influencing prognosis[13,14]. The conclusions of the reports were controversial, though most of them used the Cox proportional hazard model to find the relation between survival time and patient characteristics, and clinical and pathological factors in patients with gastric cancer.

After evaluating the clinic-pathological factors of 738 patients, Kulig *et al*[7] reported that patient age, depth of tumor infiltration, tumor location, and metastatic node ratio were identified as independent prognostic factors in a Cox proportional hazards model. In addition, Shiraishi *et al*[15] reported that independent prognostic factors of gastric cancer were serosal invasion, extragastric lymph node metastasis and liver metastasis, but survival was not significantly associated with any of the patient factors or operation factors, including the extent of lymph node dissection. In our study, age at diagnosis, past history, surgical curative degree, Borrmann type, Lauren's classification, pT stage, and pN stage were significant prognostic factors in Cox models. There was a small difference between our study and other reports. In the final model of log-normal analysis, we investigated that cancer location, distant metastasis and histologic types were significantly related to the survival. The outcomes were also verified by disease-specific survival analysis. However, the association between above factors and survival were not observed. In log-normal analysis, Pourhoseingholi *et al*[5] observed that distant metastasis, histology type and pT stage were significant prognostic factors after retrospectively studying 746 Iranian patients. Moreover, distant metastasis was a significant prognostic factor only in log-normal analysis, not in the Cox model.

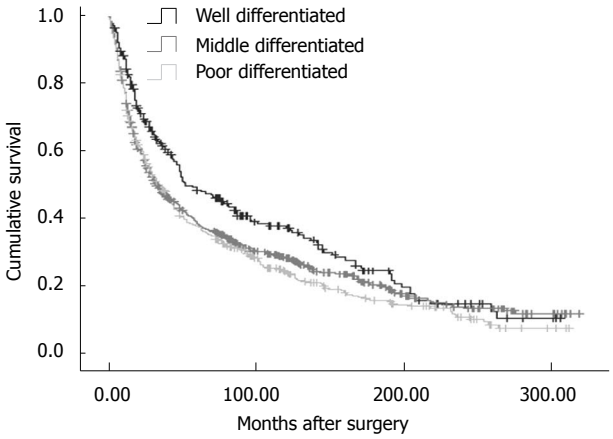

Figure 3 Disease-specific survival analysis according to histologic types (χ^2 = 12.37, *P* = 0.002, Log Rank test).

Compared to the Cox model, the evaluation criteria in our study indicated log-normal regression was more powerful not only in the full model, but also in the final one. In the final model, the selected prognostic factors in the log-normal model were different compared to those in the Cox model. Furthermore, the data strongly supported the log-normal regression in the full and final models, and might lead to more precise results as an alternative for Cox.

In conclusion, according to the results of our study, age at diagnosis, past history, cancer location, distant metastasis status, surgical curative degree, combined other organ resection, histology types, Borrmann type, Lauren's classification, pT stage, total dissected nodes and pN stage were significant prognostic factors of gastric cancer. It is suggested that log-normal regression model can be a useful statistical model to evaluate prognostic factors instead of the Cox proportional hazard model.

ACKNOWLEDGMENTS

The preparation of this manuscript has been assisted by a professional editing company, Medjaden.

COMMENTS

Background

Most of studies used the Cox proportional hazard model to find the relation between survival time and covariates of patients with gastric cancer (GC). On the other hand, some studies reported that log-normal regression could estimate

the parameter more efficiently than the Cox model. However, the efficiency of log-normal regression was still controversial.

Research frontiers

In this retrospective study, the authors elucidated the factors affecting the survival of patients with GC using log-normal regression, and to compare these results with the Cox model.

Applications

It is suggested that log-normal regression model can be a useful statistical model to evaluate prognostic factors instead of the Cox proportional hazard model.

Peer review

Overall the study was well designed, performed, and analyzed. The very minor, but important parameters should be added in analysis.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2873

World J Gastroenterol 2011 June 21; 17(23): 2873-2876
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Diaphragm disease compared with cryptogenic multifocal ulcerous stenosing enteritis

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Received: August 16, 2010 Revised: December 10, 2010
Accepted: December 17, 2010
Published online: June 21, 2011

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Key words: Non-steroidal anti-inflammatory agents; Enteritis; Gastrointestinal hemorrhage; Small intestine; Capsule endoscopy

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Chung SH, Jo Y, Ryu SR, Ahn SB, Son BK, Kim SH, Park YS, Hong YO. Diaphragm disease compared with cryptogenic multifocal ulcerous stenosing enteritis. *World J Gastroenterol* 2011; 17(23): 2873-2876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2873.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2873

Abstract

As the use of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) increases, so too do gastrointestinal ulcers, bleeding, perforation and obstruction. Diaphragm disease of the small intestine is formed by submucosal fibrosis and destruction of lamina muscularis due to chronic ulceration, which corresponds to the most severe stage of NSAID enteropathy. It may lead to stricture of the small intestine. If such ulcerations and strictures in the small intestine are multiple, differential diagnosis is between diaphragm disease and cryptogenic multifocal ulcerous stenosing enteritis (CMUSE), because the gross findings of diaphragm disease are similar to those of CMUSE. We report a rare case of diaphragm disease caused by NSAID. It has been finally confirmed by capsule endoscopy and the origin of chronic obscure gastrointestinal bleeding was found to be multiple ulcers and strictures in the small intestine. After operation, we diagnosed the patient with diaphragm disease rather than CMUSE.

INTRODUCTION

The complications of non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, antiplatelet agents, and antithrombotic agents are known to include gastrointestinal ulceration, bleeding, perforation and obstruction[1]. NSAID-induced mucosal injury can occur to the upper and lower gastrointestinal tract. The incidence of NSAID-induced enteropathy is not less than that of gastropathy. Diaphragm disease is the most severe stage of NSAID enteropathy. In ambulatory patients who took NSAIDs for > 3 mo because of arthritis, 70% had small intestinal ulcers and erosions by capsule endoscopy[2]. The diaphragm is formed by chronic fibrosis and destruction of lamina muscularis with chronic ulceration, and causes obstruction of the lumen in the small bowel[3]. However, diaphragm disease is a rare diagnosis.

Cryptogenic multifocal ulcerous stenosing enteritis (CMUSE) has multiple ulcerations and strictures in the small intestine; it is similar to diaphragm disease in terms of gross findings, and it is characterized by a chronic and

relapsing clinical course, and is more common in middle-aged patients.

We report a case of diaphragm disease in the small bowel that presented with obscure overt bleeding due to NSAID use, which was detected by capsule endoscopy. We review the differential diagnosis with CMUSE through histological findings and clinical course after surgery.

CASE REPORT

A 79-year-old woman was hospitalized for a low level of hematochezia. At admission, her hemoglobin level was 8.9 g/dL. She had also taken 20 mg piroxicam, 275 mg naproxen and 25 mg diclofenac daily for 4 years because of degenerative joint diseases. She had been hospitalized for iron deficiency anemia 5 years ago and there was no active bleeding focus upon esophagogatroduodenoscopy (EGD) and colonoscopy at that time. It was recommended that she undergo capsule endoscopy, however, she refused and was discharged.

After hospitalization, she had EGD and abdominal computed tomography (CT) to find the focus of her bleeding. No bleeding foci were revealed by EGD and abdominal CT. She had no more hematochezia after hospitalization. On day 3 of hospitalization, capsule endoscopy was performed. There were multiple, concentric circular ulcerations, with luminal narrowing in the jejunum and ileum, with many variable-sized erosions and bleeding stigmata in the ulcerations (Figure 1). The capsule in the small bowel was not excreted until 8 d after capsule endoscopy, even though she could have a meal. According to the results of capsule endoscopy, we need to diagnose differentially between NSAID-induced enteropathy and CMUSE, because of the gross similarities, such as multiple ulcerations and strictures in the small intestine. Even though the patient was an elderly woman and took NSAIDs, she was not diagnosed with NSAID-induced enteropathy because of the similar endoscopic findings to CMUSE. To confirm the diagnosis, histological examination was needed. She complained of chronic abdominal pain for 2 years, therefore, we could diagnose by capsule endoscopy that she had symptomatic multiple strictures.

To relieve the chronic abdominal pain caused by the multiple strictures in the small bowel, and to remove the retained capsule and evaluate the exact cause of the multiple ulcerations and strictures, we performed segmental resection of the ileum (Figure 2) instead of double balloon enteroscopy. Histological findings in the ileum (Figure 3) revealed typical multiple, circular ulcerations and a mucosal diaphragm, which suggested the most severe complications of chronic NSAID use. These mucosal diaphragms indicate chronic ulcers, submucosal fibrosis, reactive epithelial change, and chronic inflammation. After discharge, she had no more gastrointestinal bleeding and abdominal pain and took misoprostol at the outpatient clinic with close observation for > 1 year.

DISCUSSION

Diaphragm disease is the pathognomic characteristic of NSAID enteropathy, and was named by Bjarnason *et al*[4]. They described the clinicopathological features of NSAID-induced stricture as diaphragm disease. The histopathological characteristics of diaphragm disease are: superficial ulceration at the apex of the villi; circumferential ring-like stricture; multiple, short segment annular strictures; transmural inflammation; and submucosal fibrosis[5].

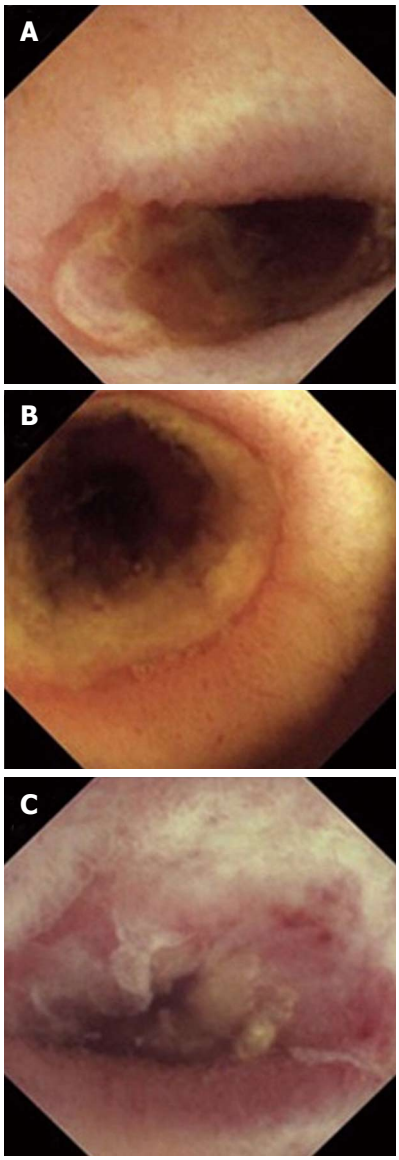

Figure 1 Capsule endoscopic findings. A: There were concentric ulcerations with luminal narrowing; B: There were many variable-sized erosions and bleeding stigmata in the ulcerations; C: There was a totally obstructed lesion in which the capsule was suspected to be retained.

Several other authors have reported diaphragm disease in patients taking NSAIDs, by enteroscopy[6] and after laparotomy[5,7]. The first diagnosis of small intestinal diaphragm disease after use of NSAID through capsule endoscopy was reported by Yousfi *et al*[3]. In their study with capsule endoscopy, there were multiple small-intestinal strictures where the capsule was retained. Thus, exploratory laparoscopy was required to remove the retained capsule, as in our case.

After long-term use of NSAIDs for > 20 years, the small intestine shows fibrotic constriction and thickened

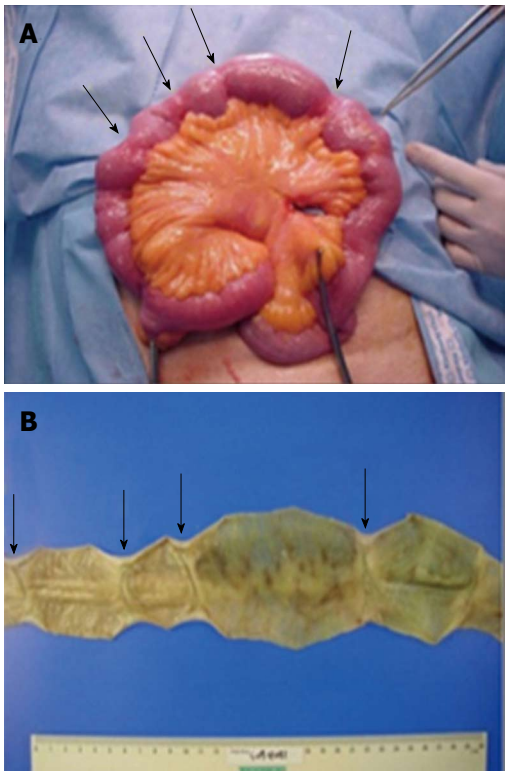

Figure 2 Specimen from segmental resection of the ileum. A: A capsule was located at 60 cm up from the terminal ileum. Forceps and hand point out the location in the ileum where the capsule was retained. There were multiple strictures in the ileum (black arrows); B: In longitudinal section, there were multiple thin, web-like mucosal septa that caused abrupt luminal narrowing in the small intestine (black arrows).

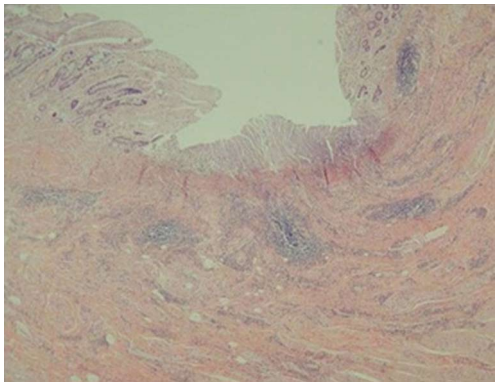

Figure 3 Histological findings of the ileum. The mucosal diaphragm reveals surface erosion or ulceration, submucosal fibrosis, reactive epithelial change, and chronic inflammation (hematoxylin and eosin stain, 40 ×).

hyperemic mucosa that lead to obstruction through local damage and healing process[8].

In NSAID-induced enteropathy, capsule endoscopy shows circumferential ulcerations, erosions and multifocal strictures[3,9]. Capsule endoscopy is a useful option to establish the etiology of obscure overt gastrointestinal bleeding, especially in elderly patients. Diagnostic yield of capsule endoscopy for finding the focus of obscure gastrointestinal bleeding is reported to be 38%-93%[10]. Diagnostic yield of capsule endoscopy for obscure gastrointestinal bleeding is not different for overt and occult bleeding[11,12]. Diagnostic yields of CT angiography and double balloon enteroscopy in finding the focus of obscure gastrointestinal bleeding were 24% and 75.7%, respectively[13,14].

Table 1 Differences between non-steroidal anti-inflammatory drug-induced enteropathy and cryptogenic multifocal ulcerous stenosing enteritis

	NSAID-induced enteropathy	CMUSE
Definition	Ulceration and stricture of small intestine induced by NSAIDs[19,20]	Unexplained small intestinal multiple stricture and ulceration[17,18]
Causes	NSAIDs	Unknown
Age	Old-aged people[26]	Middle-aged people[18]
Treatment	Stop NSAIDs, Prostaglandin (misoprostol)[21], Sulfasalazine[22], Metronidazole[23], Glutamine[24], Glucose/citrate[25]	Steroids[17]

NSAID: Non-steroidal anti-inflammatory drug; CMUSE: Cryptogenic multifocal ulcerous stenosing enteritis.

The origin of gastrointestinal bleeding can be detected in a higher percentage of patients by capsule endoscopy than by CT angiography and double balloon enteroscopy[10]. In our case, we performed capsule endoscopy to find overt obscure bleeding foci after abdominal CT scanning. There was no definite stricture or sign of significant obstruction by abdominal CT. However, the capsule became trapped in the bowel unexpectedly. The important complication of capsule endoscopy is capsule retention, for which the incidence is < 2%[15]. Intestinal stricture might be suspected, especially in patients with chronic NSAID use, ischemic bowel disease, abdominal radiotherapy, and Crohn's disease. For these patients, capsule endoscopy can be performed after abdominal CT or small bowel series to find strictures[10]. Stricture cannot be found completely by abdominal CT or small bowel series, therefore, an M2A patency capsule can be considered, which is biodegradable in the gastrointestinal tract in patients with suspected intestinal stricture[16].

Our patient had multiple ulcerations and strictures in the small intestine, therefore, diaphragm disease was needed to be differentiated from CMUSE, even though the patient was an elderly woman who was taking NSAIDs. The characteristics of NSAID-induced enteropathy and CMUSE using previously published data are summarized in Table 1. CMUSE is defined as unexplained small intestinal multiple strictures and ulcerations of unknown origin. It is characterized as an atypical type of vasculitis[17]. Its clinical features include unexplained stricture and ulceration of the small bowel, without systemic inflammation, which are found in young and middle-aged patients. CMUSE can relapse chronically or after surgery. The symptoms of CMUSE can be improved by steroid treatment[17,18].

In conclusion, NSAID-induced diaphragm disease in the small bowel can be diagnosed efficiently by capsule endoscopy, and the gross endoscopic features of diaphragm disease are similar to those of CMUSE. We

also suggest that NSAID-induced enteropathy, such as diaphragm disease, should be suspected in NSAID users, especially in elderly patients.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2877

World J Gastroenterol 2011 June 21; 17(23): 2877-2878
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Time for the world to move beyond the percutaneous endoscopic gastrostomy

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Received: December 7, 2010 Revised: December 28, 2010
Accepted: January 4, 2011
Published online: June 21, 2011

Abstract

Percutaneous endoscopic gastrostomy (PEG) is a proven feeding tube, just as the nasogastric tube is proven to be able to deliver enteral nutrition. For long-term use, both patient and caregiver want neither. What is desired is the LOOPPEG® 3G tube, more secure than the PEG, and less risky to change than the nasogastric tube. Future clinical research should focus on this high-comfort low-risk tube.

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Key words: Tube feeding; Enteral nutrition; Dysphagia; Stroke

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Pang AS. Time for the world to move beyond the percutaneous endoscopic gastrostomy. *World J Gastroenterol* 2011; 17(23): 2877-2878 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i23/2877.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2877

TO THE EDITOR

I found "Survival of geriatric patients after percutaneous endoscopic gastrostomy in Japan" by Yutaka Suzuki *et al*[1] to be an interesting and timely article.

With an aged population and several centers working together, they were able to recruit a large sample and, consequently, produce robust results. The first lesson to be learnt is that collaboration can produce better results. The second lesson is that, for the Japanese population at least, the percutaneous endoscopic gastrostomy in geriatrics is proven. To the co-authors and their institutions, I offer my heartiest congratulations. My country, Singapore, is ageing rapidly and their experiences can offer valuable lessons.

I emphasize that my subsequent suggestion for future research should not be taken as criticisms of their work, which is an unqualified success. By way of introducing my comments, I pose two questions.

First, since elderly patients in other countries might react differently from Japanese patients, should we not do a similar study of the PEG in Singapore? My answer is no, for the following reason. The difference in year (or mo) is unlikely to be important to the geriatric patient, in whom compassion is more valued than cure.

Second, since the nasogastric tube might give a better survival than the PEG, should we not do a similar study on this feeding tube? Again, my answer is no, for the following reasons. Feeding tube and survival of the elderly have a correlation but not a cause-effect relationship. Both nasogastric tube and PEG can deliver the enteral nutrition, and the choice is determined more by the risk/comfort profile of the tube. Statistical significance does not mean clinical significance. Conversely, lack of the former does not mean that the PEG is not a clinically better tube. The relationship between nasogastric tube and PEG is predictable, independent of age and race, and unlikely to be affected by the study findings, however robust.

This relationship is illustrated in Figure 1. There are enough published data to show that the nasogastric tube is low in risk and low in comfort, whereas the PEG is very comfortable but also high in risk[2]. For long-term use, both patients and caregivers want neither option! What

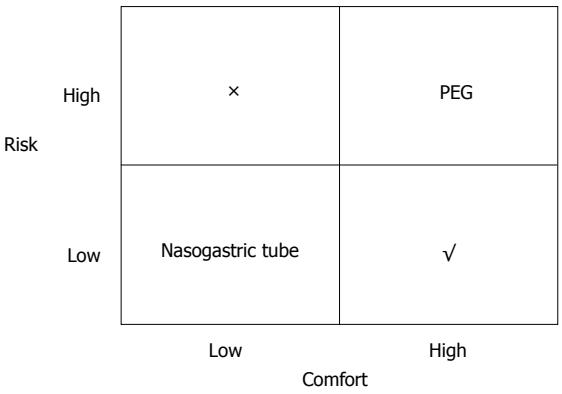

Figure 1 Risk/Comfort Chart. The desired tube is the high-comfort low-risk option (√). PEG: Percutaneous endoscopic gastrostomy.

they need is a very comfortable but low in risk option.

In my opinion, there is only one feeding tube which meets this profile. The LOOPPEG® 3G tube is comfortable because it bypasses the nose and low in risk because it cannot be dislodged[3]. Also, it is less risky to change than the nasogastric tube. Consequently, the 3G tube can be made 15 Fr or smaller because tube blockage is a non-issue, having been negated by easy tube change. Thus, the trauma of changing the tube - physical, psychological and financial - is minimized.

However, no case series has been published. It may take forever for a sizeable sample and robust results to come out from my part of the world. Hence, I hope our Japanese colleagues, having published a solid study, will take up the challenge to move beyond the PEG, research the 3G tube, and report their findings in this fine Journal.

ACKNOWLEDGMENTS

Iruru Maetani, MD, FJSIM, Professor and Chairman, Division of Gastroenterology/Department of Medicine, Toho University Ohashi Medical Center, Tokyo, Japan, encouraged me in my work on the 3G tube.

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World J Gastroenterol 2011 June 21; 17(23): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 June 21; 17(23): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, Italy

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4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

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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 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 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

World Journal of Gastroenterology

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World J Gastroenterol 2011 June 21; 17(23): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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World Journal of Gastroenterology

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

Indexed and Abstracted in
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Acknowledgments

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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 Pixudiarrhoea. *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.0000035706.28494.09]

Both personal authors and an organization as author

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]

No author given

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]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary 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

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

Conference paper

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

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 ± SD or mean ± 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 υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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

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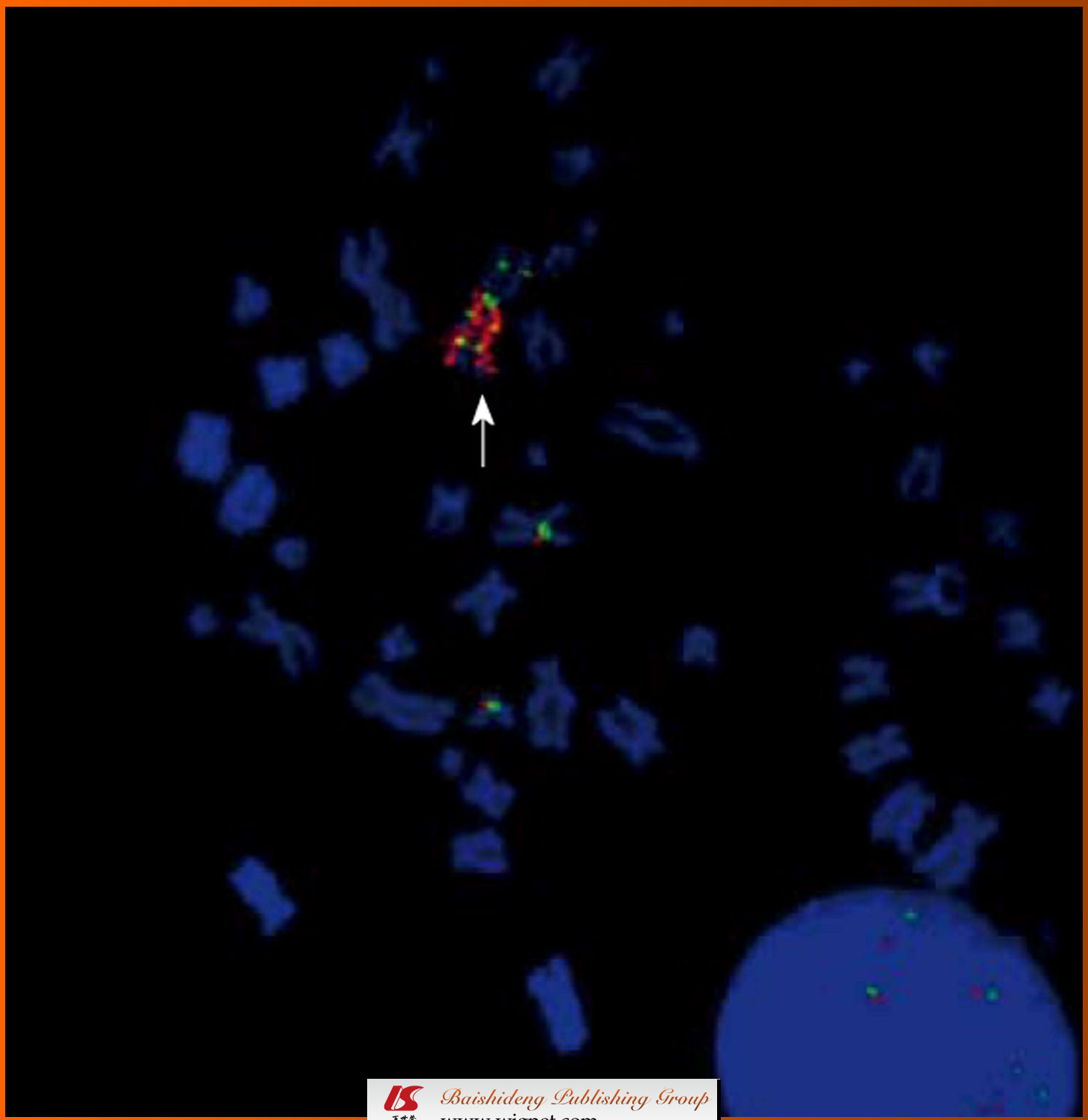
World J Gastroenterol 2011 June 28; 17(24): 2879-2976



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World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 24 June 28, 2011

EDITORIAL

2879 How to protect liver graft with nitric oxide
Ben Abdennebi H, Zaouali MA, Alfany-Fernandez I, Tabka D, Roselló-Catafau J

TOPIC HIGHLIGHT

2890 Probiotics in hepatology
Lata J, Jurankova J, Kopacova M, Vitek P

2897 Molecular biology of pancreatic cancer
Zavoral M, Minarikova P, Zavada F, Salek C, Minarik M

ORIGINAL ARTICLE

2909 Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes
Brown J, Bothma H, Veale R, Willem P

2924 Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA
Yeh CN, Lin KJ, Chen TW, Wu RC, Tsao LC, Chen YT, Weng WH, Chen MF

2933 α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon
Chen M, Sun P, Liu XY, Dong D, Du J, Gu L, Ge YB

BRIEF ARTICLE

2941 Is hyperhomocysteinemia relevant in patients with celiac disease?
Casella G, Bassotti G, Villanacci V, Di Bella C, Pagni F, Corti GL, Sabatino G, Piatti M, Baldini V

2945 Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients
Murata M, Furusyo N, Unno M, Ogawa E, Toyoda K, Taniai H, Ohnishi H, Hayashi J

2953 Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism
He XH, Li WT, Peng WJ, Li GD, Wang SP, Xu LC

2958 Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer
Deng LY, Wang JP, Gui ZF, Shen LZ

2965 $CD133^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity
Shi CJ, Gao J, Wang M, Wang X, Tian R, Zhu F, Shen M, Qin RY

CASE REPORT 2972 Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report
Camera L, Calabrese M, Sarnelli G, Longobardi M, Rocco A, Cuomo R, Salvatore M

LETTERS TO THE EDITOR 2976 Hepatocellular carcinoma and industrial epidemics
Braillon A, Dubois G

World Journal of Gastroenterology
Volume 17 Number 24 June 28, 2011

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-VI	Instructions to authors
ABOUT COVER		Brown J, Bothma H, Veale R, Willem P. Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes. *World J Gastroenterol* 2011; 17(24): 2909-2923 http://www.wjgnet.com/1007-9327/full/v17/i24/2909.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

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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
http://www.wjgnet.com

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.

PUBLICATION DATE
June 28, 2011

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

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SPECIAL STATEMENT
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2879

World J Gastroenterol 2011 June 28; 17(24): 2879-2889
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

How to protect liver graft with nitric oxide

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Supported by The Ministerio de Sanidad y Consumo (PI081988), Ciber-ehd., Instituto Carlos III and Ministerio de Asuntos Exteriores y de Cooperación/AECI (A02987/09 and A/031197/10). Zaoualí MA is a fellowship-holder from the Catalan Transplantation Society
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Received: November 17, 2010 Revised: January 18, 2011
Accepted: January 25, 2011
Published online: June 28, 2011

Abstract

Organ preservation and ischemia reperfusion injury associated with liver transplantation play an important role in the induction of graft injury. One of the earliest events associated with the reperfusion injury is endothelial cell dysfunction. It is generally accepted that endothelial nitric oxide synthase (e-NOS) is cell-protective by mediating vasodilatation, whereas inducible nitric oxide synthase mediates liver graft injury after transplantation. We conducted a critical review of the literature evaluating the potential applications of regulating and promoting e-NOS activity in liver preservation and transplantation, showing the most current evidence to support the concept that enhanced bioavailability of NO derived from e-NOS is detrimental to ameliorate graft liver preservation, as well as preventing subsequent graft reperfusion injury. This review deals mainly with the beneficial effects of promoting "endogenous" pathways for NO generation, *via* e-NOS inducer drugs in cold preservation solution, surgical strategies such as ischemic preconditioning, and alternative "exogenous" pathways that focus on the enrichment of cold storage liquid with NO donors. Finally, we also provide a basic bench-to-bed side summary of the liver physiology and cell signalling mechanisms that account for explaining the e-NOS protective effects in liver preservation and transplantation.

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Key words: Cold ischemia reperfusion injury; Endothelial nitric oxide synthase; Nitric oxide; Liver graft preservation; Ischemic preconditioning; Liver transplantation

Peer reviewer: Fabio Grizzi, PhD, Laboratories of Quantitative Medicine, Istituto Clinico Humanitas IRCCS, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Ben Abdennebi H, Zaoualí MA, Alfany-Fernandez I, Tabka D, Roselló-Catafau J. How to protect liver graft with nitric oxide. *World J Gastroenterol* 2011; 17(24): 2879-2889 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2879.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2879

INTRODUCTION

Ischemia-reperfusion (IR) injury during liver transplantation (LT) is a complex, multi-factorial process in which numerous mediators and a variety of cells interact, leading to tissue damage. It is one of the major cause of both initial poor function and primary non-function of liver allograft, and is responsible for 81% of re-transplantations during the first week after surgery. An intricate network of hepatic and extra-hepatic mechanisms is involved in the genesis of hepatic IR[1-3].

Cold storage and warm reperfusion are unavoidable steps in transplantation and all grafts undergo some degree of IR injury. The cascade of events involves microvasculature (sinusoidal endothelial cells or SEC), Kupffer cells, Ito cells, parenchyma (hepatocytes) and bile ducts. Cold ischemia during organ storage, which is intentionally applied to reduce the metabolic activity of cells in order to preserve the graft before its transplantation, has a substantial effect on graft function. Also, warm ischemia that begins at implantation has an additional negative impact on graft function and outcome. Whatever the type of attack, liver graft damage initiated during the ischemic phases is exacerbated after reperfusion with oxygen and the reintroduction of blood elements.

Although our understanding of the pathophysiology of IR injury is only partial, many response elements indicate that vascular endothelium disruption, the immune response, oxidative mediators, and several cell-death pathways all play important roles. A variety of mediators have been implicated, including reactive oxygen species (ROS) and inflammatory mediators tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, transforming growth factor-β, interferon-γ and endotheline (ET)-1. Furthermore, complement and chemokines are also released, which leads to leukocyte recruitment and activation. Upregulated adhesion molecules, including intercellular adhesion molecule 1 and E-selectin, enhance endothelial-immune cell interactions. ROS directly injure many cytoskeletal and functional cellular components, causing cell damage. After IR, direct endothelial damage and abnormal vascular tone occurs as a result of an imbalanced sensitivity to mediators of vasoconstriction and vasodilation, such as ET-1/nitric oxide (NO). Endothelial injury also causes cell swelling and narrowing of the vascular lumen, further reducing blood flow. Finally, key regulators of apoptosis, such as caspases, are upregulated resulting in increased cell death.

It is now largely appreciated that IR associated with LT leads to a rapid endothelial dysfunction characterized by a marked decrease in NO production[4-6]. The decrease in NO bioavailability occurs within the first few minutes after reperfusion, and appears to be due to decreased synthesis of NO by NO synthase (NOS), enhanced inactivation of NO by the overproduction of superoxide anion (O_2-), or both. Experimental studies emphasize that it is essential to minimize the deregulation of hepatic microcirculation during LT[7].

NO is a free-radical diatomic gas of low molecular weight with an unpaired electron[8]. It is highly lipophilic, allowing it to permeate quickly across the cell membranes. Its half-life *in vivo* is a few seconds, and it is rapidly converted to stable nitrites (NO_2-) and nitrates (NO_3-)[8]. Endogenous NO is synthesized from the amino acid precursor L-arginine in an oxidation reaction, catalyzed by the NOS enzymes. This complex reaction requires the presence of co-substrates O_2 and NAD(P)H (nicotinamide adenine dinucleotide phosphate reduced), as well as many cofactors such as flavin adenine dinucleotide, flavin mononucleotide and BH4 (tetrahydrobiopterin)[8,9]. There are three isoforms of NOS expressed in the liver in different cells and in different conditions, namely endothelial NOS (e-NOS), neuronal NOS (n-NOS) and inducible NOS (i-NOS). Both e-NOS and n-NOS are constitutively present in liver. The exact role of n-NOS in the pathophysiology of IR injury during LT remains to be established[10]. n-NOS has been observed within neurons innervating the portal tracts by histochemical methods, and several authors believe that this protein is scarcely expressed in the liver[11,12]. In contrast to inducible NOS, constitutive NOS activation requires Ca^{2+}.

Like any important signaling molecule, NO diffuses freely across cell membranes. Under physiological conditions, NO binds to soluble guanylate cyclase inside cells and then induces the production of large quantities of cGMP (guanosine 5'-monophosphate), which then triggers the signal (Figure 1)[13]. *In vivo,* NO is inactivated mainly by superoxide anion, but other pathways could be involved. The first pathway involves the autoxidation of NO to NO_2- and then to NO_3-. Other pathways may be mediated by metal-catalyzed oxidation reactions. The copper-containing protein ceruloplasmin (P-Cu^{2+}) has been shown to rapidly oxidize NO to NO_2- in physiological conditions[14,15]. In addition to the P-Cu^{2+}-mediated reaction, ferrous deoxygenated hemoglobin (Hb-$Fe^{2+}O_2$; oxyhemoglobin) rapidly converts NO to NO_3-.

e-NOS ACTIVITY AND e-NOS-DERIVED NO PRODUCTION IN LT: PATHOPHYSIOLOGICAL ASPECTS

e-NOS is constitutively expressed in venous and arterial endothelial cells and it produces small quantities of NO (at picomolar levels)[16]. It is the main source of NO in endothelial cells under physiologic conditions[9]. e-NOS is also induced in response to specific extracellular stimuli, such as shear stress[17] and metabolic stress (Figure 1)[18]. e-NOS is localized to the caveolae[19,20], which are microdomains of the plasma membrane that have been implicated in a variety of cellular functions, including signal transduction. Caveolin proteins are the major coat proteins of caveolae, and in endothelial cells e-NOS binds to caveolin-1. Caveolin-1 and other peptides from the caveola region directly inhibit e-NOS activity[21,22]. This complex membrane structure is sensitive to the fluid pressure on the membrane.

The e-NOS activation can also be triggered through the signalling pathway involving serine/threonine kinase Akt [or protein kinase B (PKB)], which in turn is stimulated by the phosphoinositide 3-kinase (PI3K)[23,24]. e-NOS is one of the targets of Akt. An important step in this activation is the phosphorylation by Akt of the serine residue in position 1179 (bovine sequence) or serine 1177 (human sequence) of the e-NOS enzyme[25,26]. Therefore, Akt-dependent e-NOS phosphorylation may be an important mechanism in the attenuation of IR injury after

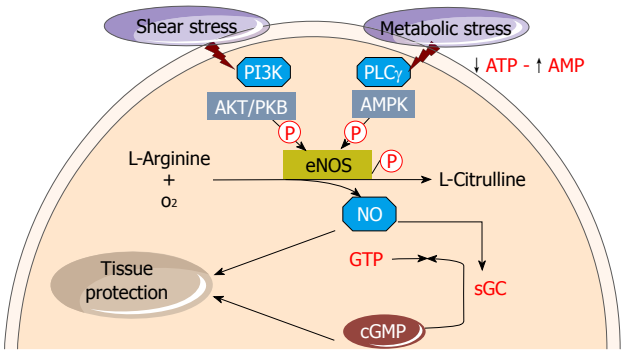

Figure 1 Endothelial nitric oxide synthase-derived nitric oxide synthesis. Shear stress leads to endothelial nitric oxide synthase (e-NOS) phosphorylation and through pathway involving phosphoinositide 3-kinase (PI3K) and Akt. Metabolic stress also phosphorylates e-NOS through the adenosine monophosphate kinase (AMPK) route. The coordination of signalling through these converging pathways allows for e-NOS activation. L-arginine is converted in the endothelium monolayer by the constitutive e-NOS to nitric oxide (NO) and L-citrulline. NO diffuses into both the vessel lumen and the vessel wall, thereby activating soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). NO in concert with cGMP involve tissue protection.

LT. Treatment of liver grafts with adenovirus encoding for myr-Akt improves biochemical and cytoprotective parameters after orthotopic LT in pigs, in comparison to uninfected groups[27].

Recent reports demonstrate that metabolic stress can elicit adenosine monophosphate protein kinase (AMPK), which stimulates phosphorylation of e-NOS protein and increases NO bioavailability in endothelial cells[18,28,29]. The e-NOS-derived NO, in turn, decreases hepatic levels of ET-1, improves hepatic microcirculation and significantly attenuates TNF-α hepatic expression and, remarkably, reduces the activation of caspase-8 and caspase-3 after OLT[30].

In addition to its key role in vascular tone regulation, studies have shown that NO is involved in several other protective routes (Figure 2). Animal studies have shown that early in the reperfusion period, tissue damage appears to be associated with decreased NO availability related to e-NOS down-regulation[27,31]. Similarly, the use of NOS inhibitors leads to IR damage[32]. It has been demonstrated that e-NOS-derived NO inhibits the production and release of several endothelial vasoconstrictor factors, including ET-1[33,34]. NO also interferes with the adhesion and aggregation of platelets, adhesion of leukocytes and monocytes to endothelial cells in vessel walls[35,36]. It also modulates the expression of pro-inflammatory molecules, such as vascular cell adhesion molecule-1 and monocyte chemoattractant protein 1. Furthermore, NO is emerging as an endogenous inhibitor of TNF-α. NO decreases endothelial permeability and also exerts anti-mitogenic effects in vascular smooth muscle cells by inhibiting their growth and proliferation.

However, other lines of evidence suggest that ROS production after LT reduces e-NOS activation, which becomes uncoupled and perturbs e-NOS-derived NO homeostasis[37] (Figure 3). In parallel, i-NOS is transcriptionally up-regulated in all liver cells, leading to the production of large amounts of NO for persistent periods[37]. Excessive NO generation can be detrimental, because it may alter systemic vascular tone and reactivity, leading to hypotension and circulatory shock[38]. In addition, the generation of peroxynitrite (ONOO-), a potent oxidant formed by reacting NO with O_2^-[39], could also cause cell injury through lipid peroxidation, direct inhibition of the mitochondrial respiratory chain[40,41], inhibition of membrane Na^+/K^+ ATPase activity, or oxidative protein modification such as the formation of nitrotyrosine[4,38]. Thus, NO acts as a double-edged sword since it has neither harmful or beneficial effects, depending on its source and the experimental conditions.

The importance of e-NOS for hepatic injury after cold storage/warm reperfusion in transplanted liver grafts has been investigated. The functions of mouse liver grafts retrieved from e-NOS-deficient donors and those from wild-type donors were compared after orthotopic transplantation[31]. e-NOS-deficient liver grafts intensified IR injury, as shown by increased ALT, necrosis and apoptosis, and elevated graft infiltration of monocytes/macrophages. In addition, both flow rate and sinusoidal diameter were diminished after transplantation of e-NOS-deficient grafts. All these alterations are detected from 4 h after LT. In another study, decreased hepatic bioavailability of NO was detected as early as 1 h after reperfusion of human liver transplants[4]. This decline was attributed to a reduction of e-NOS protein levels after reperfusion, rather than to a change in e-NOS mRNA transcription. The concerned mechanism would be a rapid turnover/degradation of the e-NOS enzyme[42]. The extent of the alteration of e-NOS protein expression depended on the duration of preservation, because the loss of e-NOS was exacerbated after 6 h of cold ischemia[43]. The stimulus for the rapid decrease in e-NOS expression is not known.

LT depletes serum arginine due to a massive release of arginase I from cold-injured liver parenchymal cells[44,45]. The depletion of arginine decreases tissue arginine availability, with subsequent down regulation of e-NOS[46,47]. In contrast, enhancement of arginine availability through arginase blockade can protect against hepatic IR injury. The results demonstrate that inhibition of arginase with nor-NOHA can partially reverse the arginine depletion seen in IR injury and improve the histopathological damage following transplantation[48].

MODULATION OF e-NOS-DERIVED NO PRODUCTION FOR LT

The quality of cold preservation is a major detriment of initial graft function and survival. While cold ischemia is considered necessary to slow tissue metabolism, it causes well-documented lesions in the SEC[49]. The SEC is the main target cell of reperfusion injury, at least during the early phase[50]. Endothelial dysfunction leads to NO deficiency, which has been implicated in many disorders.

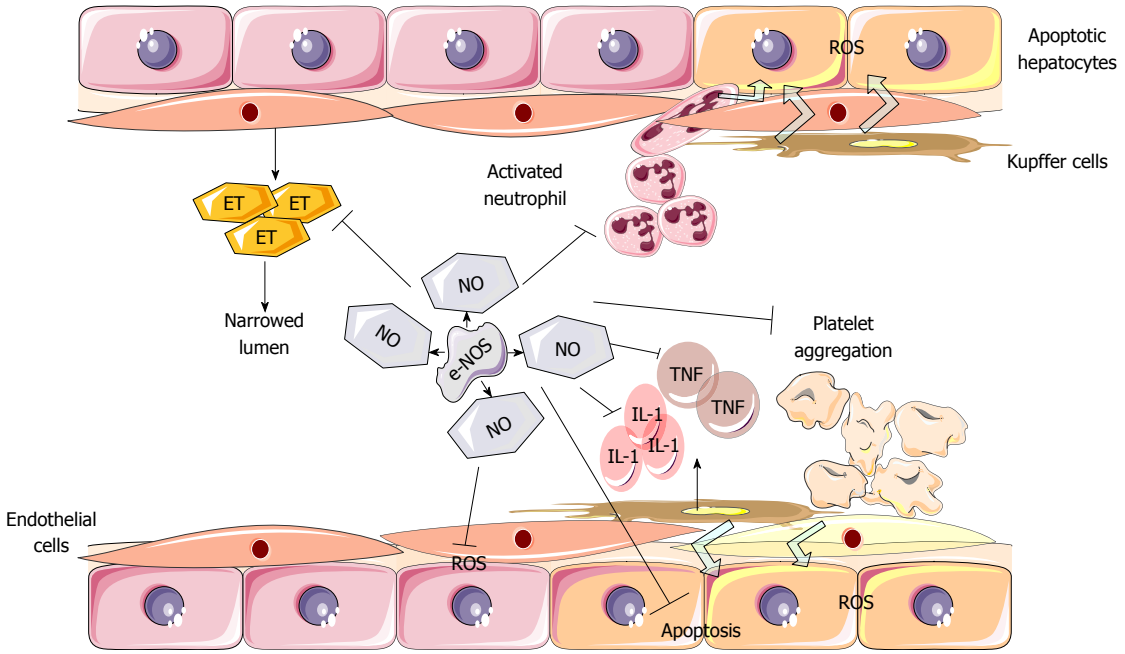

Figure 2 Protective effects of endothelial nitric oxide synthase-derived nitric oxide. The multifactorial consequences are derived from the nitric oxide (NO) generation on oxidative stress (reactive oxygen species), proinflammatory [interleukins (ILs), tumor necrosis factor (TNF)] and vasoconstrictor (endothelins) mediators. e-NOS: Endothelial nitric oxide synthase; ROS: Reactive oxygen species.

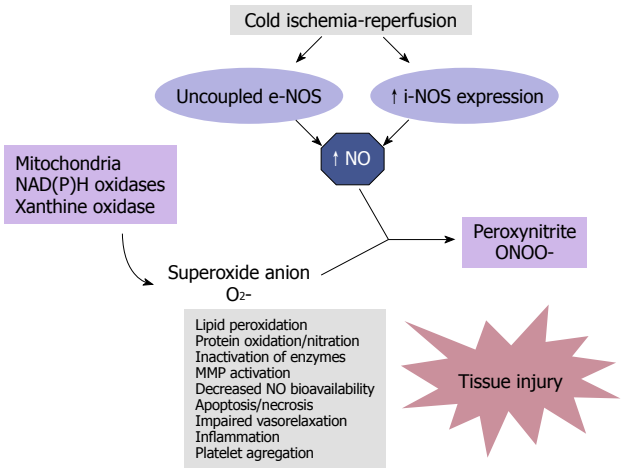

Figure 3 Tissue damage after unbalanced nitric oxide production. Cold ischemia reperfusion involves uncoupled endothelial nitric oxide synthase (e-NOS) and inducible nitric oxide synthase (i-NOS) expression. Large amounts of nitric oxide (NO) are produced under these pathological conditions. NO, in association with increased mitochondrial dysfunction and oxidative stress, reacts with superoxide anion (O_2-), to produce peroxynitrite (ONOO-). Peroxynitrite, in concert with other oxidants, induces tissue damage.

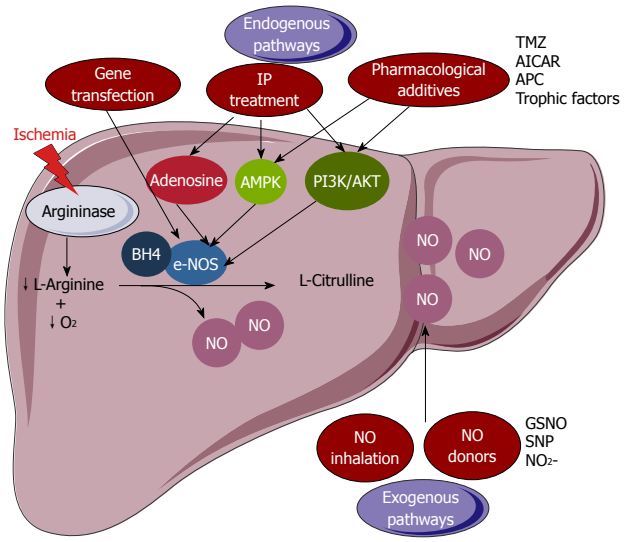

Figure 4 Modulation of the decline in endothelial nitric oxide synthase-derived nitric oxide production. endothelial nitric oxide synthase (e-NOS)-derived nitric oxide (NO) bioavailability can be compromised either by a reduced L-arginine and cofactor (tetrahydrobiopterin, BH4) availability, or by a decreased e-NOS protein activity and level. Two ways seem to be able to overcome these obstacles: the use of exogenous sources of NO (NO donors or NO inhalation) and the induction of the endogenous downstream effectors of e-NOS (pharmacological treatment or ischemic preconditioning) or transfection by an adenovirus containing e-NOS enzyme. SNP: Sodium nitroprusside; GSNO: S-nitroso-L-glutathione; AICAR: 5-amino-4-imidazole carboxamide riboside; TMZ: Trimetazidine; APC: Activated protein C; AMPK: Adenosine monophosphate protein kinase; PI3K: Phosphoinositide 3-kinase.

The morphological changes in these cells interfere with the hepatic microcirculation during reperfusion (adhesion and activation of platelets and leukocytes, and thromboses)[51]. So, improving quality of graft preservation is a means to promote its immediate function, to optimize the allocation of grafts and also to reduce the shortage of organs. Various strategies have been developed to promote immediate recovery of graft function and also to increase access to the marginal donor pool.

Many strategies could be developed to compensate for the decline in NO production during LT. We can distinguish two ways to promote NO production *in vivo*: the endogenous and the exogenous routes (Figure 4). The endogenous way consists of the use of arginine[52]

to compensate for the deficiency of this NO precursor. Other methods consist of directly influencing the activity of the e-NOS enzyme by adding to the preservation solution one of its cofactors (BH4)[53], or one of its inducers, to increase downstream effectors of e-NOS such as Akt and AMPK[54-56]. Transfection of donor liver by an adenovirus containing the e-NOS enzyme could also be envisaged before graft extraction[57,58]. Ischemic preconditioning (IP) could be an endogenous source of NO[59]. The exogenous route requires the use of an exogenous source of NO, which may be given directly by inhalation or indirectly by an NO donor such as sodium nitroprusside (SNP) or S-nitroso-L-glutathione (GSNO)[60-63].

These strategies have shown their effectiveness experimentally, but are not always applicable in human clinical contexts. NO can react with superoxide anions to form ONOO-, a highly reactive oxidant, which can induce apoptosis and cause structural and functional disorders of the graft. This explains why some authors claim that the contribution of NO during cold ischemia may be unnecessarily risky, especially if given at a high dose[64], and the use of NO in humans is controversial at present.

NO GENERATION INDUCED BY THE ENDOGENOUS PATHWAY

Pharmacological e-NOS induction during cold preservation

The potentially protective role of endogenous NO in IR-induced injury to the liver is supported by recently published studies demonstrating enhanced hepatocellular injury in post-ischemic animals that had been rendered deficient in e-NOS[65]. In addition, researchers have demonstrated that over-expression of liver e-NOS protects mice from IR-induced liver injury[27,66-68].

Endogenous e-NOS activation and e-NOS-derived NO might be a promising approach to limiting organ injury. Several pro-survival pathways such as PI3K/Akt and AMPK, which are activated following hepatic injury, are involved in the regulation of e-NOS. Pharmacological up-regulation of these pro-survival kinase cascades may provide an approach to limiting cold ischemic insult. In this regard, 5-amino-4-imidazole carboxamide riboside (AICAR), carvedilol (CVD), trimetazidine (TMZ), activated protein C (APC) and insulin-like growth factor (IGF)-1 could be promising additives in preservation solutions to improve the outcome of liver grafts after cold storage and reperfusion.

Recent studies have shown that the enrichment of University of Wisconsin (UW) with AICAR, an activator of AMPK, ameliorated long-term liver preservation[55]. The protective effect of AICAR on liver injury and function seems to be mediated by enhanced e-NOS activation and NO generation following AMPK phosphorylation, which induces vasodilatation in liver grafts[55]. The relevance of AMPK as an e-NOS upstream regulator was evidenced by the administration of AraA, a phospho-AMPK inhibitor, previous to liver graft preservation, demonstrating that AMPK inhibition reduces e-NOS activation and NO production[55]. In line with these results, Ben Mosbah *et al*[54] demonstrated that the use of CVD, a β- and α-adrenergic blocking drug, when added to UW preservation solution, protected livers through the same mechanism[54]. Livers preserved in this solution show decreased transaminases levels, improved vascular resistance, reduced mitochondrial damage and enhanced ATP levels after reperfusion.

However, the AMPK pathway is not the only e-NOS modulator. Other authors have attempted to improve the histidine-tryptophan-ketoglutarate (HTK) preservation solution by supplementation with an anticoagulant and anti-inflammatory agent, such as APC[30]. They found that the modified HTK preservation solution decreased portal pressure and improved hepatic microcirculation through increased NO hepatic levels *via* up-regulated e-NOS. In addition, the solution attenuated TNF-α expression and markedly reduced the activation of caspase-3 and caspase-8[30]. Although the exact mechanism by which APC activated e-NOS remains unclear, other *in vitro* reports postulated that APC activated e-NOS *via* phosphatidylinositol 3-kinase-dependent phosphorylation, followed by activation of PKB[69].

Recently, Institut Georges Lopez-1 (IGL-1) solution has been proposed as an effective alternative to UW liquid in clinical kidney transplantation, and in experimental orthotopic LT models[70,71]. In addition, we recently demonstrated that IGL-1 is more suitable than UW solution for fatty liver preservation and that the benefits from IGL-1 were linked to an increment of NO synthesis through e-NOS activation which, in turn, reduced oxidative stress and liver injury[72].

TMZ, which has been used as an anti-ischemic drug in the heart for over 35 years, reduced liver injury and improved liver regeneration and survival rate in an experimental model of partial hepatectomy under hepatic blood inflow occlusion[56]. Studies examining the underlying protective mechanisms of TMZ as an additive to UW solution for liver preservation suggest that AMPK up-regulation is the mechanism by which TMZ activates e-NOS and exerts its cytoprotective effect[55]. Another study demonstrated that TMZ attenuated myocardial IR injury *via* PI-3K/AKT kinase pathway activation[73]. We recently found that the addition of TMZ to IGL-1 solution has a synergistic effect on e-NOS-derived NO generation that favours HIF-1α accumulation during normothermic reperfusion[32]. Preserved HIF-1α levels contribute to the increase in the over-expression of cytoprotective proteins such as HO-1 in fatty liver grafts.

It has been established that IGF-1 up-regulates e-NOS activity by interacting with a tyrosine kinase membrane receptor which activates the AKT signalling pathway[74,75]. Furthermore, trophic factors, including IGF-1, have been added to UW solution in an attempt to improve the survival of pig orthotopic liver allografts after cold storage[76]. To this end, we explored the effects of the addition of IGF-1 to IGL-1 solution on fatty liver preservation during cold IRI. We examined the mechanisms

responsible for such effects, including AKT phosphorylation and NO generation. We have demonstrated that the beneficial action of IGF-1 as an additive to IGL-1 is mediated by AKT activation and NO generation, with concomitant prevention of pro-inflammatory cytokines, such as TNF-α[77].

Surgical e-NOS induction by IP

IP is a technique described firstly in the heart by Murry *et al*[78] in 1986, which consists of the application of short and repetitive periods of I/R before a sustained one. The protective effect of IP is not specific to the myocardial muscle, since it is observed in other organs such as skeletal muscle[79], brain[80], intestines[81], lungs[82], kidneys[83] and liver[34]. In any case, the protection induced by IP against IR injury seems to be specific to each organ and animal species, depending on the number of IR cycles applied before the sustained IR. For example, 3-4 cycles are needed for the protection of myocardium[78], whereas in the liver just one cycle of 10 min of ischemia and 10 min of reperfusion is sufficient for maximal protection[59].

In the heart, IP offers an initial protection of 2-3 h after reperfusion, and a remote protection after 12-24 h that lasts for 2 to 3 d. A similar pattern was observed in the liver, although remote protection is not yet well established. Moreover, a differential IP protection was observed in the liver, also depending on the animal species.

Molecular mechanisms responsible for hepatoprotection

The molecular basis of IP is a sequence of episodes triggered by a rapid signal, which leads to an intracellular message and to the amplification of the effector mechanisms of protection[84]. The benefits of IP are caused by the release of several inflammatory mediators such as adenosine and NO, which is followed by the activation of multiple cellular signals. NO is generated by the adenosine released (activation of adenosine A2 receptors), which in turn activates the endothelial constitutive form of the e-NOS enzyme[34,85], a few minutes after IP. The window of liver protection induced by IP is defined by two factors: (1) the concentration of adenosine must be high enough to induce NO; and (2) the concentration of xanthine must be low enough to avoid its prejudicial effects. It is well established that a high concentration of xanthine would support significant increases in superoxide anion. This would react with NO to generate peroxynitrite[86], which would cancel the beneficial effects of IP. Vasodilator effects of NO release improved liver oxygenation and microcirculation[87], and also inhibited the generation of ET, powerful vasoconstrictors generated during liver reperfusion[34].

In addition, IP preserves energy metabolism during sustained ischemia[88,89]. This is confirmed by the maintenance of ATP levels, as well as the depletion of lactate accumulated during the ischemic period. This beneficial effect is mediated by the increase of AMPK, whose activation can be mediated by NO[89].

The activation of the G protein-bound A2 receptor by adenosine stimulates the activity of many intracellular kinases, such as protein-kinase C (PKC) and p38 MAPK[90,91]. In addiction, recent studies implicate PKC in some of the beneficial effects of IP in liver. They show that PKC activation depends on the phosphorylation of different effector molecules, such as the tyrosine kinases[92] and MAPK (including p38 and MAPK[93]), and increases the tolerance of hepatocytes and endothelial cells to the ischemic insult.

Many transcription factors are involved in PKC activation, such as nuclear factor (NF)-κB, which is responsible for the protective effects of PI[94,95]. These transcription factors modulate the expression of particular genes, resulting in the synthesis of proteins such as heat shock proteins (HSP), which are understood to be effectors for the benefits of IP[96]. In the liver, IP is associated with the synthesis of many inducible forms of HSP: HSP70, HSP2 and HSP73 and heme oxygenase (HO-1/HSP32). The induction of HSP depletes the binding between pro-inflammatory transcriptional factors and improves the oxidant capacity of the cells[96-98]. Both effects could contribute to the decrease in TNF-α and to the attenuation of the inflammatory response of preconditioned livers[99,100]. It was also suggested that IP could reduce the transcription of genes, such as *c-fos* and *c-jun*, implicated in the development of the hepatic IRI, and that NFκB activation could induce the activation of signal translator and transcription activator 3, implicated in hepatoprotection and cell proliferation[89,94,96,101,102].

The beneficial role of NFκB in IP is controversial. Whereas Funaki *et al*[103] demonstrate that the protective role of IP is associated with the inhibition of NFκB activation, other authors suggest the opposite, showing that these effects are due to NFκB activation[94,95]. These differences could be attributed to differences in the models used. Besides these cellular signalization pathways, recent studies show that IP could induce the release of small quantities of ROSs[104] and TNF-α, contributing to the protective mechanisms.

Applications to LT

Several studies in animal models have demonstrated the usefulness of IP, but its application to clinical transplantation needs to be clarified. The first clinical application was carried out by Koneru *et al*[105], who used IP in deceased donor LT. They showed that deceased donor liver tolerated 5 min of hilar clamping, but IP did not decrease graft injury. More recently, Azoulay *et al*[106] demonstrated that the effects of 10 min of IP of the liver graft in the donor are associated with better tolerance to ischemia, as well as a worsened early liver function. These studies are consistent with those by Jassem *et al*[107], who demonstrated the protection of cadaver donor livers subjected to IP prior to retrieval by clamping of the hepatic pedicle for 10 min at 24 h after transplantation. This was evidenced by a significant decrease in transaminase levels, and a concomitant reduction of the non-specific inflammatory response.

Similar results were obtained by Cescon *et al*[108], in a prospective randomized study on cadaver donors, by the use of 10 min of IP followed by 15 min of reperfusion. These authors also demonstrated a significant reduction in AST, ALT and i-NOS expression levels after transplantation.

Taking all this into account, new research work is needed to establish the "effective protection" window in human LT and confirm the usefulness of IP in clinical transplantation, including the marginal graft donors, which at present are discarded for clinical transplantation purposes.

NO GENERATION INDUCED BY THE "EXOGENOUS PATHWAY" DURING COLD PRESERVATION

Some considerations

Many teams have sought to improve the performance of storage solutions by supplementation with cytoprotective agents. The bulk of the work on these specific modifications was performed on cellular models in rodents. Unfortunately, the benefits observed were not always confirmed in humans and these changes have often led to inconclusive results in clinical practice. Several NO donors have been tested experimentally for their protective effects on cold IR injury. However, due to the numerous possible reactions and related biological consequences, inappropriate NO levels can cause a series of disease states. On the other hand, insufficient NO production also has serious medical consequences. NO donor therapy should aim to achieve the production of the correct quantity of NO in the correct place for the correct length of time. The exogenous NO should acts primarily as a local mediator to respond to specific stimuli, and then it should simply dissipate through diffusion and oxidation to NO_2- and NO_3-, without the need for complex catabolism. The chemical versatility of NO has led to the synthesis of a wide range of NO donors, each with different modes and rates of NO release. NO donors are pharmacologically active substances that spontaneously release NO (direct donors) or are metabolized to NO (donors requiring metabolism)[109].

The selection of one among these NO donors for therapeutic uses is not easy, since it must meet certain requirements. The compound must be highly soluble in aqueous solutions and diffuse easily into cells, where it produces NO. It must be a proven NO donor, remain in a subtoxic range, have a prolonged half-life and mimic the effect of the endogenous NO. Several authors have amply evaluated the consequences of the use of NO donors in liver under warm ischemia conditions, but little work has been done on the effects on cold ischemia and graft preservation. In our opinion the use of such donors as additive to preservation solution is limited by their short half-life.

Kuroki *et al*[110] studied the effects of SNP on liver warm IR injury. They reported improvement of liver microcirculation and hepatocyte injury in the early period of reperfusion. In another study supporting this finding, SNP infusion after a short period of liver ischemia also decreased liver IR injury[111]. Interestingly, deleterious effects observed during cold storage conditions (like vacuolated hepatocytes, increase in intrahepatic resistance and diminution of bile production) were significantly removed after addition of 500 mmol/L SNP to the UW preservation solution[60]. The authors assumed that beneficial effects of SNP were mediated by NO release[63].

In addition, GSNO has been evaluated in the liver. GSNO may serve as an endogenous long-lived adduct or carrier of NO[112]. GSNO possesses significant antiplatelet action at doses that cause mild hemodynamic effects[113]. Thus, one possible mechanism by which the NO-donor can protect the graft against IR-induced damage could be based on its ability to block platelet aggregation, thus avoiding the intravascular coagulation that can occur during reperfusion. Quintana *et al*[60,61] evaluated the benefit of the addition in UW solution of GSNO as a NO donor. After assaying four GSNO concentrations (50, 100, 250 and 500 mmol/L), they reported an improvement in the properties of UW solution when it contained 100 mmol/L GSNO. It preserved the morphology of hepatocytes and endothelial cells and prevented the alteration of the hemodynamics and function of livers after cold preservation/reperfusion.

Some authors conclude that nitrite (NO_2-) therapy might prove beneficial in protecting organ function and integrity during periods of IR such as those encountered in organ transplantation[114-116]. Under physiological conditions of pH and oxygen pressure, NO_2- has been shown to be a non-active metabolic end product of NO oxidation with limited intrinsic biological activity[117]. However, under ischemic conditions, when e-NOS activity is strongly decreased as a result of its essential dependence on oxygen and the depletion on arginine, an increasing body of evidence indicates alternative NO production by NOS-independent routes. For instance, nitrite may be reduced back to NO by the nitrite reductase action of deoxygenated hemoglobin[118], acidic disproportionation[119], or xanthine oxidoreductase (XOR)[120,121]. In summary, the use of NO_2- in the field of cold preservation could be considered for the following reasons: (1) it is a highly stable substance with no potentially toxic effect; (2) it selectively releases NO under conditions that subsist in stored tissue, namely ischemia, hypoxia, or low pH; (3) it increases XOR activity, which may compensate for compromised constitutive e-NOS activity in terms of NO production, during hypoxia and acidosis; and (4) sodium nitrite is an FDA-approved compound. However, the use of nitrite in preservation solutions that contain allopurinol (such as UW or IGL-1 solutions) would be ineffective, since this latter inhibits XOR.

NEW DIRECTIONS FOR THE FUTURE

Along these lines we have evidenced the relevance of e-NOS system as a useful tool for liver preservation

against cold ischemia reperfusion injury. However, new potential strategies should be established in the future to increase e-NOS activity and modulate the NO availability, as well as to define the appropriate "therapeutic window" to provide the most suitable graft protection during cold storage and further during LT. A better knowledge of understanding the molecular pathways involved may lead to more efficient protective strategies to prevent early cold reperfusion injury during transplantation based on multifactorial activation of the endogenous cytoprotective e-NOS existing in preserved liver grafts.

ACKNOWLEDGMENTS

We are grateful to Robin Rycroft at the Language Advisory Service of the University of Barcelona for revising the English text. Also Catalan Society of Transplantation was acknowledged by the fellow to Mohamed Amine Zaouali.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2890

World J Gastroenterol 2011 June 28; 17(24): 2890-2896
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

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Probiotics in hepatology

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Received: December 13, 2010 Revised: February 18, 2011
Accepted: February 25, 2011
Published online: June 28, 2011

Abstract

The paper provides a basic review of intestinal microflora and its importance in liver diseases. The intestinal microflora has many important functions, above all to maintain the microbial barrier against established as well as potential pathogens. Furthermore, it influences the motility and perfusion of the intestinal wall, stimulates the intestinal immune system and therefore also the so-called common mucosal immune system, reducing bacterial translocation and producing vitamins. Immune homeostasis at mucosal level results from a controlled response to intestinal luminal antigens. In liver cirrhosis, there are many changes in its function, mostly an increase in bacterial overgrowth and translocation. In this review, probiotics and their indications in hepatology are generally discussed. According to recent knowledge, these preparations are indicated in clinical practice only for cases of hepatic encephalopathy. Probiotics are able to decrease the permeability of the intestinal wall, and decrease bacterial translocation and endotoxemia in animal models as well as in clinical studies, which is extremely important in the prevention of complications of liver cirrhosis and infection after liver transplantation. Probiotics could limit oxidative and inflammatory liver damage and, in some situations, improve the histological state, and thus non-alcoholic steatohepatitis could be considered as another possible indication.

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Key words: Intestinal microflora; Probiotics; Liver encephalopathy; Non-alcoholic steatohepatitis; Liver cirrhosis

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Lata J, Jurankova J, Kopacova M, Vitek P. Probiotics in hepatology. *World J Gastroenterol* 2011; 17(24): 2890-2896 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2890.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2890

INTRODUCTION

In recent years, probiotics have become a promising alternative for the treatment of gastrointestinal and various other diseases. Despite their initially described negative influence on the course of disease, as in the case of acute pancreatitis[1], these medicines are considered safe and their beneficial effects have also been intensively studied and described in hepatology[2].

INTESTINAL MICROFLORA

The average length of an average human adult intestine

is approximately 10 m and its irregular surface is covered with one layer of epithelial cells that represent a surface area of approximately 200 m^2. The intestinal microflora above all plays a very important role in the immune reactions of the body. During fetal development, the intestine is sterile and becomes colonized with the first microorganisms after the passage of the fetus through the birth canal. After birth, the intestine is very quickly colonized with various microorganisms, the composition of which is highly variable during the first few days of life. After the first week, the intestinal microflora achieves a stable composition that depends on the method of birth, environment and type of nutrition. In breast-fed infants, there is a predominance of bifidobacteria, while in infants on milk formula, the number of bifidobacteria can be several times lower. Breast-fed infants are therefore colonized sooner with bacterial strains whose composition resembles that of the intestinal microflora of an adult[3]. The initial colonization of the intestine also plays a very important role in further development of the individual, as the bacteria present may modulate gene expression of epithelial cells and thus create a favorable environment for themselves[4]. The primary colonizers are permanently settled in the intestine and determine intestinal colonization with further bacterial strains later in life, which are important for the final composition of intestinal microflora in adulthood.

Major changes in the features of the intestinal ecosystem occur after weaning[5]. During this period, anaerobic bacteria such as *Bacteroides spp*. and *Clostridium spp*. achieve a strong position and the intestinal ecosystem evolves into its stable form. The intestinal microflora contains a large amount of microbes that weigh more than 1 kg; this quantity exceeds the number of cells in the human body 10-fold. The microbial community of the intestine consists of more than 500 species, most of which have not been cultivated, and many that have not been identified so far. The intestinal microflora contains both bacteria that are fixed in the intestine (autochthonous, resident) and bacteria that only pass through the intestine (transient allochthonous)[6]. Most of the bacteria in the intestine form an anaerobic bioreactor that helps to digest difficult polysaccharides and synthesizes micronutrients including vitamins and short-chain fatty acids. The fermentation products of these bacteria can provide up to 10% of the daily energy needed by an individual[7]. The composition of human gastrointestinal microflora is given in Table 1.

The relationships between the host and their microflora bacteria also play an important role in postnatal development, maturation of the intestine and development of the mucosal immune system.

The intestinal microflora has many important functions, in particular to maintain the microbial barrier against established as well as potential pathogens, and furthermore, it influences the motility and perfusion of the intestinal wall, stimulates the intestinal immune system, and therefore also the so-called common mucosal immune system, reducing bacterial translocation and producing vitamins.

The digestive tract microflora is continuously influenced by numerous physical, chemical and biological factors that can affect its balance, and therefore it represents a constant potential source of digestive tract and whole-body disease. Changes in the total amount, localization, strain or species structure and in the metabolic activity of microorganisms may occur. Impairment of digestive tract microflora physiology can lead to disease or act as its cofactor (infectious, medication-associated, post-antibiotic and post-radiation diarrhea and colitis, functional diseases of the digestive tract-chronic constipation, irritable bowel syndrome, inflammatory bowel diseases, immunodeficiencies, colorectal carcinoma, some extraintestinal diseases and last, but not least, also liver diseases)[8]. In patients with liver cirrhosis, abnormal colonization of the small intestine with colonic bacteria has been reported, while the amount of these bacteria in the small intestine of healthy individuals is small. There is a reciprocal regulatory activity between intestinal microflora and the motility of the small intestine, where the motility is regulated by the presence of intestinal bacteria. Inhibition of gastric acid production induces bacterial overgrowth in the small intestine, whereas the overgrowth of its proximal part correlates with bacterial translocation into the extraintestinal space, such as the mesenteric lymph nodes, liver and spleen.

CHANGES IN INTESTINAL MICROFLORA IN PATIENTS WITH CHRONIC LIVER DISEASES

Intestinal bacterial overgrowth was described in approximately one-third of patients with alcoholic cirrhosis, ascites or advanced liver dysfunction. The main causes are considered to be anchylosis and hypochlorhydrosis, a decrease in IgA secretion and malnutrition caused by liver dysfunction, and possibly alcoholism. Also, the decrease in intestinal motility associated with cirrhotic liver damage facilitates bacterial overgrowth in the small intestine. The impaired immune mechanisms of the mucous membrane of the small intestine facilitating bacterial overgrowth can be one explanation of the repeated and common infections in patients with liver cirrhosis. In particular, the spontaneous infection of ascites-spontaneous bacterial peritonitis (SBP)-is a frequent and severe condition[9,10]. In contrast, suppression or eradication of intestinal facultative anaerobic gram-negative bacteria prevents their translocation and SBP, both in cirrhotic rats and in liver cirrhosis patients.

As a result of bacterial overgrowth, bacterial translocation may occur, and portal hypertension also plays an important role. It leads to vasodilation of the intestinal mucous membrane, edema of the lamina propria, fibromuscular proliferation and hypertrophy of the muscularis mucosae. Furthermore, the integrity of the intestinal mucous membrane is compromised; toxic influences of alcohol, disturbances in biliary secretion, malnutrition, decrease in growth factor secretion (insulin-like growth factor I),

Table 1 Composition of the human gastrointestinal tract microflora (from Nord and Kager, 1984)

Microorganisms	Numbers of microorganisms (CFU/mL or CFU/g)			
	Stomach	Jejunum	Ileum	Colon
Total bacterial count	$0\text{-}10^3$	$0\text{-}10^5$	$10^3\text{-}10^9$	$10^{10}\text{-}10^{12}$
Aerobically growing agents				
Family *enterobacteriaceae*	$0\text{-}10^2$	$0\text{-}10^3$	$10^2\text{-}10^7$	$10^4\text{-}10^{10}$
Streptococci	$0\text{-}10^3$	$0\text{-}10^4$	$10^2\text{-}10^6$	$10^5\text{-}10^{10}$
Staphylococci	$0\text{-}10^2$	$0\text{-}10^3$	$10^2\text{-}10^5$	$10^4\text{-}10^9$
Lactobacilli	$0\text{-}10^3$	$0\text{-}10^4$	$10^2\text{-}10^3$	$10^6\text{-}10^{10}$
Yeasts	$0\text{-}10^3$	$0\text{-}10^2$	$10^2\text{-}10^4$	$10^4\text{-}10^6$
Anaerobic bacteria				
Bacteroides	Rare	$0\text{-}10^3$	$10^3\text{-}10^7$	$10^{10}\text{-}10^{12}$
Bifidobacteria	Rare	$0\text{-}10^4$	$10^3\text{-}10^9$	$10^4\text{-}10^{11}$
Peptostreptococci	Rare	$0\text{-}10^3$	$10^2\text{-}10^6$	$10^{10}\text{-}10^{12}$
Clostridia	Rare	Rare	$10^2\text{-}10^4$	$10^6\text{-}10^{11}$
Eubacteria	Rare	Rare	Rare	$10^{10}\text{-}10^{12}$

changes in bile composition and flow or increased levels of nitric oxide may be present, as well as portal hypertension. The increase in intestinal permeability is conspicuously proportionate to the degree of portal hypertension, but it is independent of severity and etiology of liver impairment[11]. In patients with liver cirrhosis and portal hypertension, vascular resistance decreases and splanchnic flow increases. These changes lead to hypoperfusion and hypoxia of the mucous membrane further compromising the vascular wall. As a consequence of this, translocation of intestinal bacteria occurs easily[12].

The term bacterial translocation was used for the first time in 1979[13]. Bacterial translocation is defined as active or passive penetration of live microorganisms and their toxic products across the epithelial layer of the mucous membrane to the lamina propria mucosae. Microorganisms then migrate to the lymph nodes and/or into extraintestinal locations. Under normal conditions, this refers to the small amount of bacteria that are destroyed by the immune system of the lamina propria. Translocation is only possible when there are a high number of bacteria; the literature reports up to 10^8 bacteria in 1 g of stool[14].

Bacteria that escape phagocytes, as well as destruction by complement, can reach the circulation. *Enterobacteria, staphylococci* and *enterococci* are able to translocate, i.e. pass alive across the intestinal epithelium into the mesenteric lymph nodes, blood and other organs, while most other anaerobic microorganisms lack this ability. Bacterial translocation can be verified by positive cultivation from mesenteric lymph nodes. The main mechanisms leading to translocation include a deficit in the local immune response of the mucous membrane, a decrease in phagocytic activity of macrophages as well as neutrophils, an increase in the permeability of the intestinal barrier, and intestinal bacterial overgrowth[15].

Factors that influence bacterial translocation can be divided into 3 groups. These are the bacterial factor, comprising the nature of the translocating agent and the status of the surrounding physiological microflora, the morphological and functional state of the intestinal wall, and not least the so-called defensive factors, i.e. local and systemic antibacterial activities of the organism[16,17]. All of these systems are impaired in patients with liver cirrhosis[18].

PROBIOTICS

The history of probiotics started at the beginning of the last century with Metchnikoff[19]; however, German authors often report a study by Döderlein as the first description of a possible probiotic 16 years before Metchnikoff proposed the use of vaginal lactate-producing bacteria for the inhibition of pathogenic bacteria growth, and attributed the higher average age of certain ethnic groups to the increased intake of fermented milk products and recommended their use.

Probiotics were originally defined as "microorganisms causing growth of other microorganisms", and later on as "live microorganisms that cause or support the beneficial balance of autochthonous microbial population of the gastrointestinal tract (GIT)". These microorganisms do not have to be an essential permanent component of the GIT, but should have a "beneficial influence on the general and health status of an individual". Currently, probiotics are defined more precisely as "monocultures or mixed cultures of live microorganisms that, if administered to a person, positively influence the host by improving the properties of his/her own microflora"[20].

UTILIZATION OF PROBIOTICS IN HEPATOLOGY

In the Cochrane Library Review, there is currently no unambiguous recommendation for administration of probiotics in any indication in hepatology. According to the World Gastroenterology Organisation Practice Guideline "Probiotics and prebiotics" are probiotics in hepatology indicated only for hepatic encephalopathy[21], and in clinical practice, probiotics are now administered in principle only in the above-mentioned treatment of hepatic encephalopathy, with the disadvantage of a higher price compared to the standard treatment. The use of

probiotics in the treatment of non-alcoholic steatohepatitis and in prophylaxis of infections, or some complications in patients with liver cirrhosis, can be expected in the future.

Liver encephalopathy

It is believed that gut-produced ammonia plays a key role in the pathogenesis of hepatic encephalopathy because of the failure of the diseased liver to clear toxic products. Small intestinal overgrowth and delayed gastrointestinal transit time in cirrhotic patients plays an important role[22].

Lactulose and non-absorbable antibiotics currently hold a dominant position in the treatment of liver encephalopathy. One of the effects of lactulose may be a probiotic effect on lactobacilli that reduce the activity of bacterial ureases, resulting in a decrease in hyperammonemia. Probiotics can also have a similar effect and are already included in some recommendations for the treatment of minimal liver encephalopathy[23].

As early as the 1960s, the beneficial effect of *Lactobacillus acidophilus* was described on the course of liver encephalopathy in patients with liver cirrhosis[24]. In a more recent study on 97 patients, the beneficial effect of a synbiotic (mixture of a probiotic and prebiotic) on minimal liver encephalopathy was observed, with a decrease in ammonium levels as well as the improvement of symptoms of encephalopathy[25]. Minimal liver encephalopathy is described as an otherwise inexplicable impairment of cognitive functions such as prolonged psychomotor tempo, lack of attention, impairment of fine motor functions and the perception of visual sensations that can only be detected using special neurophysiological tests, and is present in 30%-70% of patients with liver cirrhosis without liver encephalopathy. In the treatment of advanced liver encephalopathy, a beneficial effect of *Enterococcus faecium* was observed, the administration of which led to an improvement in clinical status, electroencephalogram findings and a reduction of ammonium levels[26]; the treatment of the *Enterococcus* strain SF69[27] also had a similar effect. A mixture of probiotics[28] may have an even better effect. A combination of *Bifidobacterium* + fructo-oligosaccharides also demonstrated a significant reduction in the Trail Making Test B, a significant increase in the Symbol Digit Modalities Test and Block Design Test and improvement in some laboratory findings[29].

A study confirming improvement of liver encephalopathy during long-term administration of probiotic yogurts with the advantage of excellent adherence with potential for long-term adherence[30] is also of interest.

So far, there are only very few experimental studies, however, the studies comparing the efficacy of probiotic preparation *Golden Bifid* and lactulose on an experimental rat model of minimal hepatic encephalopathy induced by thioactamide showed excellent effects in lowering the level of ammonemia and endotoxemia, improving hepatic histopathology of rats, and decreasing the incidence of minimal hepatic encephalopathy[31].

The use of probiotics in common clinical practice as well as evaluation of the economic effect of the treatment will, however, require further studies. At the moment, a study titled "Probiotic *Lactobacillus* GG (LGG) in Patients with Minimal Hepatic Encephalopathy" is being conducted at Virginia Commonwealth University and we can only hope it will bring novel, positive results.

Effects on bacterial intestinal translocation, reduction in infections or prophylaxis of liver cirrhosis complications

Infectious complications are very frequently caused by bacterial strains that originate from the digestive tract. Bacterial overgrowth, as well as the associated translocation of microbes across the dysfunctional mucous membrane barrier, occurs for the above-mentioned reasons, especially in liver cirrhosis. The small gut of cirrhotic rats is contaminated by colonic microflora, which translocate to mesenteric lymph nodes, and is the most important cause of infection of ascites and spontaneous bacterial peritonitis. The identity of microbial strains in the gut, mesenteric lymph nodes and ascites was demonstrated by analysis of macrorestricted fragments DNA[32].

In 50%-70% of patients with liver cirrhosis, bacterial overgrowth occurs in the small intestine as a result of gram-negative colon microflora contamination. As a result, an impairment of the intestinal barrier with increased bacterial translocation occurs. Apparently, it is the most important point of entry of infection in cirrhotic patients; generally, infections are very common in liver cirrhosis and can be involved in many complications associated with the disease. In the first animal studies with short term application of *Lactobacillus* GG, the treatment did not prevent translocation of colonic microflora, although it was able to colonize the cecum in 90% of cirrhotic rats[33]. In later studies, preventive treatment with *Lactobacillus plantarum* inhibited an increase in permeability after subsequent application of *E. coli*[34], and *Lactobacillus johnsonii* La1 with antioxidants were able to decrease endotoxemia and prevent bacterial translocation in cirrhotic rats[35].

In clinical studies, administration of a symbiotic reduced the endotoxemia, which is an indicator of the degree of translocation[11]. In our study, a reduction in endotoxemia was achieved through administration of *E. coli* Nissle for 42 d[36]. The clinical significance of this has yet to be verified, but the beneficial influence on the prophylaxis of severe infectious complications, such as spontaneous bacterial peritonitis, can be expected.

In both the above-mentioned papers, an improvement in functional liver capabilities was observed, evaluated according to the Child-Pugh classification. It can be presumed that the recovery of physiological microflora in the digestive tract will reduce the liver load of toxic metabolites, above all endotoxin, which might potentially be absorbed. It stimulates secretion of cytokines. The cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 and interleukin-6 can influence the formation and degradation of the extracellular matrix, which is important for the development of liver fibrosis as well as during cirrhotic rearrangement[37]. Apart from the above-

mentioned decrease in endotoxin levels, a direct decrease in cytokine levels after administration of VSL#3 probiotic has also been reported[38]. There are some data showing, that Se-enriched *Lactobacillus* can intervene in carbon tetrachloride-induced liver injury in mice by enhancing macrophage function to maintain normal and beneficial effects, elevating antioxidant-enzyme activities, reducing lipid peroxidation reaction and inhibiting excessive release of TNF-α[39]. However, this extremely important finding will have to be confirmed. Another study with VSL#3, presenting a trend of a reduction in plasma endotoxin, on the other hand showed no change in the hepatic venous pressure gradient or intestinal permeability[40].

In liver cirrhosis of alcoholic etiology, the alcohol itself may play a role, such as increased gut permeability, endotoxemia, and TNF-α production[41]. In rats, *Lactobacillus* GG has been shown to reduce alcohol induced gut leakiness and steatohepatitis[42]. The same group also found that the mucosa-associated microflora was altered in rats on a high alcohol diet, and this dysbiosis could be counteracted by *Lactobacillus* GG or oat supplementation[43]. In a rat model of acute pancreatitis, synbiotic (*Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Bifidobacterium* in an enriched medium) and metronidazole were able to effectively protect against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption. The beneficial effect of synbiotics on liver histology seems to be correlated with endotoxemia. Metronidazole did not produce such a beneficial effect; in fact, it further worsened liver damage when alcohol was added to the background of ongoing acute pancreatic inflammation[44].

However, no large randomization study has been carried out as yet which would be relevant for clinical practice, although a recent study again confirmed the theoretic presumptions for beneficial action in this field[45]. The "Probiotics for the Prevention of Major Complications of Cirrhosis" study, carried out in the Meir Medical Center in Israel, was finished last year but its results have not yet been published.

Non-alcoholic steatohepatitis

Fatty liver disease that develops in the absence of alcohol abuse is increasingly recognized as a major health burden. Non-alcoholic steatohepatitis (NASH) was first described by Ludwig in 1980 as a disease that histologically mimicked alcoholic hepatitis and that also may progress to cirrhosis[46]. The diagnostic criteria for NASH continue to evolve and rely on the histologic findings of steatosis, hepatocellular injury (ballooning, Mallory bodies), and the pattern of fibrosis in patients with minimal intake of ethanol (< 20 g ethanol/d)[47]. Recently, NASH has been studied extensively as it is relatively frequent, and may lead to the development of liver cirrhosis. So far, treatment has not been established and probiotics may play an important role, as the bacterial overgrowth and associated increase of proinflammatory cytokines are important etiopathogenic mechanisms of NASH[48]. Clinical studies so far have been missing in this field, but the positive effect of probiotics has already been described in some laboratory studies. In mice with non-alcoholic liver steatosis, treatment with probiotics or anti-TNF antibodies improved the histological picture of the degree of damage to the liver parenchyma, led to a decrease in the alkaline phosphatase level, improved insulin resistance, and the content of total fatty acids in the liver also dropped. In another study on a model of non-alcoholic liver steatosis, treatment with the probiotic VSL#3 or TNF-α antibodies had a positive influence on histological findings, the fatty acids in hepatocytes were reduced, the ALT level decreased and the expression of TNF-α[49,50] was reduced. Similar data suggesting that VSL#3 administration could limit oxidative and inflammatory liver damage were published in a more recent study[51]. Some previous studies showed that a high fat diet caused obesity, hepatic steatosis and natural killer T-cell (NKT cell) depletion[52]. The VSL#3 was also shown to increase hepatic NKT cell number and reduce inflammatory signaling[53]. Another study indicated that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. It means, that VSL#3 effects on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver[54].

In the Cochrane Library Review, there is currently no unambiguous recommendation for administration of probiotics in NASH, even if the results from pilot studies seem promising. Randomized clinical trials are necessary to assess the clinical implication of probiotic therapy in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis[55]. Two clinical studies on the effects of probiotics on NASH are currently being conducted in Hong Kong and in Israel. Hopefully their conclusions will be encouraging.

Prophylaxis of infections after liver transplantation

In the past, several papers were published that confirmed the positive influence of probiotic administration on postoperative course after large abdominal surgery. The studies are mostly of small sample size and exhibit design flaws, but they showed statistically significant differences in infectious complications in favor of synbiotics[56-58], and the synbiotic group did require significantly less days of antibiotic therapy[59].

Patients in the postoperative period after a liver transplant are mainly at risk of infection by organisms, coming in most cases from the digestive tract. As already discussed in the theoretical part of our paper, the translocation of bacteria or their parts across the intestinal wall into the circulation occurs as a result of disturbances in barrier function of the intestine and disturbance of the immune system. Bacteria that translocated from the intestinal tract can be carried through the circulation into more distant systems and cause colonization or infection in extraintestinal locations. A prospective, randomized, double-blind study was published on 66 patients after liver transplantation, whereas half of the patients received a combination of 4 *Lactobacillus spp*. together with the standard enteral

nutrition. In the probiotic group, a significant reduction in postoperative bacterial infection (3% against 48%) was observed and the length of the antibiotic therapy was substantially reduced[60]. In our study on patients after liver transplantation, we demonstrated a correlation between an increase in endotoxin and the subsequent presence of phenotypically as well as genotypically identical strains (originally cultivated from the gastrointestinal tract) in an extraintestinal location[61]. Monitoring serum endotoxin levels can probably confirm bacterial translocation with an increased risk of infectious complications and this result further confirms the positive effect of probiotics in patients after liver transplantation. However, the conclusion is similar from the perspective of evidence-based medicine-use of prebiotics and probiotics in prevention of bacterial sepsis after liver transplantation is promising. Further randomized clinical trials are necessary.

CONCLUSION

Probiotics are becoming part of numerous therapeutic modalities in hepatology, as their effect on intestinal microflora can positively influence many liver diseases. However, verification of the efficiency of this treatment from the perspective of evidence-based medicine will be difficult, as different probiotics can be expected to have different effects in different diseases. With respect to the increasing number of studies on probiotics, the future prospects in this field are optimistic.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2897

World J Gastroenterol 2011 June 28; 17(24): 2897-2908
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Molecular biology of pancreatic cancer

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Supported by the Czech Ministry of Health Project 9809. It is a contribution No. 3 from CEGES (OPPK CZ.2.16/3.1.00/22213)
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Received: January 6, 2011 Revised: February 19, 2011
Accepted: February 26, 2011
Published online: June 28, 2011

Abstract

In spite of continuous research efforts directed at early detection and treatment of pancreatic cancer, the outlook for patients affected by the disease remains dismal. With most cases still being diagnosed at advanced stages, no improvement in survival prognosis is achieved with current diagnostic imaging approaches. In the absence of a dominant precancerous condition, several risk factors have been identified including family history, chronic pancreatitis, smoking, diabetes mellitus, as well as certain genetic disorders such as hereditary pancreatitis, cystic fibrosis, familial atypical multiple mole melanoma, and Peutz–Jeghers and Lynch syndromes. Most pancreatic carcinomas, however, remain sporadic. Current progress in experimental molecular techniques has enabled detailed understanding of the molecular processes of pancreatic cancer development. According to the latest information, malignant pancreatic transformation involves multiple oncogenes and tumor-suppressor genes that are involved in a variety of signaling pathways. The most characteristic aberrations (somatic point mutations and allelic losses) affect oncogenes and tumor-suppressor genes within RAS, AKT and Wnt signaling, and have a key role in transcription and proliferation, as well as systems that regulate the cell cycle (SMAD/DPC, CDKN2A/p16) and apoptosis (TP53). Understanding of the underlying molecular mechanisms should promote development of new methodology for early diagnosis and facilitate improvement in current approaches for pancreatic cancer treatment.

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Key words: Pancreatic cancer; Risk factors; Molecular biology; Pancreatitis; Diabetes

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Zavoral M, Minarikova P, Zavada F, Salek C, Minarik M. Molecular biology of pancreatic cancer. *World J Gastroenterol* 2011; 17(24): 2897-2908 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2897.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2897

INTRODUCTION

Pancreatic cancer is a fatal illness that is characterized by late diagnosis in the absence of early symptoms, which results in identification of the illness at an advanced stage. Despite all therapeutic efforts, the mortality of pancreatic cancer remains high, with the number of newly diagnosed patients nearly equaling that of deceased patients[1].

The incidence of pancreatic cancer is constantly on the rise, especially in regions of North America, Europe and Japan. In the United States, pancreatic cancer represents the fourth most frequent cause of death from cancer. In Europe it ranks fifth. In 2009, 42 470 new cases of this disease were diagnosed, and in the same year, 35 240 patients died of this disease[2]. One of the highest incidence and mortality rates among EU countries is observed in the Czech Republic, which, according to GLOBOSCAN 2008 data, ranks second in incidence (Figure 1A) and first in mortality (Figure 1B), followed by Hungary and Finland. In the Czech Republic, pancreatic cancer is the 10th most frequent malignancy, and accounts for 2.6% of all newly diagnosed neoplasms. In 2005, the absolute number of newly diagnosed pancreatic cancer cases was 901 in men and 876 in women. In that same year, the absolute number of deceased persons from the disease reached 1580 (819 men and 761 women)[3].

Five-year survival for pancreatic cancer is < 5%, mainly due to its late diagnosis, when it is already at an advanced stage[4]. At the time of diagnosis, < 5% of tumors are resectable. Median survival following surgical resection ranges from 13 to 21 mo. Without surgery, median survival is a mere 2.5-8 mo[2].

RISK FACTORS

Due to the relative rarity of pancreatic cancer, which is characterized by a complexity of underlying carcinogenesis, it is likely that a combination of multiple factors contributes to the initiation of the disease. Some factors, such as smoking or alcohol intake, can be controlled, while others such as age or family history cannot. Although most risk factors do not directly cause the disease, level of exposure often influences cancer development. As the treatment options are still limited and the survival prognosis remains poor, identification and evasion of the controllable risk factors becomes particularly important for individuals at high risk[5]. The most prominent pancreatic cancer risk factors are summarized in Table 1.

Smoking and alcohol

Cigarette smoking represents one of the most significant and most widely studied risk factors for pancreatic cancer. The carcinogenic effect of tobacco smoke on pancreatic tissue is explained as the direct action of N-nitrosamines or their secretion into bile and their subsequent reflux into the pancreatic duct. Active smoking

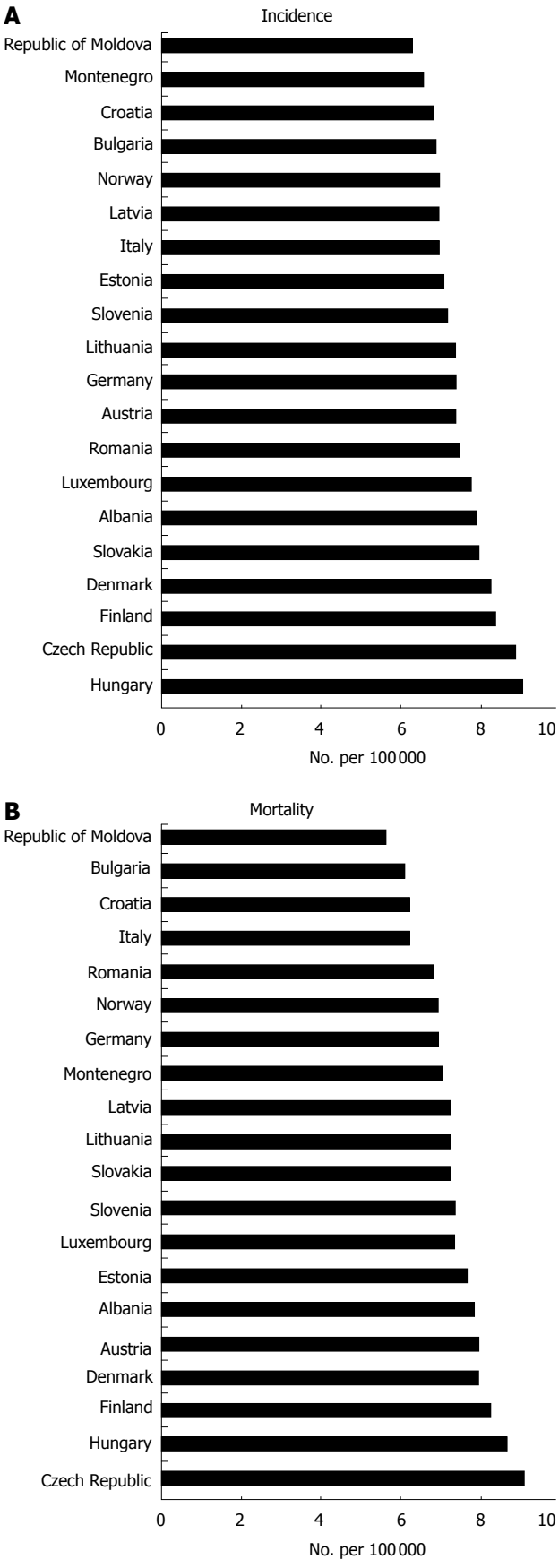

Figure 1 Standardized incidence (A) and mortality (B) rates for the 20 European countries with the highest occurrence of pancreatic cancer (Adapted from GLOBOCAN 2008).

Table 1 Pancreatic cancer risk factors

Factor	Type	Group	Maximum risk to average ratio
Smoking	Exogenous	Behavioral	3
Alcohol	Exogenous	Behavioral	Non-significant
Diet/obesity	Exogenous	Behavioral	1.72
Occupational hazard	Exogenous	Environmental	-
Radiation	Exogenous	Environmental	Inconclusive
Age	Endogenous	Biological	-
Race	Endogenous	Biological/ behavioral/ environmental	1.4
Family history	Endogenous	Genetic	32
Peutz-Jeghers syndrome	Endogenous	Genetic	132
FAMMM syndrome	Endogenous	Genetic	13.1
HNPCC	Endogenous	Genetic	7
Hereditary pancreatitis	Endogenous	Genetic	60
Chronic pancreatitis	Endogenous	Behavioral	26.3
Diabetes mellitus	Endogenous	Biological/ environmental	2.0
Hormonal	Endogenous	Biological	Inconclusive
Allergy	Endogenous	Biological	Non-significant

FAMMM: Familial atypical multiple mole melanoma; HNPCC: Hereditary non-polyposis colorectal carcinoma syndrome.

increases the relative risk of pancreatic cancer 1.5-3-fold, depending on the number of cigarettes smoked and the duration of this habit. Passive smoking has not been shown to be a risk factor[6].

A number of epidemiological studies have focused on the relationship between alcohol consumption and development of pancreatic cancer. An analysis of 14 prospective studies has not confirmed any association between alcohol consumption and a higher risk of pancreatic cancer[3]. Only a very weak association has been demonstrated in the case of alcohol consumption at a dose of $\geqslant$ 30 g/d, regardless of the alcohol source of beer, wine or spirits. There is also a significant association with body mass index (BMI), whereby a slight increase in cancer risk has been described in persons with an alcohol consumption of $\geqslant$ 30 g/d and a BMI of $\leqslant$ 25 kg/m^2. From the aforementioned, it is possible to infer that the association between alcohol consumption and development of pancreatic cancer is only implied.

This association is apparently conditional on the development of chronic pancreatitis, for which alcohol is a known inducer, and chronic pancreatitis is an independent risk factor for this cancer. It thus appears that alcohol consumption at lower doses that do not damage pancreatic tissue does not carry a higher risk for developing pancreatic cancer.

Diet and obesity

Based on a number of relevant studies, it is possible to observe an association with a diet rich in animal fats and higher consumption of meat (roasted, grilled or fried). The results of studies that focus on the effect of cholesterol, eggs, milk and dairy product consumption on increased risk of pancreatic cancer are inconsistent[7]. In contrast, a diet rich in fiber, fruit, vegetables and vitamins, especially vitamin C, is considered to be a protective factor[8]. Omega-3 unsaturated fatty acids that are contained mainly in fish oil also act protectively. A similar protective effect has been shown for substances that influence DNA methylation and synthesis, such as folates. As demonstrated in an analysis of 14 prospective studies, a positive association between alcohol consumption and cancer has been discovered for alcoholics with a low daily folate intake. Similar results have also been observed in the case of methionine. No association with coffee or tea consumption has been demonstrated[3].

Obesity is a generally recognized risk factor for pancreatic cancer in men and women[9]. In cases in which prospective studies have evaluated BMI, men with BMI $\geqslant$ 30 kg/m^2 had a higher relative risk compared to women with the same BMI[10]. One particularly interesting fact is that physical activity did not reduce the risk in persons with BMI < 25 kg/m^2, but it was indirectly proportional to the risk in persons with BMI $\geqslant$ 25 kg/m^2. Physical activity, which in its final effect leads to increased insulin resistance, decreases the degree of risk in obese patients.

Occupational hazards

The possible influence of aromatic and heterocyclic amines as well as exposure to chlorinated solvents in the carcinogenesis of pancreatic cancer remains unclear. The groups most at risk from this aspect include workers in the petrochemical and rubber industry, as well as barbers and hairdressers in whom exposure to these substances is higher compared to the general population[11]. In contrast, the influence of heavy metals and especially cadmium, in view of its accumulation in pancreatic tissue, has demonstrated an accentuation of neoplastic processes. Another element that is suspected of a carcinogenic effect on pancreatic tissue is chromium. Nonetheless, occupational exposure leads only to an imperceptible increase in the relative risk, in view of the minimal doses involved.

Radiation

A study published in 1990 has stated that ionizing radiation does not increase the risk of pancreatic cancer[9]. These findings are based predominantly on research in people who survived an atomic bomb explosion. The notion that the pancreas is relatively non-sensitive to ionizing radiation has been partially revised by a study of the increased incidence of pancreatic cancer among employees of nuclear research centers in the United States and other countries[12]. Nonetheless, this report[9] has stressed the significance of other concurrent risk factors such as smoking, diabetes, positive family history, or any other pancreatic disease.

Age and race

Age and race are the most prominent confounding factors of pancreatic cancer risk. During the first three decades, pancreatic cancer is a rarity, but from the age of 30 years onwards, its incidence increases significantly, peaking in the seventh to eighth decades, when 80% of adenocarcinomas are diagnosed. The mean age at diagnosis is 65

years. Pancreatic cancer is diagnosed before the age of 50 years only in 10% of patients[13].

With regard to racial differences, pancreatic cancer demonstrates the highest incidence in Afro-Americans in the United States, inhabitants of Northern Europe, in Polynesians in Hawaii, and Maoris in New Zealand. In the United States, the mortality of the Afro-American population is 1.4 times higher than that of the Caucasian population. This fact may be explained by a higher proportion of smokers and patients with diabetes, and at the same time, a positive family history of pancreatic cancer[14].

Hormonal factors and allergy

Pancreatic cancer demonstrates a different incidence in men and women. The cumulative risk in men is 0.2% and 0.1% in women. The lower incidence of pancreatic cancer in women may point to a link between hormonal factors and the development of cancer. The results of studies dealing with the relationship between female hormones and the development of cancer conducted to date have been inconclusive. As studies conducted to date confirm, this malignancy demonstrates minimal estrogen dependency. Parity and duration of hormonal exposure are negatively associated with the degree of risk of pancreatic cancer[15,16]. In the case of postmenopausal women, this risk is not influenced by hormonal substitution. In a number of studies, pancreatic cancer is associated with a higher number of deliveries, with earlier menarche and late menopause, higher age during the first delivery, or hormonal contraception. On the other hand, there exist studies in which the aforementioned factors were associated with a decrease in the risk of pancreatic cancer. An analysis of 10 case-control studies and five cohort studies did not demonstrate any link between hormonal factors and pancreatic cancer in women[17]. Indeed, additional factors, such as differences in life habits, may also contribute to the different cumulative risks between men and women.

Studies published to date have demonstrated a decrease in the risk of pancreatic cancer in individuals with allergies, especially respiratory allergy[18]. Longer survival has been described in allergic patients who have undergone resection procedures compared to resected patients with no allergies. The decrease in risk is most often associated with respiratory forms of allergy such as hay fever, and allergy to pollen and grass. The mechanisms of the possible protective effect of allergy in patients with a tumor is not exactly known[19].

CHRONIC PANCREATITIS

At present, acute and chronic pancreatitis are considered two pathogenically different disease entities; their relation to the genesis of pancreatic cancer is likewise quite different. Although acute pancreatitis is not considered a risk factor in terms of the index diagnosis, the concept of a causal association between alcoholic, hypercalcemic, trophic and hereditary forms of chronic pancreatitis and increased predisposition to developing pancreatic cancer is generally recognized. The obstructive type of chronic pancreatitis has been questioned as a risk factor by some authors[20].

Although no more than 5% of diagnosed cases of pancreatic cancer can be explained by recurrent attacks of chronic pancreatitis, the same genetic changes have been detected in individuals with chronic inflammation of the pancreas and pancreatic cancer. Chronic inflammation is thought to induce genetic alterations in tissue, while the ongoing healing process exposes defective cells to growth factors; the result is a pathological microenvironment in which stromal elements facilitate the neoplastic process in epithelial cells (the so-called feeder theory)[21]. According to this theory, the effector cells of the inflammatory response include active macrophages whose products, particularly the cytokines tumor-necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF) α and β induce cell proliferation, angiogenesis and desmoplastic reaction, that is, processes that form part of the pathogenesis of chronic pancreatitis and pancreatic cancer. IL-6 promotes the maturation of myeloid precursors into macrophages, and TGF-α inhibits processes leading to apoptosis and stimulates progressive fibrosis. Additionally, the same cytokine activates the transcription factor nuclear factor (NF)-κB, a mediator of inhibition of programmed cell death. Its expression has been reported in cases of chronic inflammation of the pancreas and pancreatic cancer. In addition, NF-κB upregulation promotes the production of reactive nitrogen oxide and cyclooxygenase (COX)-2 and induces IL-8 expression. There have been reports of an autocrine growth promoting effect of IL-8, which is produced at increased rates in response to hypoxia; particularly in the center of tumor foci, thus having a pro-carcinogenic and pro-metastatic effect. COX-2 responds to increased prostaglandin production in cases of inflammation and cancer, facilitates cell proliferation and angiogenesis, and is a potent inhibitor of apoptosis. Additionally, COX-2 is involved in the transformation of chemical carcinogens into their mutagenic derivatives. This particular ability of COX-2 increases the risk of pancreatic cancer in smokers. Immunohistochemical investigations have shown that COX-2 expression is bound to pancreatic islet Langerhan's cells; as a result, increased expression of the enzyme heralds islet inflammation.

One of the hypotheses of pancreatic cancer development is based on the key relations between islet of Langerhan's inflammation, insulin resistance, growth promotion, and diabetes. Additional products of activated macrophages and neutrophil granulocytes in pancreatitis and pancreatic cancer include reactive forms of oxygen and nitric oxide, whose presence is causally related to DNA structural defects[22]. The risk for developing a malignancy in individuals with chronic pancreatitis is 16 times that of the healthy population. One study has reported in patients with chronic pancreatitis, an increased incidence of both extrapancreatic tumors (relative risk, 1.5) and pancreatic malignancy (relative risk, 18.5). When one considers relevant only conditions that develop during 4 years of chronic inflammation, then the relative

risk increased by a factor of 15.6 for smokers, whereas there was no increase in non-smokers[23]. A prospective study of the French Cancer Registry, including 85% of cases of chronic pancreatitis of alcoholic etiology, has reported a relative risk of 19.0[24]. In a Czech study of 213 patients, 11 of whom had cancer, the prevalence of pancreatic cancer was 5.1%. The interval from establishing the diagnoses of chronic pancreatitis and pancreatic cancer was 6-13 years. The cumulative risk for malignancy in patients with chronic pancreatitis was shown to increase in a linear manner, and was 1.8 and as high as 4.0 after 10 and 20 years, respectively[25]. The priority of current clinical research is to identify patients with sporadic chronic pancreatitis who are at increased risk of developing pancreatic cancer.

GENETIC SUSCEPTIBILITY

Family history of pancreatic cancer

Familial pancreatic cancer (FPC) is defined as two or more first-degree relatives with pancreatic cancer. As an independent nosological unit, FPC represents only 3%-10% of the total number of pancreatic cancers. Nonetheless, the relative risk in such cases is 4.6-32 times higher, depending on the number of afflicted persons within the family[26]. Only 20% of FPC patients demonstrate a genetic abnormality. Nonetheless, individuals from families with FPC should undergo genetic testing for the presence of hereditary breast and ovarian syndrome (BRCA1, BRCA2). These mutations are most often identified in FPC. The relative risk of pancreatic cancer in carriers of the BRCA1 mutation increases to 2.26 and in the case of the BRCA2 mutation to 3.5-8[27].

Pancreatic cancer forms a component of a whole range of hereditary diseases and syndromes.

Cystic fibrosis is an autosomal recessive disease that is caused by mutations in the *CFTR* gene, and is characterized by the production of viscous mucus, which apart from blocking the airways, also leads to obstruction of the pancreatic duct, which increases the risk of inflammation. Patients with CF are at increased risk of chronic pancreatitis and of pancreatic tumors[28].

Familial atypical multiple mole melanoma (FAMMM) is an autosomal dominant disease that is characterized by the occurrence of > 50 atypical nevi and malignant melanoma in two or more first or second-degree relatives. Approximately 10% of melanomas have a familial incidence and the mutation of the *CDKN2A* gene is identified in -40% of these families[29].

Peutz-Jeghers syndrome is an autosomally dominant hereditary disease with characteristic hamartoma polyps of the gastrointestinal tract, and mucocutaneous melanin pigmentation. Almost half of these patients are carriers of a germinal *STK11/LKB1* gene mutation. Thus, afflicted individuals have a 36% risk (cumulative lifetime risk) of developing pancreatic cancer[30].

Hereditary non-polyposis colorectal carcinoma syndrome (HNPCC) is another hereditary cancer syndrome, for which the incidence of pancreatic cancer is typical. This syndrome is caused by mutations in mismatch repair (MMR) genes *MSH2*, *MLH1*, *MSH6* and *PMS2*. The average risk in carriers of MMR gene mutations is 5%-10%. Pancreatic cancer is approximately seven times more frequent in carriers of *MMR* gene mutations, and in these individuals, it is 15 times more often diagnosed before the age of 60 years[31]. Apart from the aforementioned, pancreatic cancer can occur in association with other diseases such as Li-Fraumeni syndrome, ataxia-teleangiectasia syndrome, multiple endocrine neoplasia type I syndrome (MENI) or Von Hippel-Lindau syndrome.

Hereditary pancreatitis

Hereditary pancreatitis is currently considered to be an independent nosological unit. This is an autosomally dominant disease with 80% penetrance. In patients with hereditary pancreatitis, trypsin becomes activated while still in the pancreas. This accounts for partial digestion of the pancreatic tissue, which causes irritation and inflammation. A strong genetic association exists with mutations found in the *PRSS1*, *SPINK1* and *CFTR* genes[32]. Patients with this hereditary pancreatitis have a 40-60-fold higher risk of developing pancreatic cancer. If such predisposed individuals are smokers, then the development of pancreatic cancer, or rather its diagnosis, shifts to younger age categories, in which it occurs up to two decades earlier than in non-smokers. Similarly, alcohol consumption also leads to earlier diagnosis of cancer, also 20 years earlier[2].

DIABETES MELLITUS

A mutual association between pancreatic cancer and diabetes mellitus has long been monitored. However, the issue of mutual linkage is complicated by the fact that, while long-term diabetes is considered to be an etiological factor of the cancer, newly developed diabetes is an early manifestation of the cancer[33].

The pathogenesis of diabetes associated with cancer and the biochemical mediators involved have not been completely elucidated. Its development due to the mere destruction of pancreatic tissue by the tumor or as a consequence of chronic pancreatitis is less probable. The high prevalence of diabetes and disorders of glucose tolerance in small, early carcinomas (< 20 mm), and primary detection of diabetes nearly 2 years before the diagnosis of carcinoma, points to the influence of humoral markers rather than to local effects of the tumor. Further research is necessary to clarify the pathogenesis of carcinoma-associated diabetes, and to uncover new markers that can differentiate it from type 2 diabetes[33]. Newly developed diabetes during a period of < 2 years prior to the diagnosis of carcinoma is a promising sign of the presence of a completely asymptomatic carcinoma. This is why screening of sporadic, early pancreatic cancer in persons with newly diagnosed diabetes is being considered. The interval between primary detection of diabetes and the diagnosis of carcinoma ranges between 5 and 29 mo[34].

Primary detection of hyperglycemia and diabetes represent a reference point for the timely diagnosis of sporadic pancreatic carcinoma before symptoms develop. It is well known that the symptoms of pancreatic cancer occur only weeks or months before diagnosis, which usually means that an advanced, non-resectable tumor is present and expected survival is only 4-6 mo. If we monitor glycemia in patients with small, resectable carcinomas (< 20 mm), then most suffer from disorders of glucose tolerance. Studies have demonstrated in 55%-65% of patients with resectable carcinoma, a disorder of glucose tolerance or newly developed diabetes during a period of < 2 years prior to the diagnosis of carcinoma[34,35]. Approximately half of patients with sporadic carcinoma suffer from diabetes, and in almost 50%, diabetes is diagnosed at the time of carcinoma diagnosis. It is highly probable that diabetes precedes the diagnosis of the malignancy by several months or even years. The aforementioned facts indicate the application of pancreatic cancer screening in asymptomatic individuals with newly diagnosed diabetes[33].

Studies conducted to date have shown that the prevalence of diabetes (determined on the basis of the oral glucose test, fasting blood glucose, and meeting American Diabetes Association criteria) in patients with pancreatic cancer is 45%-65%. In the original study, diabetes was newly diagnosed concurrently with carcinoma in 40% of cases[34]. In other studies, the percentage of newly diagnosed diabetes was as high as 74%-88%. In summary, it could be concluded that the majority of diabetes associated with pancreatic cancer represents *de novo* diabetes, that is, diagnosed during a period of 2 years preceding pancreatic cancer, and almost half of patients with early carcinoma have diabetes. Moreover, diabetes that develops in this way usually improves following pancreatic resection.

MOLECULAR MECHANISMS

Model of pancreatic neoplasia

Molecular mechanisms of solid cancer are very complex with different mechanisms taking place and affecting the tissue at different stages of the disease. Detailed molecular mechanisms of initiation, development and progression of pancreatic cancer have been thoroughly studied since the basic principles of the disease were revealed in the 1970s and 1980s[36-40]. The classic model of pancreatic cancer development describes morphological as well as molecular transformation from precursor lesions into invasive carcinoma[41]. The standard nomenclature and diagnostic criteria for classification of duct lesions has primarily been based on grades of pancreatic intraepithelial neoplasia (PanIN)[42]. The grades 1A, 1B, 2 and 3 represent growing cytological atypia characterized by loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. Each PanIN stage is characterized by a distinct pattern of molecular processes that are characterized by genetic irregularities that affect specific genes and genetic pathways.

Proto-oncogenes and tumor suppressors

In the PanIN model, genetic alterations have a fundamental role affecting key guardians of cellular signaling, which induces instability of entire molecular systems such as cell growth, division, apoptosis and migration. Proto-oncogenes code for proteins that act as positive regulators for these systems, such as growth factors, signal transducers, transcription factors or apoptotic inhibitors. Their mutated forms, oncogenes, are often present in cancer cells. The mutation causes the protein products of oncogenes to be permanently activated, which results in uncontrolled cell proliferation. Oncogenic mutations have a dominant character; therefore, deficiency of one allele (i.e. heterozygous mutation) is sufficient for a fatal outcome. There are several key proto-oncogenes involved in pancreatic cancerogenesis, including KRAS, CTNNB1 (β-catenin), PIK3CA or AKT1. The most common oncogenic mutation types are point mutation, deletion, gene amplification, and gene rearrangement.

Tumor suppressor genes code for proteins that act against cell proliferation, such as signaling inhibitors, negative transcription factors, activators of apoptosis, or members of DNA repair systems. As a result of genetic alteration, their normal function may be reduced or eliminated completely. Mutations in tumor suppressor genes have a recessive character; hence, the cell looses their function only when both alleles are affected. In the most common case, described as a double hit model, one allele is initially mutated while the other is subsequently mutated or lost completely[43]. A separate mechanism of tumor suppressor deactivation is by hypermethylation[44]. In pancreatic cancer, the frequently affected tumor suppressors include TP53, APC, SMAD4 and TP16. The genes most frequently mutated in pancreatic cancer are listed in Table 2[45,46].

Signaling pathways in pancreatic cancer c-MET/HGF signaling pathway

The c-MET/HGF (hepatocyte growth factor) signaling pathway is a key factor in early progression of pancreatic cancer. The pathway is responsible for invasive growth through activation of key oncogenes, angiogenesis and scattering (cell dissociation and metastasis). c-MET is a proto-oncogene that encodes an HGF receptor that has a primary function in embryonic development and wound healing[47]. Although c-MET mRNA is present at very low levels in normal human exocrine pancreas, it is upregulated in a majority of pancreatic cancers[48-50], as well as in pancreatitis-affected epithelial cells[51]. Overexpression of c-MET is also observed in regenerating tissue affected by acute pancreatitis[52], and it is seen as an early event in pancreatic cancerogenesis[51]. HGF is a primary ligand of c-MET. Upon c-MET/HGF interaction, several different signaling pathways are activated, including the Ras, phosphoinositide 3-kinase (PI3K), Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and β-catenin (Wnt) pathways.

Ras/Raf/MAPK pathway

The Ras/Raf/mitogen-activated protein kinase (MAPK) pathway is one of the most studied and best described signaling pathways in cancer[53]. The role of Ras/Raf/

Table 2 Genes most frequently mutated in pancreatic cancer[45,46]

Gene symbols	Protein name	Mutation frequency (%)	Type	Main signaling or system
KRAS	K-ras	58	Proto-oncogene	*Ras/Raf/MAPK*
TP53	Tumor protein p53	37	Tumor suppressor	*Apoptosis Cell cycle control*
CDKN2A	Tumor protein p16 (INK4A)	29	Tumor suppressor	*Cell cycle control*
CTNNB1	β-catenin	24	Proto-oncogene	*Wnt*
SMAD4 DPC4	Smad Dpc4	22	Tumor suppressor	*TNFbeta/SMAD*
APC	Apc	16	Tumor suppressor	*Wnt*
PIK3CA	Phosphoinositide 3-kinase	5	Proto-oncogene	*PTEN/PI3K/AKT*

DPC: Deleted in pancreatic cancer; APC: Adenomatous polyposis coli; MAPK: Mitogen-activated protein kinase.

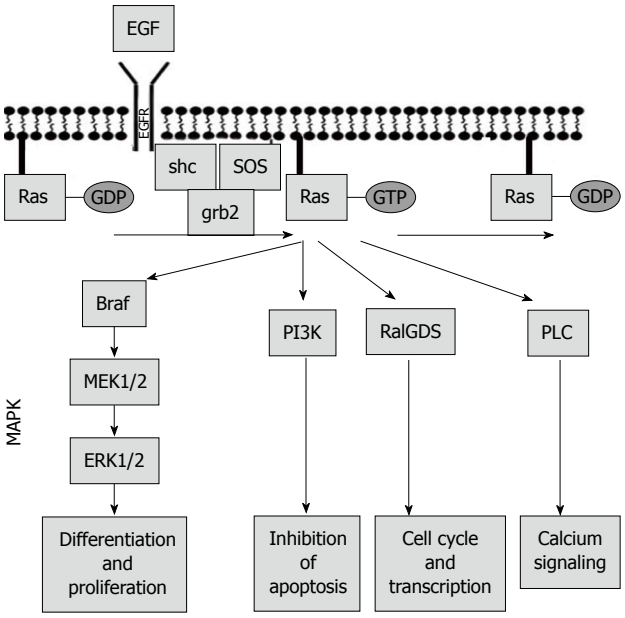

Figure 2 Ras/Raf/mitogen-activated protein kinase pathway.

MAPK signaling is critical for many cancerogenic processes, including cell growth and division, cell differentiation and migration, tissue healing and repair, and angiogenesis. The central regulator of the signal transduction from extracellular to intracellular environment is Ras protein, which is localized at the inner wall of the cellular membrane. Under normal physiological conditions, the hydrophobic Ras protein is inactive and bound to GDP. An extracellular signal coming through growth factor transmembrane receptors (such as growth factor receptors or cytokine receptors) promotes release of a guanidine exchange factor SOS, which initiates removal of GDP from Ras protein and its subsequent activation upon binding to GTP. Activated GTP-Ras complex triggers a kinase activity of Raf kinase, which ultimately results in activation of an MAPK, an important regulator of DNA transcription and mRNA translation. Mutations that affect any of the Ras/Raf/MAPK members produce an increase in tumorigenicity. Aside from Raf and MAPK, there are other downstream effectors of Ras protein, including PI3K, thus providing crosstalk between multiple pathways (Figure 2).

PTEN/PI3K/AKT pathway

PTEN/PI3K/AKT is a significant signaling pathway that is fundamentally based on regulated activation of AKT oncogene through its localization at the cell membrane[54]. The two important protein families involved in the membrane localization of AKT are PI3K and PTEN phosphatases.

PI3K is able to phosphorylate certain membrane-bound lipids known as phosphoinositides. The PI3K-mediated phosphorylation may progress in three stages, which produces phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol (3,4)-bisphosphate (PIP_2), and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The phosphorylated forms, PIP_3 and, to a lesser extent, PIP_2, attract important protein kinases to the cell membrane. The most prominent is AKT, a family of serine/threonine protein kinases that trigger a number of key cellular processes, including glucose metabolism, cell proliferation, apoptosis, transcription, and cell migration. AKT activity is strongly dependent on its proper localization on the cell membrane. The positioning of AKT at the membrane is achieved through its strong binding to PIP_3. In pancreatic carcinogenesis, AKT1 acts as an oncogene that upholds cell survival by overcoming cell cycle arrest[55-57], blocking apoptosis[58-60], and promoting angiogenesis[61]. PTEN is a phosphatase that acts in opposition to PI3K. It has tumor suppression ability by converting PIP_3 back to PIP_2 and to PIP, hence disrupting membrane localization and reducing activity of AKT[62]. In most cancers, expression levels of PI3Ks and AKT are high, while PTEN is often deactivated by mutation, or deleted completely. Through its key role in pancreatic carcinogenesis, PI3K/AKT/PTEN signaling is an important target for anticancer therapy.

JAK/STAT pathway

The JAK/STAT signaling pathway has an important role in regulation of DNA transcription by transmission of chemical signals from cytokine receptors into the cell nucleus. The signal is passed upon phosphorylation of receptor tyrosine residues by JAK prompting activation and dimerization in a family of STAT proteins. Activated STAT dimers initiate DNA transcription inside the nucleus. It is known that inhibition of JAK/STAT signaling induces apoptosis in various human cancers, and is there-

fore, a primary focus for potential new drug candidates[63]. A recent study has reported reduced growth of pancreatic cancer cells *in vitro* when exposed to benzyl isothiocyanate (BITC), due to its suppression of STAT3 signaling and subsequent induction of apoptosis. This is suggested as a possible explanation of the anticarcinogenic effect of cruciferous vegetables (such as broccoli, cauliflower, cabbage or horseradish) that are rich in BITC[64].

TGF-β/SMAD signaling

TGF-β is a ligand that binds to type II cytokine receptor dimer, which then binds and activates type I cytokine receptor dimer, which triggers phosphorylation of receptor-regulated SMADs (R-SMADs), mainly SMAD2 and SMAD3. In phosphorylated form, the R-SMADs form a complex with SMAD4, which accumulates in the nucleus and interacts with other factors to stimulate transcription of genes that are important for cell cycle arrest and migration. SMAD4 is therefore a key mediator for TGF-β signals. Due to its frequent absence in proliferating pancreatic cancer tissue, it is also known as DPC or "deleted in pancreatic cancer"[65]. Relatively high frequency of SMAD4 mutations and loss of heterozygosity at the DPC4 locus (18q21.1) strongly suggest that the protein is a primary tumor suppressor that is involved in pancreatic cancerogenesis. However, reinstating SMAD4 expression results in tumor growth suppression only *in vivo* and not *in vitro*. It has also been found that SMAD4-independent pathways may be responsible for tumorigenic effect of TGF-β signaling[66].

Wnt signaling

Wnt signaling is crucial to formation and maintenance of endocrine pancreas[67]. During pancreatic carcinogenesis, Wnt triggers transcription of a number of genes that have a direct impact on cell proliferation, differentiation and migration[68]. Activation of Wnt signaling is by interaction of a family of membrane-bound receptors known as Frizzleds with Wnt ligands. Once activated, the downstream signals may proceed through separate pathways. In a canonical pathway, signal transduction is mediated by stabilization and translocation of β-catenin from the cytosol into the nucleus followed by its interaction with T-cell factor (HMG box) which activates transcription of target genes. The localization of high expression levels of β-catenin at the nucleus has been experimentally confirmed for various high grade PanIN lesions, as well as in advanced pancreatic cancer[69]. In a non-canonical, β-catenin-independent pathway, other signal mediators are involved, which block the β-catenin-assisted transcription. The nuclear localization of β-catenin and high expression levels of WNT5a, a gene involved in non-canonical Wnt pathways, suggests involvement of both pathways in pancreatic cancer progression[68].

CDKN2A and cell cycle control

The cell cycle control genes have profound importance in cancer and CDKN2A is one of key factors in its negative control. The CDKN2A has two promoters and alternative splicing sites that result in two alternative protein products: cyclin-dependent kinase inhibitor p16INK4a and p53-activator p14ARF. Although both proteins are active in negative control of the cell cycle, only the function of p16INK4a is frequently lost in pancreatic tumors due to point mutations, deletions or hypermethylation[70]. p16INK4a protein (also known as p16) inhibits key elements of cell cycle progression at the G1 checkpoint. p16 inactivation is an early event in pancreatic carcinogenesis, and low levels of p16 expression are associated with larger tumors, risk of early metastases and poor survival[71].

MOLECULAR DIAGNOSTICS

A whole range of findings regarding the molecular biological basis of malignant transformation in pancreatic cancer has been published in recent years, and certain progress has been achieved also in the diagnosis, staging and treatment of localized tumors. In the fields of prevention, early diagnosis, screening and treatment of advanced tumors, which represent the majority of newly diagnosed cases, research has failed to provide any fundamental discoveries that would significantly affect the prognosis of patients with pancreatic cancer. Better understanding of the mechanisms of molecular genetics involved in pancreatic carcinogenesis has enabled the identification of a number of hereditary syndromes that in probands represent an increased risk of cancer. An overview is shown in Table 3[72].

Carbohydrate antigen (Ca) 19-9 retains its dominant role among tumor markers. It is the only marker to have been applied in clinical practice, where it is used to detect early recurrence in patients with an already known diagnosis and those undergoing treatment[73]. Use of Ca 19-9 as a screening test has yielded unsatisfactory results. This marker is not specific for pancreatic cancer and may be elevated in various cholestatic syndromes, and not necessarily a tumor. Moreover, the levels of secreted Ca 19-9 are affected by positivity of the Lewis antigen a and b[74]. Table 4 presents an overview of other biomarkers being studied as potentially useful in the diagnosis of pancreatic cancer[75].

Currently, none of the listed biomarkers meets the criteria of utility for the detection of early carcinoma. Even the promising marker PAM4 has demonstrated a sensitivity of 54% in early stage 1a carcinoma and 75% in stage 1b carcinoma[75]. The common denominator of the failure of all biomarkers in early detection lies in their low sensitivity; in some cases, associated with difficult or an invasive collection of biological material.

Detection of early pancreatic cancer in the general population using currently available means is impossible. Interest is now focusing on at-risk groups; especially those in whom the risk of developing this cancer is at least 10-fold higher compared to the general population. This risk may be stratified into low, intermediate and high[76]. Table 5 summarizes this risk stratification.

Several studies published recently have attempted to detect early and resectable carcinoma in high-risk groups with the aid of imaging methods[72,76]. Screening exami-

Table 3 Overview of hereditary syndromes predisposing to pancreatic cancer

Syndrome	Gene	Life-time risk	Relative risk
FAMMM	CDKN2A	10%-15%	20-34
HBOC	BRCA2	5%	10
HBOC	BRCA1	Not known	2
Hereditary pancreatitis	PRSS1/TRY1	30%-50%	50
Lynch syndrome	MLH1/MSH2	Not known	Not known
Peutz-Jeghers syndrome	STK11/LKB1	36%	136
FAP	APC	Not known	4
Li-Faumeni syndrome	p53	Not known	Not known
FPC	Not known	Up to 50%	18-57

APC: Adenomatous polyposis coli; FAMMM: Familial atypical multiple mole melanoma; FAP: Familial adenomatous polyposis; FPC: Familial pancreatic cancer; HBOC: Hereditary breast and ovarian syndrome. Adapted from[72].

Table 4 Overview of biomarkers in pancreatic cancer

Biomarker	Sensitivity (%)	Specificity (%)
CEA	45	75
Carcinoembryonic antigen-related cell adhesion molecule-1	85	98
Ca 19-9	80	73
SPan-1	81-94	75
DUPAN-2	48-80	75-85
Macrophage inhibitory cytokine 1	90	62
Alpha4GnT	76	83
PAM4	77	95
Pancreatic juice DNA methylation	82	100
Fecal K-ras	77	81

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; DUPAN-2: Pancreatic cancer-associated antigen 2; PAM4: Peptidylglycine alpha-amidating monooxygenase 4.

Table 5 Level of risk according to cumulative risk factors

Factors	Risk level
Race/sex: Male; black; Ashkenazi Jewish descent. Exposures: obesity; smoking; diabetes mellitus; *Helicobacter pylori* infection. Family history: Cancer history in a first-degree relative; history of pancreatic cancer in one first-degree relative. Inherited conditions: Hereditary non-polyposis colorectal cancer; familial adenomatous polyposis; *BRCA1* mutation carrier	Low (less than 5-fold)
Family history: History of pancreatic cancer in two first-degree relatives. Inherited conditions: cystic fibrosis; *BRCA2* mutation carrier. Comorbidity: Chronic pancreatitis	Moderate (5- to 10-fold)
Inherited conditions: FAMMM kindreds with p16 germline mutation and at least one case of pancreatic cancer in a first-degree or second-degree relative; hereditary pancreatitis; Peutz-Jeghers syndrome; *BRCA2* or *BRCA1* mutation carrier with at least one case of pancreatic cancer in a first-degree or second-degree relative. Family history: Three or more first-degree; second-degree or third-degree relatives with pancreatic cancer	High (greater than 10-fold)

FAMMM: Familial atypical multiple mole melanoma.

nations usually include some type of imaging method, such as endoscopic ultrasound, magnetic resonance imaging, computer tomography, or genetic tests. Successful identification of small tumors or precursor lesions, cystic tumors and intraductal papillary mucinous neoplasia is now possible in the at-risk population to a higher degree than in the general population. In cases in which solid ductal adenocarcinoma is uncovered, these lesions are usually resectable. Nonetheless, according to the available literature, recurrence has been demonstrated in all such patients over a period of several months.

In recent years, clinical research has focused on identifying patients with chronic pancreatitis with a high risk of developing pancreatic cancer. As discussed above, the following risk factors have been identified to date: smoking[23], duration of chronic pancreatitis[25], status after surgery for chronic pancreatitis in symptomatic individuals (recurrent pain, jaundice, weight loss, loss of appetite)[77], presence of a mutated form of the K-ras oncogene in a sample obtained using fine-needle aspiration biopsy or pancreatic duct brush cytology[78], loss of suppressor gene p16 expression[79], and polymorphism of the uridine diphosphate glucuronyltransferase gene (presence of the UGT1A7 allele causing low detoxification activity of the enzyme)[80].

In view of the completely different pathogenesis of acute and chronic pancreatitis and thus the different relationship to the development of pancreatic cancer, acute pancreatitis currently is not considered to be a risk factor for the development of pancreatic cancer. On the contrary, the association between alcoholic, hypercalcemic, tropical and hereditary chronic pancreatitis and the increased risk of pancreatic cancer is generally valid. The risk of developing pancreatic cancer in patients with chronic pancreatitis is up to 16-fold higher compared to the healthy population. K-ras mutations are detectable in nearly 80% of patients with carcinoma. Detection of K-ras mutations in patients with chronic pancreatitis may thus be used in combination with other methods as a screening test for the detection of early carcinoma[81].

As described above, much attention is also being paid currently to the relationship between newly diagnosed type 2 diabetes mellitus and pancreatic cancer, whereby diabetes is considered to be an early manifestation of pancreatic cancer, preceding the usual clinical manifestations of this malignancy. Patients with newly diagnosed diabetes will probably be considered to be at higher risk than they are today and will be screened. However, patients with diabetes who are suitable for screening will need to undergo multilevel selection, and the diagnosis of diabetes itself will represent the first filter of such a process. The second level should be the presence of one of the current biomarkers, or preferably the identification of a new marker with a higher predictive value. No

such marker is currently available, therefore, predictive computer models are also being envisaged.

Recent studies have demonstrated in pancreatogenic diabetes mellitus a protective effect of metformin for decreasing the risk of developing pancreatic cancer in patients with chronic pancreatitis. In contrast, treatment with insulin or its secretagogues increases the risk of carcinoma in these patients[82].

CONCLUSION

Uncovering the importance of basic risk factors such as chronic pancreatitis and diabetes mellitus, along with a detailed knowledge of fundamental molecular processes is expected to assist in reducing mortality of pancreatic cancer through development of new approaches for detection of early stages of the disease. This will mainly be applied to evaluation of the survival prognosis and rational selection of therapy; most importantly, with respect to options for radical surgical treatment. In addition, identification of specific genetic aberrations may serve as key molecular markers as predictors of response for targeted therapies. The response prediction should not only prolong survival, but also improve the quality of life for most advanced stages of the disease.

ACKNOWLEDGMENTS

Authors would like to thank Dr. Lucie Benesova for her assistance with detailed description of signaling pathways and Professor Marcela Kopacova for helpful discussions and comments on the manuscript.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2909

World J Gastroenterol 2011 June 28; 17(24): 2909-2923
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes

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Supported by The Cancer Association of South Africa, the National Health Laboratory Services Research Trust and the Medical Research council of South Africa
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Received: October 7, 2010 Revised: October 30, 2010
Accepted: November 6, 2010
Published online: June 28, 2011

Abstract

AIM: To identify molecular markers shared across South African esophageal squamous cell carcinoma (ESCC) cell lines using cytogenetics, fluorescence *in situ* hybridization (FISH) and single nucleotide polymorphism (SNP) array copy number analysis.

METHODS: We used conventional cytogenetics, FISH, and multicolor FISH to characterize the chromosomal rearrangements of five ESCC cell lines established in South Africa. The whole genome copy number profile was established from 250K SNP arrays, and data was analyzed with the CNAT 4.0 and GISTIC software.

RESULTS: We detected common translocation breakpoints involving chromosomes 1p11-12 and 3p11.2, the latter correlated with the deletion, or interruption of the *EPHA3* gene. The most significant amplifications involved the following chromosomal regions and genes: 11q13.3 (*CCND1*, *FGF3*, *FGF4*, *FGF19*, *MYEOV*), 8q24.21(*C-MYC*, *FAM84B*), 11q22.1-q22.3 (*BIRC2*, *BIRC3*), 5p15.2 (*CTNND2*), 3q11.2-q12.2 (*MINA*) and 18p11.32 (*TYMS*, *YES1*). The significant deletions included 1p31.2-p31.1 (*CTH*, *GADD45α*, *DIRAS3*), 2q22.1 (*LRP1B*), 3p12.1-p14.2 (*FHIT*), 4q22.1-q32.1 (*CASP6*, *SMAD1*), 8p23.2-q11.1 (*BNIP3L*) and 18q21.1-q21.2 (*SMAD4*, *DCC*). The 3p11.2 translocation breakpoint was shared across four cell lines, supporting a role for genes involved at this site, in particular, the *EPHA3* gene which has previously been reported to be deleted in ESCC.

CONCLUSION: The finding that a significant number of genes that were amplified (*FGF3*, *FGF4*, *FGF19*, *CCND1* and *C-MYC*) or deleted (*SFRP2* gene) are involved in the Wnt and fibroblast growth factor signaling pathways, suggests that these pathways may be activated in these cell lines.

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Key words: Esophagus; Cancer; Single nucleotide polymorphism arrays; Fluorescent *in situ* hybridization

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Brown J, Bothma H, Veale R, Willem P. Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes. *World J Gastroenterol* 2011; 17(24): 2909-2923 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2909.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2909

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a major cause of cancer-related death in the world and it is char-

acterized by a peculiar epidemiology with worldwide geographic pockets of high incidence. In South Africa, the region of the Eastern Cape shows the highest incidence and ESCC represents the leading cancer affecting men and the second most common cancer in woman with a prevalence of 31.3 and 18 per 100 000 individuals, respectively[1]. The Gauteng (Johannesburg) and the Western Cape regions are also affected but to a lesser extent[2,3]. The tumors are diagnosed at advanced stages of the disease and patients have a poor survival rate. The etiology of this cancer is unresolved and while the most common risk factors associated with ESCC include smoking and alcohol consumption, these factors are surprisingly lacking in some parts of the world that have a high incidence such as in Iran and northern parts of China[4]. Additional risk factors have been proposed to play a role in some regions. In particular, exposure to fumonisin, a Fusarium fungi toxin that grows on maize, was reported in South Africa and China[5,6] as well as human papillomavirus (HPV) infection[7]. Poor nutrition is associated with ESCC in most parts of the world[4], and chronic inflammation was described in endemic parts of South Africa[8]. The respective part played by environmental risk factors and potential genetic susceptibility remains unclear and it is possible that different combinations of factors may be at play in different parts of the world.

It is widely accepted that recurrent chromosomal breakpoints in malignancies often pinpoint genes involved in the initiation or progression of cancer[9]. A major limitation in assessment of the chromosome complement in ESCC specimens is the difficulty in obtaining metaphases from fresh tumors, and established cell lines provide a unique resource for such investigations. The number of ESCC cell lines that have been reported to date remains limited and were all established in China[10-12] and Japan[13]. These cell lines have been investigated with one or several low resolution molecular cytogenetic techniques including cytogenetics, fluorescence *in situ* hybridization (FISH), multicolor FISH (M-FISH) or SKY and conventional comparative genomic hybridization (CGH). Various clonal aberrations have been identified and the most common abnormalities across studies involved over representation of chromosomes 1q, 3q, 11q and 8q as well as breakpoints in the centromeric or near centromeric regions of chromosomes 1, 3 and 8[10-12,14].

Five cell lines have previously been established from South African ESCC patients[15,16] but apart from cell line SNO, which was karyotyped, these cell lines were never characterized for their genetic constitution. We used conventional cytogenetics, FISH, and M-FISH to identify common chromosome structural abnormalities across these cell lines. Affymetrix 250K single nucleotide polymorphism (SNP) arrays were performed to investigate DNA copy number changes and the common aberrations detected by M-FISH and conventional cytogenetics. Here we describe clonal aberrations shared by these cell lines and highlight preferential targets for chromosomal rearrangements and copy number changes. These are the first ESCC cell lines from Africa to be genetically characterized to our knowledge.

MATERIALS AND METHODS

Cell lines

The five esophageal carcinoma cell lines used in this study were previously isolated from male patients with moderately differentiated ESCCs[16]. These cell lines were previously described and are designated as WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines[15,16].

Cytogenetics

Cell lines were cultured at 37℃, 5% CO_2 in Dulbecco's Modified Eagles medium: HAMS F12 (3:1) (GIBCO®, Invitrogen Corporation, USA) containing 10% fetal calf serum, 100 μg/mL streptomycin (ICN, Costa Mesa, CA, USA) and 100 IU/mL of penicillin (ICN, Costa Mesa, CA, USA). When the cultures were half confluent, the cells were incubated with a final concentration of 0.44 μg/mL of Karyomax® Colcemid® (Invitrogen Corporation, USA) for 4 h. Cells were then harvested by standard cytogenetic procedure. Slides were either prepared for metaphase analysis using GTG banding by standard procedures or for FISH preparations.

M-FISH

M-FISH was performed using the SpectraVysion™ Assay (Vysis®, Abbott Molecular Inc, IL, USA) according to the manufacturer's protocol. The slides were analyzed on an Olympus BX41 fluorescent microscope with six single band pass filters for visualization of the six fluorophores. Genus™ CytoVision 3.0 software (Applied Imaging Corporation, San Jose, California, USA) was used for image acquisition and analysis. Ten metaphases were analyzed per cell line.

FISH

FISH was performed on metaphase chromosomes from all cell lines in order to confirm or refine translocation derivative breakpoints and clarify their composition. Probes specific for the short and long arms of chromosome 3, the short arm of chromosome 1 and the long arm of chromosome 22 (Qbiogene, Strasbourg, France) as well as the Vysis® Cep 3 Alpha and Cep 1 Alpha SpectrumOrange probes (Abbott Molecular Inc., IL, USA) were hybridized to further map the breakpoints. The Vysis® LSI IGH and Vysis® LSI RARA, both dual color break apart rearrangement probes (Abbott Molecular Inc., IL, USA) were used to confirm the involvement of chromosomes 14 and 15 in translocation derivatives seen in cell lines WHCO1 and WHCO3 respectively. In order to establish if the *EGFR* (epidermal growth factor receptor) gene was involved in a marker chromosome 7 the Vysis® LSI EGFR SO/CEP 7 SG probe (Abbott Molecular Inc., IL, USA) was hybridized to metaphase chromosomes.

FISH was also performed on interphase nuclei from all cell lines using the Vysis® LSI C-MYC SpectrumOrange

probe and the Vysis® LSI t(11;14) dual color probe (Abbott Molecular Inc., IL, USA). These probes target the *C-MYC* gene (Spectrum Orange) on 8q24 and the *CCND1* gene (Spectrum Orange) on chromosome 11q respectively and were used to confirm SNP array copy number results. A hundred interphase nuclei were analyzed in each cell line. All FISH experiments were performed according to the manufacturer's instructions and analyzed on an Olympus BX41 fluorescent microscope equipped with appropriate fluorescence filters. The Genus™ CytoVision 3.0 software was used for image acquisition and analysis.

DNA isolation

DNA was extracted from the cell lines and from six blood specimens obtained from healthy and population-matched volunteers using standard phenol-chloroform extraction methods. DNA was quantified on the ND-1000 Spectrophotometer (Nanodrop® Technologies, Rockland, DE) and quality assessed by gel electrophoresis.

SNP arrays and data analysis

Cell lines and control DNA were hybridized to the Affymetrix® GeneChip® Human Mapping 250 K Nsp Arrays (Santa Clara, CA). The GeneChip® mapping 500 K protocols were used. The control group used as a reference for analysis was DNA samples extracted from whole blood from black male volunteers.

Data analysis was performed using the Affymetrix®, Genotyping Console™ 2.0 and the copy number analysis tool (CNAT 4.0) software (Affymetrix®, Santa Clara, USA). Subsequently data were analyzed with third party software, Genepattern[17]. The signal intensities from the CEL files were normalized by the PM-MM (perfect match minus mismatch) probe intensity and quantile normalization against the median intensity of the controls. The raw copy number was then estimated as a ratio against the signal intensities of the normal reference samples. The log2ratios were smoothed using GLAD (Gain and Loss analysis of DNA)[18].

To determine the significant common regions of amplification and deletion (i.e. driver aberrations as opposed to random passenger aberrations) across the five cell lines, the GISTIC (Genomic Identification of Significant Targets in Cancer) algorithm[19] was applied to the smoothed data. The algorithm first scores the regions of copy number change according to their frequency and amplitude, which indicates the likelihood of these regions to represent a driver aberration (G-score). The statistical significance of each G-score is calculated by comparison of these scores against a null model of random aberrations. This significance is represented as a q-value (False discovery rate), which is the likelihood that the data was generated by chance. The most probable locations for oncogenes or tumor suppressor genes are identified by calculating the minimal common regions of aberration, which are most significantly altered i.e. high amplitude change. The regions of aberrations with high G-scores and minimal q-values (less than 0.25) are therefore more likely to contain target genes that have a significant role in carcinogenesis. The chromosomal regions are then classified as high or low level where low level amplification is considered for a log2ratio greater than 0.1 but smaller than 0.9 and high level amplification is considered for a log2ratio > 0.9 (0.9 corresponds to at least 3.7 copies per diploid cell). Similarly low level deletions (hemizygous) were considered for a log2ratio of -0.1 and homozygous deletions were considered for a log2ratio < -1. The GISTIC algorithm has been used for copy number analysis in previous studies[20,21].

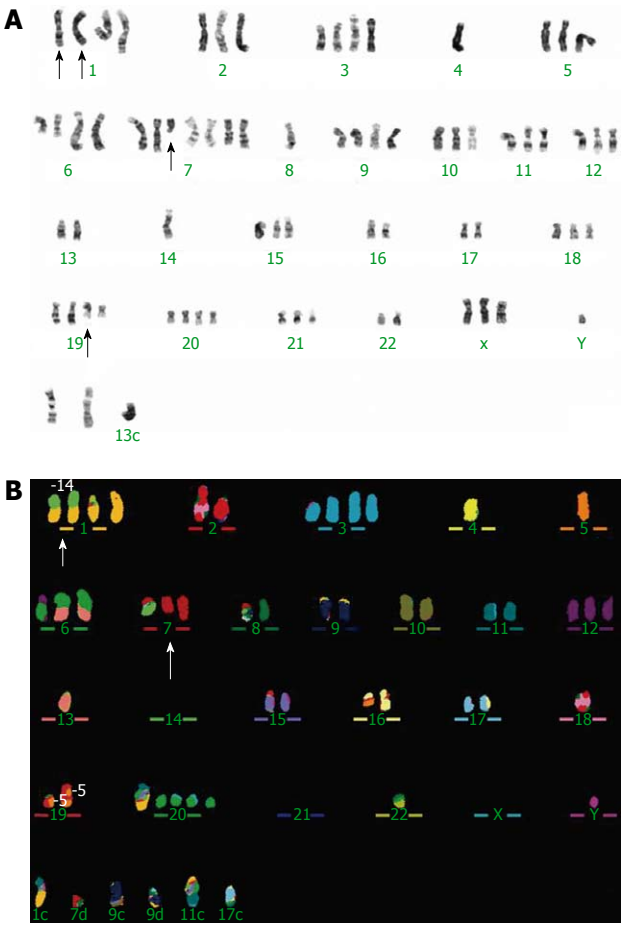

Figure 1 G-banded and multicolor fluorescence *in situ* hybridization representative karyotypes of cell line WHCO1. A: G-banded karyotype; B: Multicolor fluorescence *in situ* hybridization (M-FISH) karyotype, the arrows indicate the marker chromosomes, der(1) t(1;14)(p11;q11) and del(7)(q21).

RESULTS

Cytogenetics and M-FISH

Cytogenetic analysis revealed complex numerical and structural chromosome aberrations in all cell lines with a high variability observed between cells from the same cell line (Figures 1 and 2). The G-banded karyotype of cell line WHCO1 and a corresponding M-FISH karyotype that illustrates the degree of variation from one metaphase to another are shown in Figure 1. The M-FISH data confirmed the complexity of the karyotypes and revealed

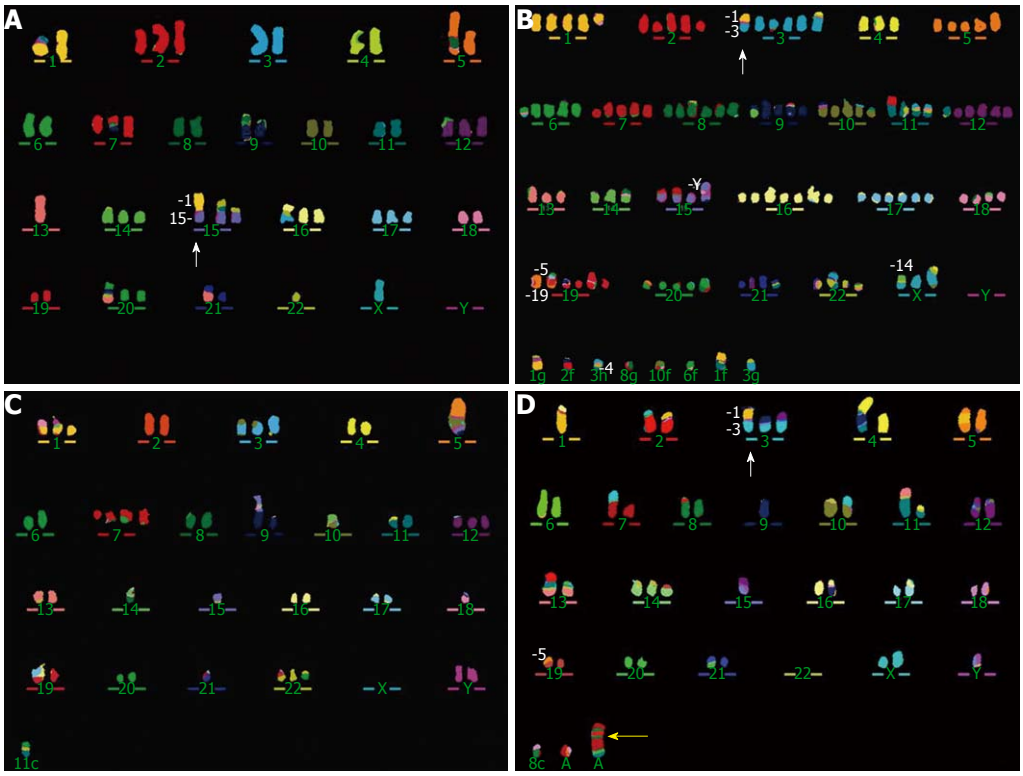

Figure 2 Multicolor fluorescence *in situ* hybridization representative karyotypes showing complex rearrangements in four cell lines. A: Cell line WHCO3, the white arrow indicates the der(15)t(1;15)(q11;p11); B: Cell line WHCO5, the white arrow points to the der(3)t(1;3)(p11-12;q11); C: Cell line WHCO6; D: Cell line SNO, the white arrow points to the der(3)t(1;3)(p11-12;p11) and the yellow arrow indicates the marker chromosome 7, mar(7), which involves the *EGFR* (epidermal growth factor receptor) locus (Figure 6).

the genetic composition of recurrent chromosome markers that could not be identified on G-banded metaphases (Figure 2); some of these markers are further discussed below. The detailed composite karyotypes obtained from twenty metaphases in each cell line are summarized in Table 1. All cell lines were near diploid except for cell line WHCO5 which was near tetraploid (Figure 2). Across the five cell lines a total of 97 translocations, 19 trisomies and 11 monosomies were detected. The breakpoints amounted to 203, with 78 of these clustering around the centromeric regions of chromosomes. The chromosomes involving the highest number of abnormalities were chromosomes 1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20 and 22. G-banded metaphases also revealed the presence of isochromosomes involving the D group acrocentric chromosomes in all the cell lines. In particular isochromosome 13q, i(13)(p10) was common to cell lines WHCO3, WHCO5 and WHCO6, isochromosome 14q, i(14)(p10) was common to cell lines WHCO1, WHCO5 and WHCO6, and isochromosome 15q, i(15)(p10) was seen in cell lines WHCO3 and WHCO5.

The combined results of cytogenetics, FISH and M-FISH revealed a common translocation derivative, der (3)t(1;3)(p11;q11) that combined chromosome 3 and chromosome 1 short arms in cell lines WHCO5 and SNO (Figures 2-4). The SNP array copy number data did not bring further information on these breakpoints, possibly due to a lack of SNP probes in this region.

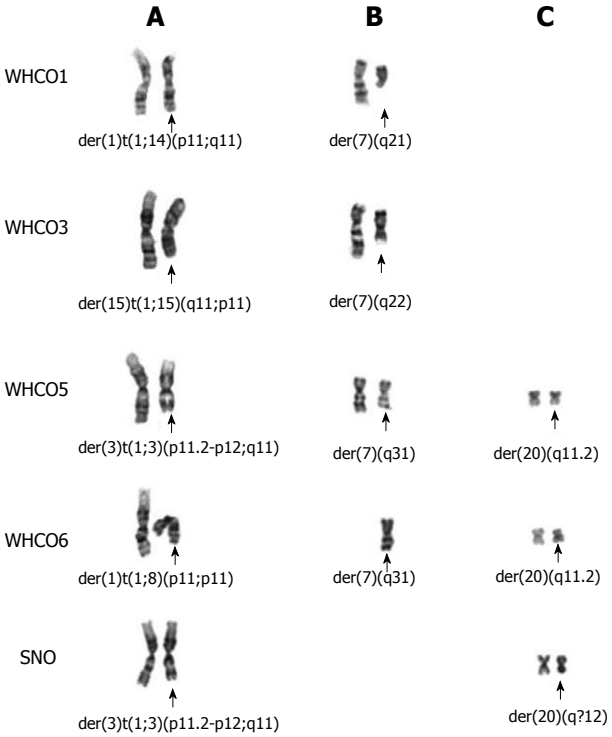

Figure 3 Partial G-banded karyotypes of the five cell lines showing shared chromosomal aberrations across cell lines. Chromosome derivatives from unbalanced translocations involving (A) chromosome 1p11-12 breakpoints in four cell lines, 1q11 in cell line WHCO3, (B) deletions of chromosome 7q in four cell lines, and (C) deletions of chromosome 20q in three cell lines.

Table 1 Composite karyotypes

Cell line	Composite combined cytogenetics and M-FISH karyotype
WHCO1	42~73, XY, +X, +X [4], +1 [10], del 1(?) [4], + der(1)t(1;14)(p11;q11) x2 [18], t(1;14;22)(?;?;q?) [4], t(1;22)(?;q?) [6], + 2 [8], t(2;8)(?;?) [4], +3 [8], +del 3(p21p13) x2 [4], der(3;22)(q10;q10) [4], -4 [8], t(5;21)(?;?) [8], + der(5)t(5;?)(q23;?) [4], + del(6)(q?21) x2 [4], t(6;12)(?q;?p) [16], t(6;13)(?q25;?q14) x2 [18], +7 [4], + del(7)(q21) [4], + der(7;14)(p10;q10) [20], der(7)t(7;18)(p21;q23) [16], der(7)t(7;19)(p22;p13) [4], der(8)t(8;?19)(q?24;q?11) [4], t(8;22)(?;?) [18], +9 [10], + der(10)t(9;10)(q13;q?11.2) [4], +11 [8], t(11;17;20)(?;?;?) [4], +12 [10], t(13;22)(?;?) [4], add(14)(q?) [4], i(14)(q10) [6], der(15;19)(q10;q10) [20], der(16)t(5;16)(?;?) [8], +18 [4], der(19)t(5;19)(p?12;q11) [12], der(19) t(5,19)(q33;q13.4) [18], + der(19)t(9;19)(q?13;q?13) x2 [4], t(19;21)(?;?) [14], +20, +20 [20], + der (20)t(1;11;20)(?;?;?) [4], +1~7mar [20] [cp20]
WHCO3	46~50, X, -Y, +2 [12], der(5)t(5;8,18)(q?;?;?) [20], + del 7(q22) [4], der(7)t(7;9)(?;?) [6], t(7;9;16;18)(?;?;?;?) [14], t(7;15)(?;?) [4], +12 [6], der(12)t(6;12)(?;?) [16], i(13)(q10) [6], t(13;14)(?;?) [6], t(13;14;20)(?;?;?) [6], +14 [12], + der(15)t(1;15)(q11;p11) [6], + der(15)t(1;15;11)(?;?;?) [10], der(15;22)(q10;q10) [20], i(15)(q10) [4], der(16)t(3;16;22)(p?11.2;?;q?10) [20], +17 [10], der(?20)t(9;13;20)(?;?;?) [10], der(21)t(13;21)(?;?) [12] [cp20]
WHCO5[1]	99~108, XY, t(X;4;10.22)(?;?;?;q?) [14], t(1;19)(?;?) [8], t(1;18)(?;?) [6], del 2 [2], der(2)t(2;9)(q12;q13) [10], t(2;9)(?q31;?q34) [8], +der(3)t(1;3)(p11-12;q11) [12], t(3;11;13;22)(?;?;?;?) [8], t(3;11;22)(?;?;?) [16], t(3;22)(p11;q11) [12], der(5;20)(p10;p10) [4], t(6;13)(?;?) [4], del(7)(q31) [4]; t(8;14;18)(?;?;?) [16], t(8;18)(?;?) [10], der(9)t(9;14)(?;?) [16], t(9;15)(q?;q?) [10], t(9;19)(?;?) [6], t(12;19)(?;?) [4], i(13)(q10) [6], i(14)(q10) [8], der(15)t(Y;15)(?;?)[12], der(15)t(7;15)(?;?) x2 [14], i(15)(q10) [4], der(19)del(19)(q13.?2)t(5;19)(p?12;p11) [12], del(20)(q11.2) [12] [cp20]
WHCO6	44~54, Y, -X, der(1)t(1;8)(p11;q11) [14], t(3;10)(?;?) [4], t(3;10)(?q13.3;p10) [6], t(4;10)(?;?) [4], t(5;10)(?;?) [10], t(5;22)(?;q?) [4], +6 [2], del(6)(q?21) [4]; t(6;11)(p12;q13) [6], der(22)t(6;22)(?;?) [6], +7[7], del(7)(q31) [8]; +8 [8], t(9;15)(?;?) [10], t(10;14)(?;?) [6], -11 [6], t(11;22)(p?;q?) [6], +12 [10], i(13)(q10) [6]; i(14)(q10) [6], +16 [8], t(17;19)(?;?) [10], +18 [4], del(20)(q?11.2) [6]; -21 [12], t(21;22)(?;?) [6], der(22)t(6;22)(?;?) [6], [cp14]
SNO	29~43, XY, +X, del X(?) [14], der(Y;15)(q10;q10) [12], t(1;16)(?;?) [14], der(2)t(X;2)(?;?) [16], der(2)t(1;2)(?;?) [18], der(3)t(1;3)(p11-12;q11) [20], t(3;9)(?;?) [4], t(3;10)(?;?) [14], t(3;12)(?;?) [20], t(3;20)(?;?) [8], t(4;9;11)(?;?;?) [20], t(4;11)(?;?) [12], der(5)t(1;5)(?;?) [20], -6 [18], del(6)(q?23) [20], der(7)t(3;7)(?q25;p22) [18], t(7;20;11;8;2)(?;?;?;?;?) [18], der(8)t(2;8)(?;?) [14], t(8;18)(?;?) [8], -9 [10], t(9;18)(?;?) [4], t(10;22)(q?;q?) [12], t(11;13)(?;?)[8], t(12;15)(?;?)[8], t(12;21)(?;?)[20], t(13;11;20)(?;?;?) [14], der(14;22)(p10;q10) x2 [20], t(14;19)(?;?) [20], -16 [14], der(16)t(9;16)(q?22;q?13) [20], der(17)t(6;17)(?;?) [12], -18 [16], -19 [16], der(19)del(19)(q13.?2)t(5;19)(q?12;q10) [16], +20 [10], del(20)(q?12) [16]; der(20;21)(q10;p10) [6], -21 [18], -22 [20] [cp20]

[1]Only structural rearrangements are listed due to the complexity of this cell line.

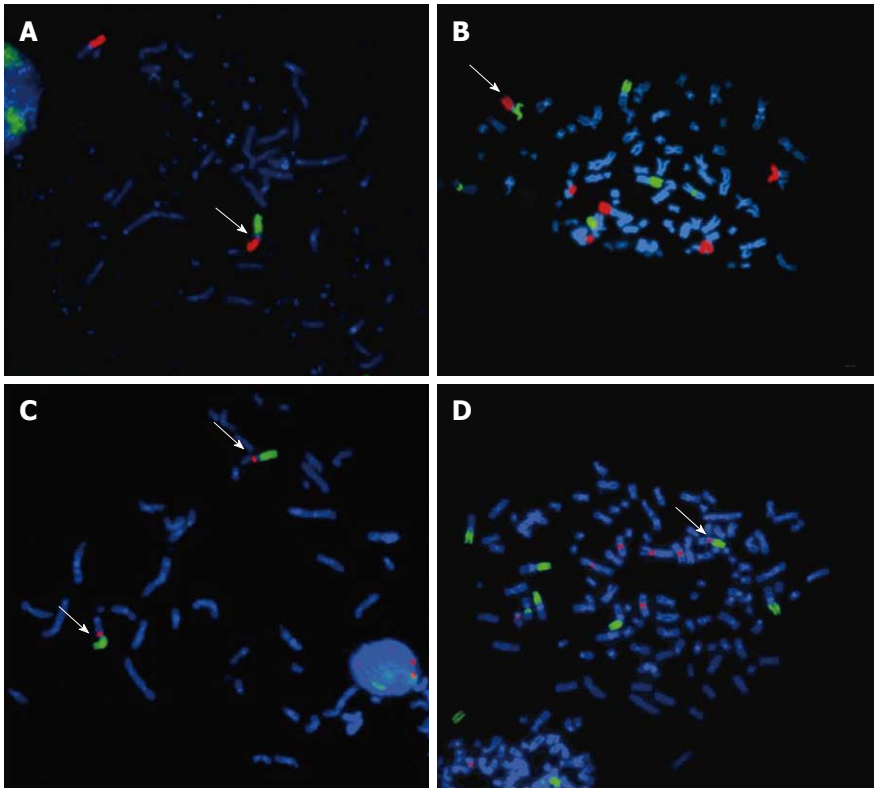

Figure 4 DAPI stained metaphasic chromosomes showing the der(3)t(1;3)(p11-12;q11) in cell lines SNO and WHCO5. A, B: Arm-specific paint for chromosomes 1p (green) and 3p (red) in cell lines SNO and WHCO5 respectively showing the derivatives der(3)t(1;3)(p11-12;q11) arrowed; C, D: Arm specific paint for chromosome 1p (green) and Cep 3 α probe (red) in cell lines SNO and WHCO5 respectively showing that the centromere of chromosome 3 is retained on the derivatives der(3)t(1;3)(p11-12;q11).

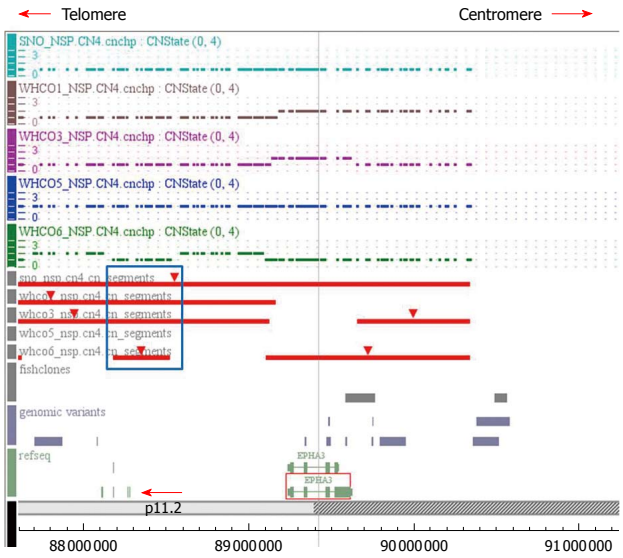

Figure 5 CNAT plot of single nucleotide polymorphism hybridization results showing the map of the region of deletion on chromosome 3p11.2-12.1 in cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO. The red bars indicate the segment of deletion as determined by CNAT 4.0. The dotted lines indicate the copy number status for each single nucleotide polymorphism probe for each cell line. The blue block indicates the minimal common region of deletion and the red arrow indicates the two genes in this region, *c3orf38* and *CGGBP1*.

Interestingly, the 1p11 breakpoint was also involved in cell lines WHCO1 and WHCO6 in differing unbalanced translocations (Figure 3). The M-FISH and FISH results with probes for the respective partner chromosomes and centromeric Cep1, confirmed the interpretation of these derivatives as der(1)t(1;14)(p11;q11) in cell line WHCO1 and der(1)t(1;8)(p11;p11) in cell line WHCO6 (results not shown). In contrast, a translocation derivative also involving the chromosome 1 pericentromeric region in cell line WHCO3 (Figure 3) was shown not to involve 1p11 but 1q11, and was interpreted as der(15)t(1;15)(q11-12;p11) (result not shown).

Translocation derivatives involving chromosomes 3 and 22 were seen in cell lines WHCO3 and WHCO5 and were interpreted as der(16)t(3;16;22)(p11;?;q?11) and t(3;22)(p11;q11) respectively (Table 1). The array data showed corresponding hemizygous deletions at 3p11.2, in cell line WHCO3. Interestingly deletions at 3p11.2 were also observed in cell lines WHCO1, SNO and WHCO6. The minimal region of overlap was 343 kb (88 184 220-88 527 215 bp) in size and involved *c3orf38* and CGG triplet repeat binding protein (*CGGBP1)* genes. In addition the *EPHA3* gene was deleted in cell lines WHCO6 and SNO (Figure 5). The *EPHA3* gene encodes a tyrosine kinase, which is mutated in lung and breast cancers[22-24].

Cell lines WHCO1, WHCO3, WHCO5 and WHCO6 all showed a deletion of chromosome 7 long arm with varying breakpoints, q21 to q31, on G-banded metaphases (Figure 3). However GISTIC analysis of the SNP array data identified a significant common focal deletion (q-value of 0.09) of approximately 5.16 Mb at 7q33-q34 (133 721 542-138 880 555 bp) in only three of these cell lines. This region contained 26 genes including the potential target gene homeodomain-interacting protein kinase-2 (*HIPK2)* the product of which activates p53 expression and is pro apoptotic[25].

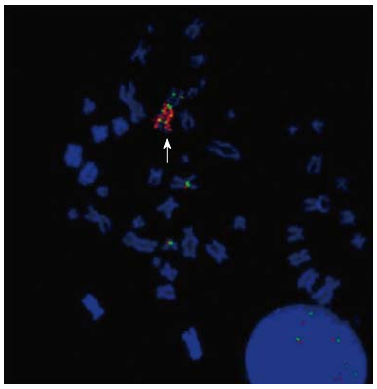

Figure 6 DAPI stained metaphase from cell line SNO showing fluorescence *in situ* hybridization results with the locus specific EGFR probe (red) and Cep7 α (green). The arrow indicates the chromosome 7 marker with a homogeneously stained region that contains the *EGFR* locus.

Although deletions at 20q11.2 were detected in cell lines WHCO5, WHCO6 and at 20q12 in cell line SNO on G-banded metaphases (Figure 3), copy number analysis (CNAT) revealed that there was in fact amplification of 20q sequences in all cell lines implying that complex rearrangement occurred for these sequences to be relocated elsewhere in the genome. Two minimal regions of amplification were identified, the 20q11.21-q11.22, of approximately 1.12 Mb in size (31 799 867-32 906 584 bp), which contained 14 genes and a smaller region at 20q13.12 of 149.48 kb which contained five genes. Both these regions were amplified in all cell lines, yet these amplifications were not found to be significant on GISTIC analysis.

Cell line SNO showed a large marker chromosome 7 on metaphases analyzed by M-FISH. This marker was interpreted as a possible inverted duplication of chromosome 7p sequences (Figure 2D). FISH with an *EGFR* probe, revealed a high amplification of the *EGFR* gene in 14% of the cells (Figure 6). A high level amplification at 7p13-7p11.2 (genomic location of *EGFR*) was confirmed by GISTIC (q-value 0.14) on array analysis in this cell line, while low level amplification was observed in the remaining four cell lines in agreement with the presence of 4 to 7 copies on FISH analysis.

Copy number changes

In view of the high cell to cell heterogeneity in each cell line, and in order to separate the potential driver aberrations out of the background of aberrations that may have occurred by chance, we used the software GISTIC specifically designed for this purpose. Target genes, defined as genes whose alteration confers a cell growth advantage, are likely to reside in the regions amplified or deleted to the highest degree in a majority of cells and within a common region of overlap amongst all cell lines. Fourteen common regions of amplification and 20 regions of deletion were

Table 2 Amplification peaks as detected by the GISTIC algorithm

Cytoband	Location (kb)	Approx size (Mb)	Frequency (*n*/5)	Mean log2ratio[1]	q-value	Genes[2]
1p34.2	Chr1:39027237-40780163	1.75	3	1.10	0.18	*MYCL1*
3q11.2-q12.2	Chr3:95917505-101945216	6.02	4	1.40	0.17	*MINA*
5p15.2	Chr5:10051329-11800765	1.75	2	1.36	0.09	*CTNND2*
7p11.2-p13	Chr7:45294289-57299457	12.01	1	1.24	0.13	*EGFR*
8q24.21	Chr8:127445828-129661846	2.22	5	2.10	0.0007	*MYC*
9q31.1	Chr9:100905143-102152148	1.25	1	2.57	0.15	
10q12.33-q21.3	Chr10:17811791-65388337	47.58	1	3.05	0.13	
11q13.3	Chr11:68753086-69985447	1.23	4	1.81	5.76E^{-05}	*CCND1, CTTN, FGF3, FGF4, FGF19, MYEOV*
11q22.1-q22.3	Chr11:100815801-103042620	2.23	2	2.35	0.03	*BIRC2, BIRC3, YAP1*
18p11.32	Chr18:1-1118244	1.12	2	1.04	0.13	*TYMS, YES1*
20p11.1-p11.22	Chr20:22140447-26145930	4.01	2	1.24	0.13	*PYGB*
22q11.21	Chr22:16558724-17937900	1.38	2	1.20	0.21	*BID, CLDN5*
22q11.21-q11.22	Chr22:18577713-20667607	2.10	2	1.20	0.13	*CRKL, MAPK1*
22q12.3	Chr22:31889314-32003182	0.11	2	1.15	0.13	*LARGE*

[1]The mean log2ratio for the samples with a log2ratio > 0.9 (equivalent to 3.7 copies per diploid cell); [2]The selected genes from the peak region.

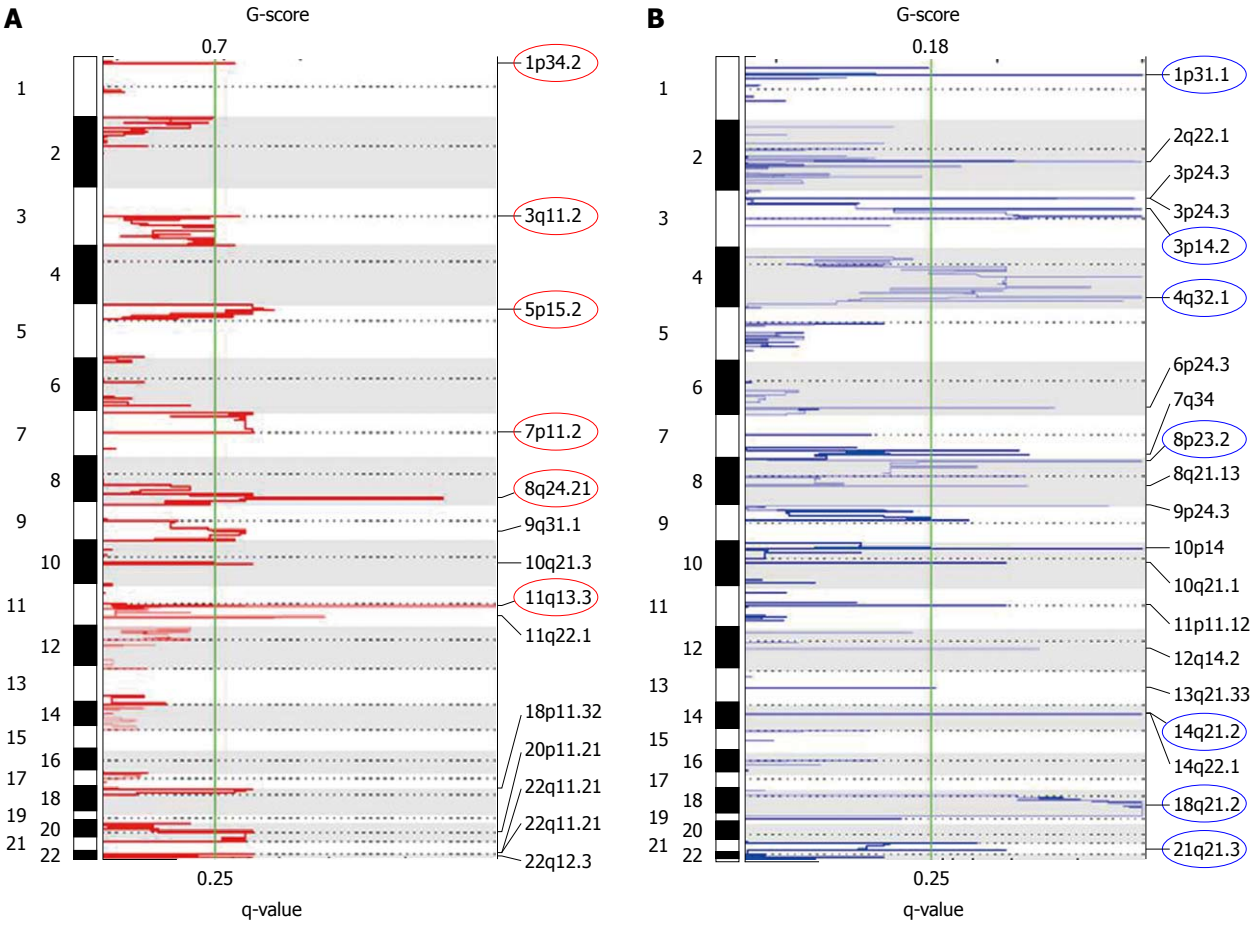

Figure 7 Plots of recurrent genomic amplifications (A) and deletions (B) detected in the esophageal squamous cell carcinoma cell lines from GISTIC analysis of single nucleotide polymorphism array data. The x-axis shows the G-score (top) and false discovery rate (q value; bottom). The green line indicates the false discovery rate cut off of 0.25. The circles indicate the peaks of the most significantly aberrant chromosomal regions.

identified (Figure 7, Tables 2 and 3). The most significant chromosomal regions of amplification were, in descending order of significance: 11q13.3 and 8q24.21, in four and five cell lines respectively, 11q22.1-q22.3 and 5p15.2 both detected in two cell lines. Chromosomal regions of less significant amplification were 10p12.33-q21.3, 18p11.32, 20p11.1-p11.22, 22q11.21-q11.22, 22q12.3, 9q31.1, 22q11.21, 3q11.2-q12.2 and 1p34.2 (Figure 7). Similarly, chromosomal regions of deletion were seen in the following order of significance: 1p31.1-p31.2, 2q22.1,

Table 3 Deletion peaks as detected by the GISTIC algorithm

Cytoband	Location (kb)	Size (Mb)	Frequency (*n*/5)		Mean log2ratio[1]	q-value	Genes[2]
			Hemi	Homo			
1p31.1-p31.2	Chr1:66691991-71187083	4.50	3	1	-3.03	0.02	*CTH*
2q22.1	Chr2:141590067-141951947	0.36	3	2	-1.66	0.19	*LRP1B*
3p26.3-q29	Chr3:1-199344050	199.34	2	1	-2.90	0.022	
3p12.1-p14.2	Chr7:60424050-85108679	24.70	4	1	-2.99	0.02	*FHIT, ADAMTS9*
4q22.1-q32.1	Chr4:91972774-162358674	70.40	3	2	-2.19	0.02	*CASP6, SMAD1*
6q24.3-q27	Chr6:147967444-170914576	22.95	2	1	-2.22	0.07	
7q33-q34	Chr7:133721542-138880555	5.16	2	1	-1.72	0.09	*HIPK2*
8p23.2-q11.1	Chr8:4078057-47043375	43.00	4	1	-2.47	0.02	*BNIP3L, INDO*
8p23.3-q21.13	Chr8:1-146308819	146.31	1	1	-2.27	0.09	
9p24.2-p24.3	Chr9:1151516-2459741	1.31	2	1	-2.53	0.03	
10p12.31-p14	Chr10:10309026-19155158	408.85	2	1	-2.74	0.02	
10q11.23-q22.1	Chr10:50393324-70694787	20.30	1	1	-2.35	0.12	
11p11.12-q12.2	Chr11:50256798-61426521	11.20	1	1	-1.90	0.12	
12p13.33-q24.33	Chr12:1-132078379	132.10		1	-2.80	0.08	
13q21.33-q34	Chr13:68772537-113042980	44.30	1	1	-1.70	0.24	
14q21.2	Chr14:42828345-44176016	1.35	3	1	-4.50	0.02	
14p13-q32.33	Chr14:1-105311216	105.30		1	-5.10	0.02	
18q21.1-q21.2	Chr18:46081464-51919972	5.80	5		-1.03	0.02	*DCC, SMAD4*
21p13-q21.3	Chr21:1-29932926	29.90	3			0.12	*BAGE*

[1]The mean of the log2ratio of those samples with log2ratios < -1.3 (< 0.9 copies per diploid cell); [2]The selected genes within the deletion peaks.

Table 4 Fluorescence *in situ* hybridization for detection of *CCND1* and *C-MYC* amplification

Cell line	FISH signals	Amplitude[1]	Log2Ratio
CCND1			
WHCO1	5-15	2	1.56
WHCO3	> 20	2	2.57
WHCO5	10-20	1	0.78
WHCO6	4-8	1	0.55
SNO	15-20	1	0.79
C-MYC			
WHCO1	4-6	1	0.12
WHCO3	15-> 20	2	1.83
WHCO5	10-> 20	2	1.83
WHCO6	10-> 20	2	2.06
SNO	4-> 20	2	1.71

[1]Amplitude threshold where log2ratio < 0.1 = 0, log2ratio > 0.1 < 0.9 = 1 and log2ratio > 0.9 = 2 as determined by SNP array copy number analysis. FISH: Fluorescence *in situ* hybridization.

3p12.1-p14.2, 4q22.1-q32.1, 8q11.1-p23.2, 14q21.2, 18q21.1-q21.2 and 21p13-q21.3 detected in all or four cell lines. Other regions of less significant deletion, and only seen in one or two cell lines, included: 10p12.31-p14, 14p13-q32.33, 3p26.3-q29, 9p24.2-p24.3, 6q24.3-q27, 12p13.33-q24.33, 7q33-q34, 8p23.3-q24.3, 10q11.23-q22.1, 11p11.12-q12.2 and 13q21.33-q34 (Figure 7). Together the regions of amplification and deletion encompassed a total of 4595 genes.

Significant gains

We selectively describe below the five regions of amplification that were the most significant on GISTIC analysis (q-value < 0.25) (Figure 7). Chromosomes 11q13.3 (68 753 086-69 985 447 bp) and 8q24.21 (127 445 828-129 661 846 bp) were the two most amplified and most significant regions with a q-value of 5.76E^{-05} and 0.0007 respectively. The 11q13.3 region was 1.23 Mb in size and was highly amplified in cell lines WHCO3, WHCO5, WHCO6 and SNO while amplified to a lesser degree in cell line WHCO1. This region harbors seven candidate genes including the cyclin D1 (*CCND1*), the cortactin (*CTTN*), the protein tyrosine phosphatase, receptor type, polypeptide, interacting protein α 1 (*PPFIA1*), *FGF3*, *FGF4* and *FGF19* and the myeloma overexpressed (*MYEOV*) genes, which could all play a role in ESCC oncogenesis. FISH validated these findings and confirmed the amplification of *CCND1* (Table 4, Figure 8). The common amplicon of 2.22 Mb at 8q24.21 was highly amplified in cell lines WHCO1 and WHCO3 and moderately amplified in cell lines WHCO5, WHCO6 and SNO. This amplicon involved the oncogene v-myc myelocytomatosis viral oncogene homolog (*C-MYC*) and the family with sequence similarity 84, member B (*FAM84B*) gene. Locus specific FISH confirmed the amplification of *C-MYC* in cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO (Table 4, Figure 8).

A second focal region of high amplification on chromosome 11 was observed at 11q22.1-q22.3 (2.23 Mb in size) in cell lines WHCO5 and SNO. This region included the regulators of apoptosis *BIRC2* (*cIAP1*) and *BIRC3* (*cIAP2*), the matrix metalloproteinases (*MMP*) and the Yes associated protein (*YAP-1*) genes all potential target genes. The *BIRC2* gene was previously described as a target of amplification /increased expression in cervical cancers[26] and the *YAP-1* gene product is a cellular adaptor protein, which can induce *BIRC2* expression. *YAP-1* was reported to be over expressed in hepatic and mammary cancers[27]. In turn, the MMP genes, which include

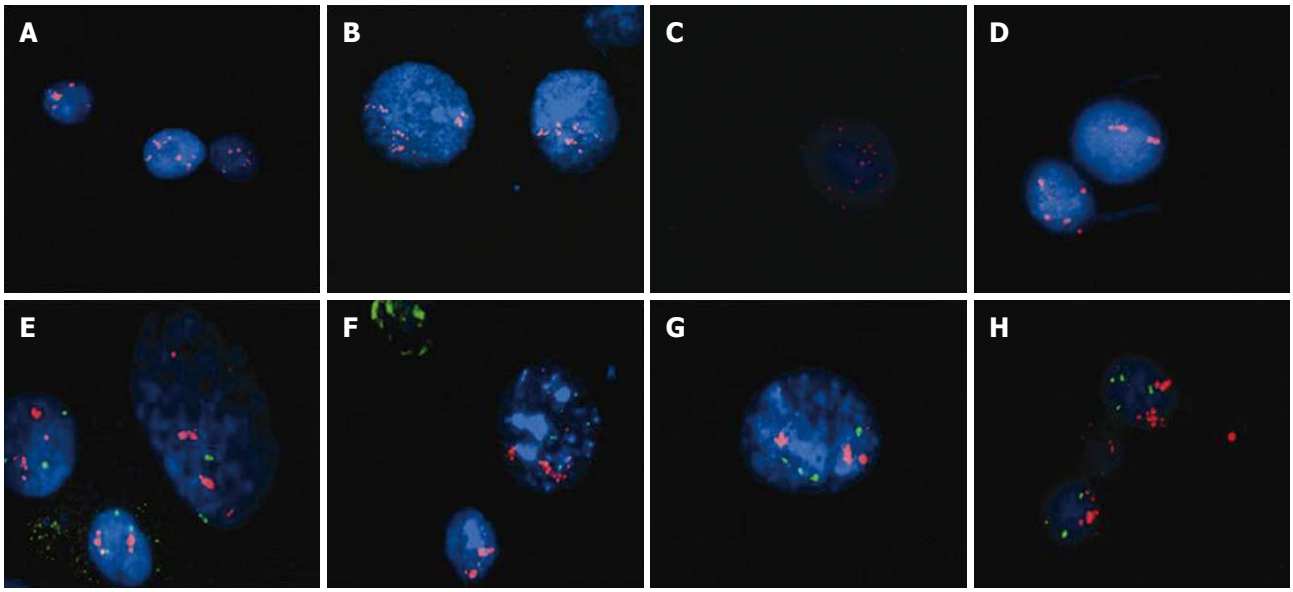

Figure 8 DAPI stained interphase nuclei hybridized with locus specific probes for C-MYC Spectrum Orange (red) (A-D) and *CCND1* (red)/*IGH* (green) (E-H). C-*MYC* amplification can be seen in cell lines WHCO1 (A), WHCO3 (B), WHCO5 (C) and SNO (D). *CCND1* amplification was detected in cell lines WHCO3 (E), WHCO5 (F), WHCO6 (G) and SNO (H).

MMP1, *MMP7*, and *MMP13*, have been shown to be co-expressed in early stage ESCC correlating with a poorer prognosis[28].

A 1.75 Mb region on chromosome 5p15.2 (10 051 329-11 800 765 bp) was highly amplified in cell lines WHCO6 and WHCO5 and moderately amplified in cell lines WHCO1 and SNO. This region hosts the potential target gene, delta catenin (*CTNND2*), overexpressed in prostate cancer[29].

Four cell lines, WHCO6, WHCO3, WHCO5 and SNO, had focal gain on chromosome 3q. The minimal common region of amplification mapped at 3q11.2-12.2, and was 6.02 Mb in size (95 917 505-101 945 216 bp) (q-value = 0.17). This region is commonly amplified in a variety of cancers[20,30] including esophageal squamous carcinoma[31] and it involved the potential oncogene, MYC induced nuclear antigen (*MINA*).

The 18p11.32 sub-band was highly amplified in cell lines WHCO3 and WHCO6 and moderately amplified in the remaining 3 cell lines. This region of 1.12 Mb in size (1-1 118 244 bp) has previously been described in ESCC[32] and involves the potential oncogenes *TYMS* and *YES-1*. Both genes have been implicated in gastro intestinal cancer. The *TYMS* gene codes for a thymidylate synthase involved in DNA synthesis and targeted by the chemotherapy agent fluorouracil (5FU). *TYMS* overexpression leads to 5FU treatment resistance[32] and affects colorectal cancer treatment[33]. *YES-1* is a homologue of the Yamaguchi sarcoma virus v-*yes* amplified and overexpressed in gastric cancers and ESCC[32].

Significant losses

The most significantly deleted chromosomal regions were 1p31.1-p31.2, 2q22.1, 3p12.1-p14.2, 4q22.1-q32.1, 8p23.2-q11.1, 14q21.2 and 18q21.1-q21.2 (in descending order of significance) in all or four of the cell lines (Figure 7). Less significant regions detected in less than four cell lines are depicted in Table 3. Three small regions of deletions involved chromosomes 1p, 2q and 18q. The 4.5 Mb deletion on chromosome 1 short arm, 1p31.2-p31.1 (66 691 991-71 187 083 bp) was seen in four cell lines (homozygous deletion in cell line SNO and hemizygous in cell lines WHCO1, WHCO5, WHCO6) with a high significant q-value of 0.02. Three genes with a reported tumor suppressor activity were involved in this deletion, the cystathionine γ-lyase (*CTH*), the growth arrest and DNA damage-45 α (*GADD45α*) and the DIRAS family, GTP-binding RAS-like 3 (*DIRAS3*) genes. The *DIRAS3* gene was shown to be down regulated in hepatocellular carcinoma and breast cancers[34,35] and is postulated to have a tumor suppressive activity. Both the *CTH* and *GADD45* genes were shown to negatively control cell growth[36,37].

Chromosome 2, sub-band q22.1 (141 590 067-141 951 947 bp) was lost in all cell lines (homozygous deletion in cell lines WHCO3 and WHCO5 and hemizygous in the other 3 cell lines) (q-value = 0.019). The low density lipoprotein 1B (*LRP1B*) tumor suppressor gene, deleted in lung cancer[38,39] maps in this region.

A chromosome 18q sub-band, q21.1-q21.2 (46 081 464-51 919 972 bp) (5.8 Mb) was hemizygously deleted in all five cell lines, involving both the *SMAD4* and deleted in colorectal carcinoma (*DCC*) genes.

Three large regions of deletion involved chromosomes 3p, 4q and 8p. First, the 24.7 Mb region of deletion at 3p12.1-p14.2 (60 424 050-85 108 679 bp) was significant (q-value of 0.02) in all cell lines (homozygous deletion in cell line SNO). This region houses the FRA3B associated gene, Fragile Histidine triad (*FHIT*), whose deletions were previously detected by MLPA analysis in these cell lines[40]. The potential tumor suppressor *ADAMTS9* gene, a me-

talloproteinase family member involved in inhibition of angiogenesis[41] was also involved in this deletion.

Second, the 4q22.1-q32.1 (91 972 774-162 358 674 bp) (75 Mb) region was homozygously deleted in cell lines WHCO3 and WHCO5 and hemizygously deleted in the three other cell lines with a q-value of 0.02. This region encompasses many genes but of interest are the Bone morphogenetic protein receptor 1B (*BMPR1B*), the caspase 6 (*CASP6*), the secreted frizzled-related protein 2 (*SFRP2*) and the SMAD protein 1 (*SMAD1*) genes, all potential tumor suppressor genes.

Lastly, a chromosome 8p23.2-q11.1 (4 078 057-47 043 375 bp) (43 Mb) deletion was seen in all cell lines (homozygous in cell line WHCO3 and hemizygous in the other 4 cell lines) (q value of 0.02), including five potential target genes, the BCL2/adenovirus E1B 19kDa interacting protein 3-like (*BNIP3L*), the leucine zipper tumor suppressor 1 (*LZTS1*) and the three tumor necrosis factor related superfamily genes *TNFRSF10A, TNFRSF10B* and *TNFRSF10C*.

DISCUSSION

We have characterized the karyotype and genomic constitution of five ESCC cell lines established in SA using a combination of traditional cytogenetics, M-FISH, FISH and SNP arrays. The number of ESCC cell lines genetically described worldwide is limited. Only eight ESCC cell lines have been investigated previously with traditional cytogenetics to our knowledge[10,12,42] and 10 with M-FISH[11,43,44], these are the only two techniques that can detect recurrent translocation breakpoints. In this study the five cell lines had complex karyotypes and were hyperploid with WHCO5 being near tetraploid. There was a high level of intra cell-line heterogeneity. The chromosomes most frequently involved in translocations were chromosomes 1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20 and 22. These features were comparable to ESCC cell lines previously characterized[10-12,42,43] and across all studies that involved karyotyping, including the karyotyping of fresh ESCC tumor samples[14], chromosomes 1, 3 and 8 were the most commonly affected by translocation breakpoints[10-12,43].

Forty percent of translocation breakpoints occurred in near centromeric regions (Table 1). These were represented by unbalanced whole arm chromosome translocations, frequently involving chromosomes 1 and 3, and by isochromosomes for the D group acrocentric chromosomes 13, 14 and 15. Indeed, frequent centromeric breakpoints have been described in squamous carcinoma including ESCC[10,12] with up to 60% of all breakpoints being in centromeric regions[43] supporting the idea that centromeric disruption is a frequent event in epithelial cancers. It has been suggested that environmental factors may preferentially interact with centromeric sequences[45], and clastogenic compounds, such as mitomycin C, induce breaks in centromeric of chromosomes 1, 9 and 16[46]. Smoking is a major risk factor associated with ESCC and nicotine is known to induce single strand DNA breaks[47]. Although active smokers exhibit an increased number of breaks at fragile sites[48], it is not known if centromeric regions are also targeted.

Two chromosomal breakpoints were shared across cell lines. First, chromosome 1p11 was involved in a translocation in four cell lines, translocation t(1;3)(p11.2-12; q11) in cell lines SNO and WHCO5 and translocations with differing partners in cell lines WHCO1 and WHCO6. Second, chromosome 3p11-12 was involved in translocations in two cell lines and deletions in, or near the *EPHA3* locus was seen in four cell lines.

This gene codes for a receptor tyrosine kinase, with a tumor suppressor activity[49]. It was found to be mutated in lung and breast cancers[22-24] and interestingly, found to be deleted in 18.2% of ESCC patients in a previous study[31].

Breakpoints in, or near the centromeric regions of chromosomes 1 and 3 have previously been reported in several ESCC cell lines[10-12], as well as in fresh ESCC tumor samples that were karyotyped[14], and in ESCC cell lines obtained by *in vitro* transformation with HPV[50]. This strongly points to 1p11 and 3p11 translocation hotspots in ESCC that may affect genes and/or regulatory sequences not identified by SNP array. Deletions affecting or near the *EPHA3* gene may point to a role for this gene which was previously found to be deleted in 18.2% of ESCC patients[31].

We knew from previous studies that these ESCC cell lines, all overexpress the *EGFR* gene[16]. *EGFR* DNA amplification observed in cell line SNO is likely to contribute to *EGFR* overexpression whilst other factors are likely to be involved in the other four cell lines where low levels of *EGFR* amplification were observed.

In view of the high clonal heterogeneity observed in each cell line, we used the GISTIC software in addition to CNAT to analyze SNP array data and evidence the significant targets of amplification and deletions. The most stable genetic rearrangements are thought to reflect a proliferative cell growth advantage. In this context, the GISTIC algorithm allowed us to prioritize amplicons and regions of deletions in term of their likelihood to host driver genes. The five most interesting significant regions of amplification included chromosomal regions: 11q13.3, 8q24.21, 11q22.1-q22.3, 5p15.2 and 3q11.2-q12.2in decreasing order of significance.

The 8q24.21, 11q13.3 and 3q11.2-q12.2 regions have all previously been reported in a variety of carcinomas[51-53] and they often co-exist with one another. Genomic amplification at 8q24 occurs in a large variety of cancers[51,53-56], and most amplicons described in the literature involve both the *C-MYC* and *FAM84B* genes, as was observed in this study in four ESCC cell lines. In previous reports the target of amplification has been attributed to either both genes[54], or to one or the other[55,56] based on their respective increased transcription.

The 11q13.3 amplicon covered a large region containing a number of potential target genes. The *CCND1* and *MYEOV* genes (11q13.3) were co-amplified in four cell

lines. Co-amplification of these genes has been reported in multiple myeloma, breast cancer and ESCC[14]. *CCND1* is a downstream effector in the Wnt2/β-catenin pathway and the most frequent target of amplification in several ESCC studies[14,53,57]. While the *MYEOV* gene has been associated with cell proliferation in colon cancer[58], its amplification in ESCC is not always matched by increased transcription due to its silencing by epigenetic mechanisms[59]. The cortactin gene, *CTTN,* involved in cell motility[60], was previously shown to be over expressed in ESCC pre-cancerous lesions, as well as in carcinogen induced murine ESCC supporting a role for this gene in ESCC carcinogenesis[61]. The three fibroblast growth factor (FGF) genes, *FGF3*, *FGF4* and *FGF19* were part of the 11q13.3 amplicon. FGF and Wnt signaling pathways cross talk in a number of carcinogenesis scenarios[62]. Activated FGF receptors activate the FRS-GR2-GAB1-PI3K-AKT signaling cascade, and downregulate GSK-3β protein activity, thus hampering β-catenin phosphorylation and degradation[62]. In particular, FGF19 ligand downregulates GSK-3β activity, which results in the release and nuclear accumulation of β-catenin. Nuclear β-catenin activates the transcription of downstream genes including *C-MYC* and *CCND1*[63].

In addition to the amplification of Wnt pathway activators, the *SFRP2* tumor suppressor gene locus was deleted at chromosome 4q22.1-q32.1 (Figure 7). The *SFRP2* gene encodes a frizzled-related protein and is part of the SFRP family of Wnt inhibitors. Loss of *SFRP2* is detected in medulloblastoma and is suggested to contribute to carcinogenesis through loss of inhibition of the Wnt pathway[64].

The copy number data therefore suggests that the Wnt signaling pathway may be at work in these ESCC cell lines through one or the combined effects of genes activating the β-catenin transcriptional activity and/or the FGF signaling pathways as well as deletions of genes, at 4q22.1-q32.1, inhibiting this pathway. Amplicons at both 8q24 and 11q13.3-13.4 have been described in a variety of squamous cell carcinoma[54,65-67] suggesting that the activation of pathways through the combined effects of genes at 8q24 and 11q13.3-13.4 contributes to the development and aggressiveness of SCC.

In addition to the *SFRP2* gene, the three tumor suppressor genes *BMR1B, SMAD1* and *CASP6* were also targets of deletion at 4q22.1-q32.1. Both *BMR1B* and *SMAD1* genes have previously been reported to have decreased expression in gliomas correlating with poor survival[68], and *BMPR1B* decreased expression in breast cancer is associated with increased cell proliferation and poor prognosis[69]. The *CASP6* gene encodes the pro-apoptotic caspase-6 protein[70].

Although large 3q amplicons are commonly observed in squamous carcinoma[71,72] in this study the 6 Mb, 3q11.2-12.2 amplicon was focal and involved the *MINA* gene. This gene has previously been reported to be over-expressed in 83% of ESCC in one study and its inhibition was shown to suppress ESCC cell proliferation[73].

A 43 Mb region of deletion at 8p23.2-q11.1 (4 078 057-47 043 375 bp) was observed in the five cell lines and involved five potential target genes. These included the *BNIP3L* gene deleted or downregulated in prostate cancer and malignant melanomas, respectively[65,74], the *LZTS1* gene, deleted in oral squamous cell carcinomas and downregulated in breast carcinomas[75,76]; and the three *TNFR* genes, *TNFRSF10A*, *TNFRSF10B* and *TNFRSF10C,* whose epigenetic inactivation was reported in gastric cancers[77]. An 8p loss, was previously detected by conventional CGH in a study performed on 29 South African black and colored ESCC patients[78]. Chromosome 8p22 loss has also been reported in prostate, breast, lymphoma, hepatocellular and colorectal cancers[79-83].

Active smoking is linked to increased fragile site expression[84] and is also one of the primary risk factors associated with ESCC in South Africa[85]. We previously hypothesized that deletions affecting anti-oncogenes located at fragile sites may contribute to the etiology of ESCC in South Africa and reported *FHIT* intragenic deletions in these cell lines and a small cohort of patients[40]. *FHIT* gene deletions were confirmed here supporting its role in ESCC carcinogenesis.

Deletion at 18q23 involved the *SMAD4* and *DCC* genes in all cell lines. Both genes have previously been reported to be down regulated in ESCC either by deletion, mutation or methylation[86]. Decreased expression of the *SMAD4* gene, a tumor suppressor of the transforming growth factor β family signaling pathway, has been associated with ESCC tumor invasion[87]. The *DCC* gene was shown to be frequently methylated in ESCC tumor specimens[88].

In summary, breakpoints at 1p11 and 3p11 were recurrent in the five ESCC cell lines and may point to genes such as *EPHA3* that may be involved in ESCC carcinogenesis. Copy number alterations involved both amplicons previously reported in squamous cell carcinoma (8q24, 11q13 and 3q11) as well as novel regions of significant amplification (11q22.1-q22.3, 5p15.2 and 18p11.32). The finding that a significant number of genes that were amplified (*FGF3, FGF4, FGF19, CCND* and *C-MYC)* or deleted (*SFRP2* gene) are involved in the Wnt and FGF signaling pathways suggest that these pathways may be activated in these ESCC cell lines. These results warrant expression studies of these genes in both cell lines and patients' specimens. Of interest, should *FGF* gene expression be increased, ESCC patients may benefit from the respective FGF targeted therapies recently developed[89,90].

ACKNOWLEDGMENTS

We thank Elsabe Scott for culturing the cell lines.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is a major cause of cancer death in the world and has a peculiar epidemiology with worldwide geographic

pockets of high incidence particularly in Asia and Africa. In South Africa, in the Eastern Cape region, ESCC represents the leading cancer affecting men and the second most common cancer in woman. There is a need to identify biomarkers to better understand the pathophysiology of this cancer and inform future diagnostic and therapeutic strategies for these patients. Established cell lines provide a unique resource to investigate both the presence of chromosomes translocations and copy number imbalances.

Research frontiers

A limited number of ESCC cell lines, all established in Asia, were reported to date. These have been investigated with molecular cytogenetic techniques of limited resolution and no key pathways have been reported previously.

Innovations and breakthroughs

This is the first comprehensive molecular cytogenetic study of five ESCC cell lines established in South Africa. The authors combined high-resolution whole genome array copy number analysis with conventional cytogenetics and multicolor fluorescence *in situ* hybridization to assess common chromosomal imbalances. Interestingly, a significant number of genes that were amplified (*FGF3*, *FGF4*, *FGF19*, *CCND1* and *C-MYC*) or deleted (*SFRP2* gene) are involved in the Wnt and FGF signaling pathways. In addition, a deletion within or near the *EPHA3* gene was present in 4 of these cell lines, corresponding to a translocation breakpoint at 3p11.2 shared in some cell lines.

Applications

These results suggest that the Wnt and FGF pathways may be involved in the initiation or progression of ESCC. They also point to the *EPHA3* gene as an added potential key gene. Further study on patients' specimens and functional studies will determine the significance of these genes in ESCC pathogenesis.

Terminology

High-resolution 250K single nucleotide polymorphism (SNP) arrays cover at least one SNP per 100 kb of DNA, using an average of 24 probes per SNP. The copy number data are derived from the summary of non-polymorphic SNPs and examined as a ratio to a reference genome. Changes in intensity ratios are indicative of amplification/deletions. The Wnt and fibroblast growth factor (FGF) signaling pathways are involved in tissue homeostasis as well as in cell proliferation and differentiation. Although their mechanisms differ, these two pathways cross talk through GSK3β inhibition.

Peer review

The manuscript by Brown and coworkers demonstrates that a significant number of genes that were amplified or deleted are involved in the Wnt and FGF signaling pathways in five cell lines established from South African ESCC patients. They suggest that these pathways are activated in these cell lines. The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting and clinically important.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2924

World J Gastroenterol 2011 June 28; 17(24): 2924-2932
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA

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Supported by Chang Gung Medical Research, Program grant 350363, National Science Council grants to Yeh CN; National Science Council grant 98-2314-B-027-001 to Weng WH
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Received: September 16, 2010 Revised: November 15, 2010
Accepted: November 22, 2010
Published online: June 28, 2011

Abstract

AIM: To characterize a culture model of rat CCA cells, which were derived from a transplantable TTA-induced CCA and designated as Chang Gung CCA (CGCCA).

METHODS: The CGCCA cells were cultured at *in vitro* passage 12 times on a culture dish in DMEM medium. To measure the doubling time, 10^3 cells were plated in a 96-well plate containing the growth medium. The cells were harvested 4 to 10 d after seeding, and a standard MTT assay was used to measure the growth. The phenotype of CACCA cell and xenograft was determined by immunohistochemical study. We also determine the chromosomal alterations of CGCCA, G-banding and spectral karyotyping studies were performed. The CGCCA cell line was transplanted into the nude mice for examining its tumorigenicity. 2-Deoxy-2-(^{18}F)fluoro-D-glucose (FDG) autoradiography was also performed to evaluate the FDG uptake of the tumor xenograft.

RESULTS: The doubling time for the CGCCA cell line was 32 h. After transplantation into nude mice, FDG autoradiography showed that the tumors formed at the cell transplantation site had a latency period of 4-6 wk with high FDG uptake excluding necrosis tissue. Moreover, immunohistochemical staining revealed prominent cytoplasmic expression of c-erb-B2, CK19, c-Met, COX-Ⅱ, EGFR, MUC4, and a negative expression of K-ras. All data confirmed the phenotypic features of the CGCCA cell line coincide with the xenograft mice tumors, indicating cells containing the tumorigenicity of CCA originated from CCA. In addition, karyotypic banding analysis showed that the diploid (2n) cell status combines with ring and giant rod marker chromosomes in these clones; either both types simultaneously appeared or only one type of marker chromosome in a pair appeared in a cell. The major materials contained in the marker chromosome were primarily identified from chromosome 4.

CONCLUSION: The current CGCCA cell line may be used as a non-K-ras effect CCA model and to obtain information and reveal novel pathways for CCA. Further applications regarding tumor markers or therapeutic targeting of CCA should be addressed accordingly.

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Key words: Cholangiocarcinoma; Rat cell line; Establishment; Characterization; Thioacetamide

Peer reviewer: Giuseppe Garcea, 13 Kinchley Close, Bradgate Heights, Leicester, LE3 9SE, United Kingdom

Yeh CN, Lin KJ, Chen TW, Wu RC, Tsao LC, Chen YT, Weng WH, Chen MF. Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA. *World J Gastroenterol* 2011; 17(24): 2924-2932 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2924.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2924

INTRODUCTION

Cholangiocarcinoma is a malignant neoplasm derived from bile duct epithelium (i.e. cholangiocytes). It is characterized by a great diversity of symptoms commonly occurring in the late course of the disease, and therefore making treatment puzzling. The biological behavior of the tumor and early intrahepatic and/or extrahepatic spread limit the efficacy of surgical management to perform a curative resection; although liver transplantation may provide an alternative option for CCA treatment, high rates of recurrence still limit liver transplantation for most CCA patients[1] and usually lead to a poor prognosis. Three- to five-year survival rates, even with resection, remain dismal[2-7]; in addition, neither radiation therapy nor chemotherapy significantly improves long-term survival rates. This cancer is related to a wide range of risk factors, such as infestation with liver flukes, primary sclerosing cholangitis, and hepatolithiasis, that cause the incidence rates of CCA to vary greatly among different areas of the world[8]. However, many data have shown that the incidence and mortality rates of CCA have been rising worldwide over the past several decades, particularly the intrahepatic CCA[9-11].

Therefore, our goal is to identify potential possible diagnostic biomarkers as the investigation of the molecular pathophysiology associated with this disease becomes more and more important and necessary. In our previous study, a thioacetamide (TAA)-induced CCA rat model was successfully established, and serves as a powerful pre-clinical platform for therapeutic and chemoprevention strategies for human CCA[12]. Herein, we further developed the rat CCA tumor cells as a cell line designated as Chang Gung CCA (CGCCA), and then transplanted the cells into a xenograft of nude mice to further confirm the characteristics of this cell line. A series of IHC studies, including CK19, c-Met, COX-Ⅱ, and MUC4, were performed to determine the phenotype of the cell line. The genotype was examined by cytogenetic studies, and the 2-Deoxy-2-(^{18}F)fluoro-D-glucose (FDG)-avid character of the CGCCA xenograft of the nude mice was demonstrated by animal PET. All of the evidence proved that the CGCCA cell line rat was derived from the original primary tumor formed by the TAA carcinogen. Our current work supports the view that the systematic cell cultures may provide a relevant CCA model to study the complex mechanisms involved in CCA by revealing the potential pathogenesis of this disease. In addition, we may be able to determine the possible diagnostic markers for the early detection and diagnosis of this disease.

MATERIALS AND METHODS

Animal study

This study was approved by the experimental animal ethics committee at the Chang Gung Memorial Hospital, Taiwan. The investigation conformed to the US National Institute of Health (NIH) guidelines for the care and use of laboratory animals (Publication No. 85-23, revised 1996). Male Sprague-Dawley (SD) rats weighing 319 ± 14 g were used in the experiments. The rats were housed in an animal room with a 12:12 hour light-dark cycle (light from 08:00 AM to 08:00 PM) at an ambient temperature of 22 ± 1℃. Food and water were available *ad libitum*.

Establishment of TAA-treated rats CGCCA cell line

The cell isolation procedure as described by Lai *et al*[13] was applied with minor modifications in this study. In brief, hyperplastic bile ductular epithelial cells were obtained from the liver of the male Sprague-Dawley (SD) rats treated with TAA 300 mg/L daily after 25 wk of exposure. The isolation of the CGCCA cell line was established from the nest of cholangiocarcinoma by employing differential cell harvesting with 0.05% trypsin and 0.53 mmol/L EDTA (Life Technologies), and then combined with subsequent serial propagation suspended in a cell culture medium composed of DMEM with 100 U/mL penicillin and 100 U/mL streptomycin (basal medium) plus 10% fetal bovine serum[13].

Estimating growth kinetics of cells in vitro

The CGCCA cells were cultured at *in vitro* passage 12 times on a culture dish in DMEM medium containing 10% fetal bovine serum. To measure the doubling time, 10^3 cells were plated in a 96-well plate containing the growth medium as described above. The cells were harvested 4 to 10 d after seeding, and a standard MTT assay was used to measure the growth according to the instruction manual (MTT Cell Growth Assay Kit; #CT01; Chemicon). The doubling time of the cell population was estimated based on the slope angle of the linear regression model for the four time points.

Determine phenotype of CACCA cells and xenograft by immunohistochemical study

CGCCA rat cells were grown on a miniature cell culture vessel chamber, which permits cells to be grown, fixed, stained, and analyzed all on the same slide (#154461 Lab-Tek II Chamber Slide System; Nalge Nunc International, USA). A total of 2000 cells per well were grown over night, rinsed with PBS twice, fixed with 4% PFA for 1 min, and perforated with 1% Tween 20 for 1 min. In addition, we further compared the cells with the xenograft mice tumors. The mice tissues were obtained for the immunoreactivities study once the mice tumors reached a

diameter of 1.0 cm. The slides were then stained according to the routine IHC staining as described previously[14]. In brief, the primary antibodies CK19 (MAB-1675; Millipore; Temecula, CA), K-ras (clone sc-30; Santa Cruz Biotechnology Vision Corporation; Fremont, CA), c-erb-B2, COX-Ⅱ, EGFR, MET, and MUC4 were diluted at 1:200 and 1:400, respectively (clone sc-284; Santa Cruz Biotechnologies; Santa Cruz, CA; M-3563; Dako Cytomation; RB-9072-9; Lab Vision Corporation; Fremont, CA; clone sc-161; Santa Cruz Biotechnology Vision Corporation; Fremont, CA; and 35-4900; Zymed S; San Francisco, CA) and incubated overnight at 4℃. The slides were then washed three times with TBST, mounted, and analyzed under microscope by authors blindly before visualization with the DAKO LSAB2 System (Peroxidase; DAKO A/S; No. K0675). Control slides were incubated with the secondary antibody only.

Cytogenetic study of the CGCCA cell line

To determine the chromosomal alterations of CGCCA, G-banding and spectral karyotyping (SKY) studies were performed. The CGCCA cells were grown under the conditions as described above. After the cells were harvested, a metaphase chromosome spread was prepared for G-banding and SKY analysis[15]. At least ten metaphases were analyzed after G-banding (data not shown). For SKY analysis, 22 differentially labeled chromosome-specific painting probes and Cot-1 DNA were denatured and hybridized to the tumor metaphase chromosomes according to the protocol recommended by the manufacturer (Applied Spectral Imaging; Migdal Haemek, Israel) with some modifications as previously described[15]. Image acquisitions were performed using a SD200 Spectracube system (Applied Spectral Imaging) mounted on a Leica DM2500 microscope with a custom-designed optical filter (SKY-1; Chroma Technology; Brattleboro, VT). The clonality criteria and the karyotype description followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN 2005)[16].

Heterotransplantation of CGCCA cells in nude mice

Seven male nude mice (BALB/cA Jcl nu/nu) (6 to 8 wk old; 20 to 22 g body weight) were purchased from Clea Japan (Ohita, Japan). The CGCCA cell line was transplanted into the nude mice for examining its tumorigenicity. Each of the 7 mice received a 50 μL subcutaneous inoculation that contained ten million CGCCA cells suspended in the thigh area. Tumor size was measured with a digimatic caliper, and tumor volume was calculated according to the subcutaneous tissue on the flank of each mouse using the formula $V = 1/6\pi abc$ (a, b, and c indicate the diameter in each axis). The xenograft developed after implantation of CGCCA; 4 to 6 wk static later, all animals were sacrificed by CO_2 asphyxia. The xenograft was dissected, and the histopathology of the xenograft was evaluated as described[12]. The xenografted tumors were compared with the original cell line for morphology changes.

FDG autoradiography

FDG positron emission tomography (PET) is an important imaging technique for the evaluation CCA in humans[17]. To evaluate the different levels of fluorodeoxyglucose uptake of the CGCCA cell line xenograft of the nude mice, FDG autoradiography was performed 4-6 wk after tumor implantation. Animals were food-deprived for 8 h, and 37 MBq of FDG was given intravenously. The animals were sacrificed by decapitation after deep anesthesia with isoflurane 45 min after radiotracer injection. The details of the quantitative autoradiography procedures have been described by the authors[18]. In brief, the target tissues, including liver, tumor, and thigh muscle, were quickly removed and placed on dry ice for fixation. The frozen samples were cut to a 10-μm thickness and mounted on glass slides. The slides were exposed to a phosphor image plate (IP) for 2 h and digitized using FLA5100 scanner (Fuji; Japan, Tokyo). The radioactivities of the adjacent tissue sections (assumed to possess the same radiotracer distribution) were measured by γ counter and decay corrected to the time of injection. The correlations between the radioactivities and IP signal intensities were established. All IP signal intensities were converted to radioactivities by using the following calibration curve. Regions of interest (ROI) were manually drawn around the edge of the tumor xenograft activity by visual inspection. The mean activities were recorded from the entire ROI. The percentage injected dose per gram (%ID/g) was calculated as follows: %ID/g = activity in a gram of tissue (*Ci*)/injected dose × 100%. The slices were stained with hematoxylin-eosin (HE) for histologic examination.

Statistics analysis

All data are presented as mean ± SD. Group comparisons of FDG uptake among tissues were determined using analysis of variance (ANOVA). All statistical analysis was performed using SPSS computer software (Chicago, IL) and a P value of < 0.05 was considered statistically significant.

RESULTS

Estimating growth kinetics of cells in vitro

To establish a TAA-induced rat cell line, 25 wk of orally administered TAA rat liver tissues were harvested for culturing. CGCCA rat cells showed a typical growth curve that included lag, logarithmic, and stationary phases (as shown in Figure 1). The doubling time of the CGCCA cell line was calculated to compare the growth of 10^3 cells. Under the series surveillance, a cell population doubling time of 32 h was determined (6 points at day 4, 5, 6, 7, 8, and 10). When the CGCCA cell line underwent the 26th *in vitro* passage, we harvested cells to perform the cytogenetic or IHC experiments in the current study. The cell line has been maintained in culture for approximately a year so far. Therefore, viable CGCCA cells have been cryopreserved at almost every *in vitro* passage time, from which new cultures may be established.

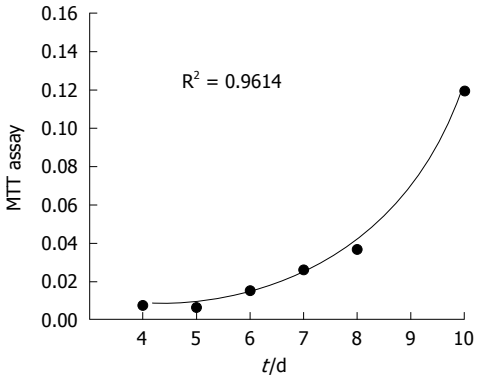

Figure 1 MTT assay. Twenty-five weeks orally administrated TAA rat liver tissues cells have been harvested and successfully cultured. The cells followed a typical growth curve; lag, logarithmic, and stationary phases during culturing were estimated. Under series surveillance, a cell population doubling time of 32 h was determined (6 points at day 4, 5, 6, 7, 8, and 10).

Tumorigenicity study of CCA in transplanted mice

To examine whether the CGCCA cell line contained the tumorigenicity of CCA, the cells were transplanted into the thigh area of seven recipient nude mice and given a 100% incidence of CCA. In addition, their morphological features were essentially identical to those of the parental tumor from which the tumorigenic CCA cells were originally isolated. Tumors formed at the cell transplantation site had a latency period of 4-6 wk, as represented by the photomicrographs shown in Figure 2.

Autoradiography

All seven mice developed a large tumor 4-6 wk after tumor cell implantation. Necropsy and histology confirmed the presence of TAA-induced CCA in 7 mice (Figure 2). Central necrosis could be observed in all large tumors suggesting the fast growth of this malignant tumor. Of note, all tumor cells, excluding necrosis tissue, possessed high FDG uptake. The quantitative uptake value of fluorodeoxyglucose in muscle, liver, and tumor were 0.67 ± 0.17, 2.23 ± 0.85, and 5.00 ± 2.15 %ID/g, respectively (Figure 3A). The tumor to liver and tumor to muscle ratios of FDG uptake were 2.25 ± 0.43 and 7.48 ± 1.78, respectively (Figure 3B). These data are highly consistent with our previous *in vivo* findings, indicating that FDG metabolic activity is significantly higher in the CGCCA xenograft in the nude mice.

The rat CGCCA cell line and heterotransplantation of nude mice tissues were evaluated by immunohistochemistry (IHC) stainings.

To demonstrate the phenotype characteristics and histopathology of CGCCA cells, IHC staining was performed on either the cell line or mouse xenograft tissues. The CGCCA cells revealed prominent cytoplasmic expression of CK19 (biliary cytokeratin 19), as well as c-erb-B2, c-Met, COX-Ⅱ, EGFR, and MUC4 (Figure 4C-H). However, the CGCCA cells revealed a negative expression of K-ras (Figure 4A and B). All of the data from the neoplastic glandular epithelia of the TAA-induced rat CGCCA cell line were highly consistent with previous *in vivo* findings, indicating that these proto-oncogenes are concordantly overexpressed. The tumor tissues from the heterotransplanted nude mice retained the characteristic traits of their *in vitro* cell counterparts. Phenotypically, xenograft highly expressed cytoplasmic immunostaining of CK19, mucin-producing tubular adenocarcinomas closely resembling in their histological and phenotypic features those of the parent tumor; besides, COX-Ⅱ, MET, and MUC4 revealed strong and diffuse cytoplasmic immunostaining as well (Figure 4I-L).

Cytogenetic study of the CGCCA cell line

Spectral karyotyping (SKY) and G-banded analyses were performed to determine the genetic alterations in the CGCCA cell line using the 26th week's TAA-induced rat CGCCA cell line. The cytogenetics study revealed a number of chromosomes ranging between 50 to 56 diploidy (2n) karyotype with complicated genetic abnormalities of marker chromosomes. Obviously, at least two clones were identified in this cell line. In the clones, the loss of whole chromosome 8 and 20 was observed in all analyzed cells; multiple translocated chromosomes formed at the site of chromosome 2q10, such as t(2;3), t(2;5), t(2;3;10); similarly, at the site of chromosome 4q10, commonly formed t(4;13) and t(4;11;4) fusion chromosomes were observed as well. Further, i(6)(q10) was frequently observed in most of the clones. Notably, two major similar contents of ring and giant rod marker chromosomes were involved in these clones; they either simultaneously appeared in a cell or only one type of marker chromosome appeared as a pair to be observed (Figure 5).

DISCUSSION

CCA is the second most common primary hepatic tumor after hepatocellular carcinoma, which is also known as a slowly progressing bile-duct cancer. CCA usually originates in the liver or the intrahepatic bile ducts, and it is characterized by a poor prognosis. Incidence rates account for between 5% and 30% of primary liver cancers and vary geographically[19]. For instance, peripheral CCA represented 3.58% of all primary liver cancers reported by the Japan Liver Cancer Society[1]. According to the report of Bartlett[20], the mortality of intrahepatic CCA has increased 15-fold, and it is currently a more common cause of mortality than hepatocellular carcinoma. New insights into the pathogenesis of this disease, either by using a panel of systematic *in vitro* cell lines for revealing the obscure mechanisms of CCA, or animal models for development of novel therapeutic strategies, are urgently required for clinical-therapeutic purposes.

To our knowledge, there are two published human CCA cell lines available for the purpose of study; however, both have limited availability due to their long-term passage *in vitro*, and they have been shown to be markedly aneuploid. With respect to experimental animal CCA culture models, two published reports have described

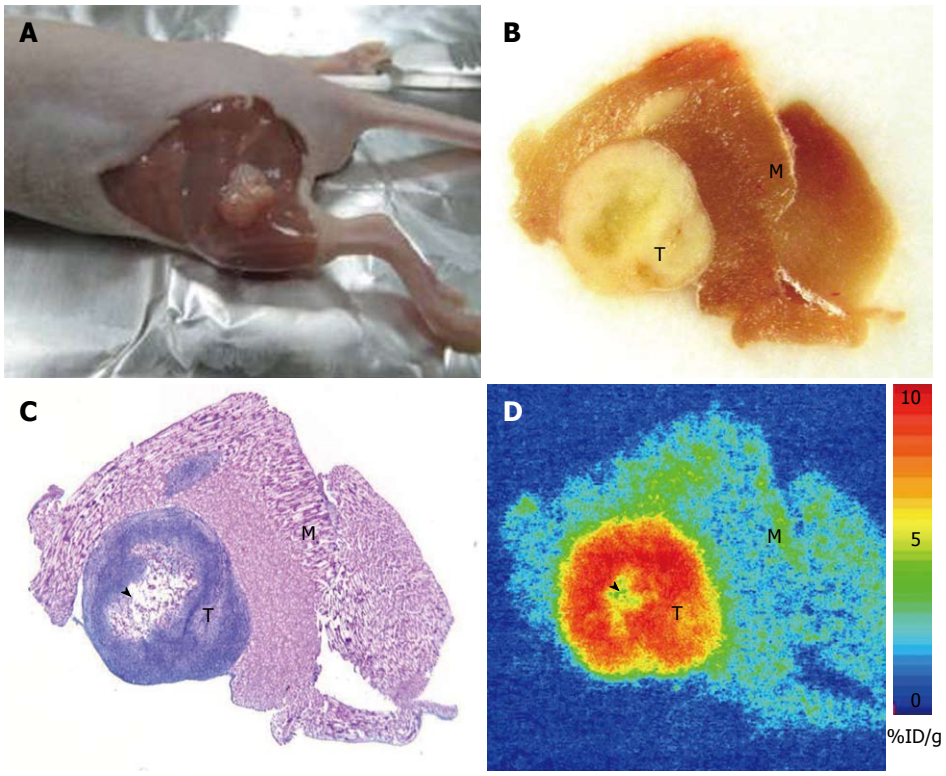

Figure 2 The tumors formed at the cell transplantation site of the CCA xenograft with central necrosis in all large tumors suggesting the fast growth of this malignant tumor at the thigh of the nude mice had a latency period of 4-6 wk and, as represented by (A) gross picture, (B) the photomicrographs of the necropsy, (C) histology, and (D) autoradiography (D). Of note, all tumor cells excluding necrosis tissue possessed high 2-Deoxy-2-(^{18}F)fluoro-D-glucose uptake.

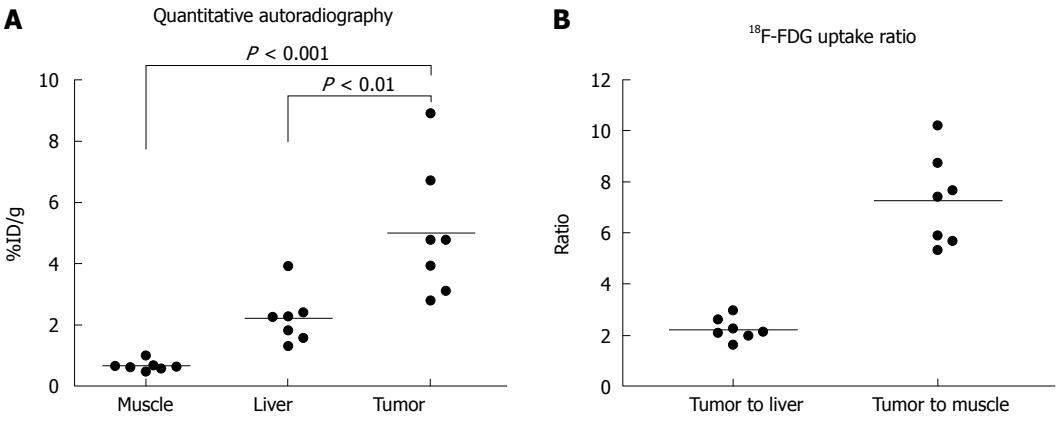

Figure 3 The mean quantitative uptake values of fluorodeoxyglucose in the muscle, liver, and tumor represented by injected dose per gram of tissue (%ID/g) were (A) 0.67 ± 0.17, 2.23 ± 0.85, and 5.00 ± 2.15, respectively. The tumor to liver and tumor to muscle ratios of FDG uptake were (B) 2.25 ± 0.43 and 7.48 ± 1.78, respectively.

the process of establishment; one was developed from liver fluke-associated CCA induced in a hamster model[21]; the other was developed from furan-fed rat CCA culture model[22]. We have been working for several years and have succeeded in establishing a TAA-induced rat intestinal-type CCA model. This model provides a unique and useful study tool for CCA investigation. The important aspects of the cellular and molecular pathogenesis of CCA that are potentially relevant to the human disease have been addressed in previous reports as well[12,23]. In the current study, we further aim to establish a CCA cell line from a TAA rat CCA model and report its identical karyotype as well as immunohistochemical characteristics. This systematic *in vitro* cell line panel will be used as a valuable study tool for revealing the obscure mechanisms of CCA, and searching for the potential biomarkers for CCA tumorigenesis.

Regarding the growth kinetics of the CGCCA cell, CGCCA cells exhibit a 32-h doubling time. This is longer than C611B cells derived from a furan rat model, which exhibited a cell doubling time of approximately 24 h and were aneuploid[13]; but comparable with the hamster liver

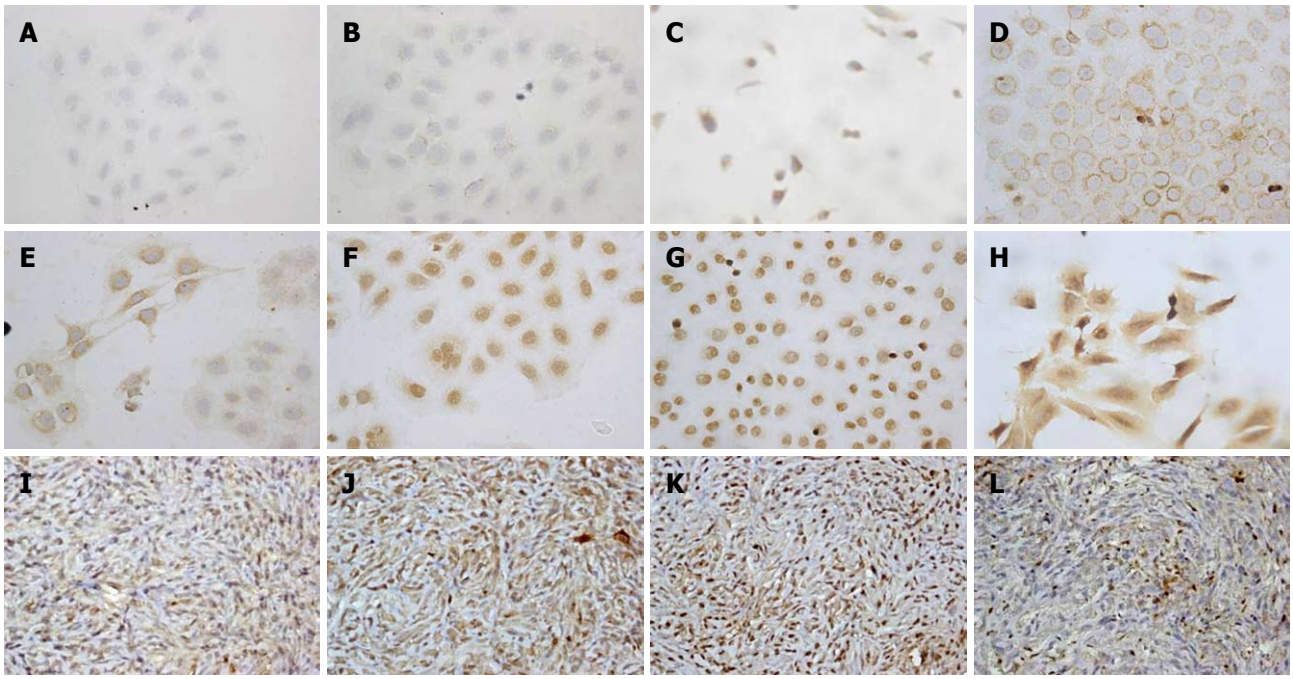

Figure 4 Immunohistochemical staining (× 400) analysis of Chang Gung CCA cells (upper two panels, A-H) and xenograft tissues of CCA mouse models (lower panel, I-L). A: Negative control; B: Negative expression of K-ras; C: EGFR weakly expressed in a cytoplasmic distribution; D-G: Her-Ⅱ, Biliary cytokeratin (CK19), COX-Ⅱ, and Met diffusely expressed in a cytoplasmic distribution; H: MUC-4 strongly and diffusely expressed in a cytoplasmic and membranous distribution; I-L: The results revealed that CK-19, COX-Ⅱ, Met, and MUC4 are diffusely expressed in a cytoplasmic distribution in the rat CCA xenograft.

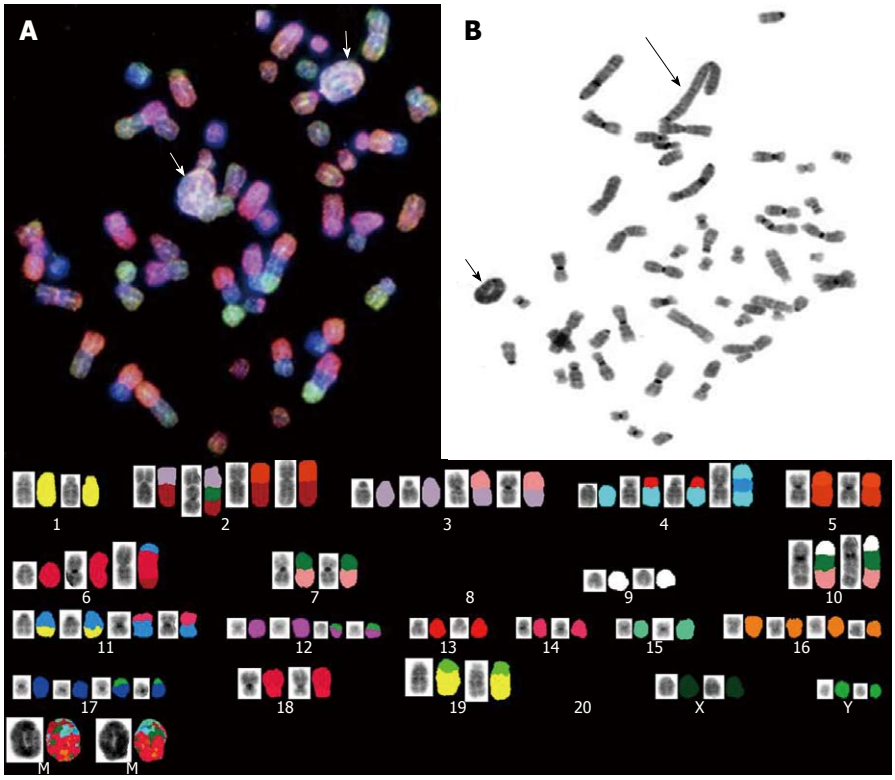

Figure 5 Examples of the karyotype spectral karyotyping analysis revealed by the 26th passage cell culture; the analysis established that, from the 25th week, TAA-induced CCA rat cells presented either (A) a ring chromosome (short arrows) or (B) giant rod chromosome (long arrow); complicated chromosomal alterations could be observed in most of the chromosomes and are illustrated at the bottom.

fluke-associated CCA cells[21]. While skeptics question whether human population doubling times can be slower than animal cell lines, after comparing the population doubling times with some human CCA cell lines cultured

under similar conditions, the human cell lines presented range from 50 to 180 h[24-27].

Regarding phenotype, the strong and diffuse expression of biliary cytokeratin (CK19) confirms the bile ductular ontogeny of the CGCCA cells and xenograft. The molecular alterations involved in CGCCA cells are similar to TAA rat models previously described[12,22,28,29]; the receptor tyrosine kinases c-Met, c-erb-B2 (also known as HER-2/neu), and EGFR and their interact elements, COX-Ⅱ, MUC4, were over-expressed in the current neoplastic cells, either in CGCCA cells or xenograft tissues. It is well known that receptor tyrosine kinases have become important therapeutic targets for anti-tumor molecularly targeted therapies. There is increasing evidence to suggest that, when the HGF/SF interacts with the receptor tyrosine kinases c-Met, the activation could lead to a plethora of biological and biochemical effects in the cell[30] and may play an important role in the development and/or progression of human CCA[31-34]. Other molecular alterations include the overexpression of COX-2[5,35,36] and MUC4, which acts as a ligand for c-erb-B2 and was also confirmed to be overexpressed in the CGCCA cells in our current study. Furthermore, we recently demonstrated that MUC4 could be an independent risk factor of poor prognosis in clinical patients with the mass-forming type of intrahepatic CCA who underwent hepatectomy[37].

Notably, negative K-ras expression was observed in the current CGCCA cells. It is known that very high frequency multiple K-ras gene mutations at codon 12 are commonly detected in CCA (15/15 showed one mutation, and 9/15 showed more than two mutations)[38]. Evidence has further indicated that the mutation status of the K-ras gene affects the response of cetuximab, an epidermal growth factor receptor (EGFR) inhibitor[39]. Therefore, K-ras is considered to be one of the important factors involved in the stepwise progression of neoplastic cells to full malignancy. It is also proven to be related to the higher incidence of bile duct cancers that arise distally in the common bile duct[40,41]. Therefore, the current CGCCA cell line could offer a valuable suitable model that could avoid the influence from K-ras expression.

Nevertheless, evidence of the overexpression of these typical oncoproteins in the CGCCA cell line provides strong resemblances with the human CCA disease, as well as an avenue for future pathogenetic or pharmacologic studies.

In addition, there is evidence that FDG PET imaging may be useful in the diagnosis and management of both hilar and peripheral cholangiocarcinomas in humans[17]. We have previously shown that the FDG uptake pattern in TAA-induced rat CCA was similar to that observed in other human studies[18]. In the present study, our CGCCA xenograft closely mimicked the TAA-induced rat CCA with regard to fluorodeoxyglucose uptake as evaluated by FDG autoradiography. The xenograft had a high tumor to liver and tumor to muscle FDG uptake ratio, which makes *in vivo* tumor detection possible by FDG microPET. The data of the animal PET with regard to the TAA mice models were highly consistent with our previously published *in vivo* findings, indicating that FDG metabolic activity is significantly higher in the CGCCA xenograft in the nude mice[18].

The results of cytogenetic analysis with regard to CGCCA cells revealed complicated chromosomal alterations, and the hyperdiploid karyotype has been characterized in the CGCCA cell line. Basically, the hyperdiploid karyotype is thought to arise from the maintenance of heterozygosity. According to the marker chromosomes observed in the CGCCA cell line, at least three clones could be identified in current cell line, suggesting that the hyperdiploidy does not arise from a near haploid precursor. In addition, although hyperdiploid clones have been identified, all the clones tend to show a pattern of chromosome loss with all copies of chromosomes 8 and 20; further, the gains are more often tetrasomic than trisomic for the chromosomes 2, 3, 4, 11, 12, 16, and 17 (Figure 5). Notably, the marker rings and/or giant rod chromosomes could be observed in every cell as well; the materials contained in the marker chromosomes of the ring or rod shapes varied from cell to cell; however, the major materials mostly came from chromosome 4 as was demonstrated by SKY (Figure 5). To our knowledge, rings are rare in benign tumors, whereas they are common in certain invasive tumors. In addition, the ring chromosomes are even common in certain tumors, especially in subgroups of sarcomas where they may be used as diagnostic indicators for these lesions as described by Gisselsson *et al*[42]. The other additional anomalies, such as translocations and other structural chromosome abnormalities, were present in approximately half of the cells; apparently, the presence of non-random alterations in every clone is most likely the primary change event; in addition, the duplication of chromosome 4 fragments are the most common additional change; deletion of chromosomes 8 and 20, and other random structural abnormalities, such as der(2)t(2;3)(q10;q2), der(2)t(3;10;2), der(2)t(2;5)(q10;q1), der(4)t(4;13)(q10;q?) × 2, der(4)t(4;11;4), are probably a secondary event (Figure 5).

However, although the presence of non-random translocations, such as der(3)t(3;7)(q10;q13), der(3)t(3;7)(q10;q13), der(5)t(5;8)(q10;q1), der(7)t(7;10)(q10;q1), der(11)t(11;1)(q10;?), der(11)t(11;14)(q10;?), der(11)t(11;14)(q10;?), der(17)t(17;Y), der(18)t(18;6)(p10;q1), der(19)t(19;1)(q21;qter), indicates the occurrence of translocation and hyperdiploidy, none of them are known have clinical prognostic implications so far. Therefore, to improve the classification of the disease according to translocation rather than diploidy group in order to assign the correct prognostic implications, further cytogenetic studies to compare the disease stages combined with a series of clinical data for the evaluation of cases are necessary.

Accordingly, our findings suggest that the genes involved in the ring marker chromosomes could play a role in the early stage of tumor development. The correlation

between the conjecture of the cytogenetic changes and tumor progression was also consistent with our previous PET findings. We detected that a 100% initial visual yield of invasive CCA was observed by the 22nd week in rats; however, a 50% yield rate of invasive CCA was observed by the 16th week, and the occurrence of biliary dysplasia and invasive CCA precedes the development of hepatic fibrosis by 4 wk[12].

In conclusion, the current CGCCA cell line was well established and characterized in order to obtain information regarding diagnostically useful tumor markers, which could shed light on a dark area of CCA tumorigenesis for a future understanding of human clinical therapeutics.

COMMENTS

Background

Cholangiocarcinoma is characterized by a great diversity of symptoms commonly occurring in the late course of the disease, and therefore making treatment puzzling. In addition, neither radiation therapy nor chemotherapy significantly improves long-term survival rates. However, many data have shown that the incidence and mortality rates of CCA have been rising worldwide over the past several decades, particularly the intrahepatic CCA. Therefore, the goal is to identify potential possible diagnostic biomarkers as the investigation of the molecular pathophysiology associated with this disease becomes more and more important and necessary. Herein, the authors developed the rat CCA tumor cells as a cell line designated as Chang Gung CCA (CGCCA).

Research frontiers

Positive immunostaining of CK19, c-Met, COX-Ⅱ, and MUC4 determined the phenotype of the cell line. The genotype was examined by cytogenetic studies, and the 2-Deoxy-2-(^{18}F)fluoro-D-glucose-avid character of the CGCCA xenograft of the nude mice was demonstrated by animal PET. All of the evidence proved that the CGCCA cell line rat was derived from the original primary tumor formed by the TAA carcinogen.

Innovations and breakthroughs

The current work supports the view that the systematic cell cultures may provide a relevant CCA model to study the complex mechanisms involved in CCA by revealing the potential pathogenesis of this disease. In addition, the authors may be able to determine the possible diagnostic markers for the early detection and diagnosis of this disease.

Applications

The current CGCCA cell line was well established and characterized in order to obtain information regarding diagnostically useful tumor markers, which could shed light on a dark area of CCA tumorigenesis for a future understanding of human clinical therapeutics.

Peer review

The authors describe the characterization of a new cholangiocarcinoma cell line model and report its successful implantation into nude mice. The paper is well-written, clear and concise.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2933

World J Gastroenterol 2011 June 28; 17(24): 2933-2940
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon

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Received: July 1, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: June 28, 2011

Abstract

AIM: To investigate the role of α-fetoprotein (AFP), a cancer-associated fetal glycoprotein, in glucocorticoid-induced precocious maturation in rat colon.

METHODS: Colons from suckling Sprague-Dawley rats were used in this study. Corticosterone acetate at a dose of 100 μg/g body weight was given to normal pups on days 7, 9 and 11 after birth to induce hypercorticoidism. Control animals were injected with identical volumes of normal saline. Some rats receiving corticosterone 7 d after birth were also treated with mifepristone (RU38486), a glucocorticoid cytoplasm receptor antagonist to investigate the effects of glucocorticoids (GCs). The morphological changes of the crypt depth and villous height of the villous zone in colon were observed as indices of colon maturation. Expression levels of AFP in colons were detected by reverse transcriptase polymerase chain reaction and Western blotting. To identify the cellular localization of AFP in developing rat colons, double-immunofluorescent staining was performed using antibodies to specific mesenchymal cell marker and AFP.

RESULTS: Corticosterone increased the crypt depth and villous height in the colon of 8- and 10-d-old rats with hypercorticoidism compared with that in the control animals (120% in 8-d-old rats and 118% in 10-d-old rats in villous height, $P = 0.021$; 145% in 8-d-old rats and 124% in 10-d-old rats in crypt depth, $P = 0.017$). These increases were accompanied by an increase of AFP expression in both mRNA and protein (2.5-folds in 8-d-old and 2.5-folds in 10-d-old rats higher than in control animals, $P = 0.035$; 1.8-folds in 8-d-old and 1.3-folds in 10-d-old rats higher than in control animals, $P = 0.023$). Increased crypt depth and villous height and increased expression of AFP in the colon of rats with hypercorticoidism were blocked by mifepristone. Both had positive staining for AFP or vimentin, and overlapped in mesenchymal cells at each tested colon.

CONCLUSION: GCs promote the development of rat colon. AFP appears to be involved, in part, in mediating the effects of GCs in the developmental colon.

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Key words: Glucocorticoids; α-fetoprotein; Precocious maturation; Colon; Rat

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Chen M, Sun P, Liu XY, Dong D, Du J, Gu L, Ge YB. α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon. *World J Gastroenterol* 2011; 17(24): 2933-2940 Available from: URL: http://www.wjgnet.

com/1007-9327/full/v17/i24/2933.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2933

INTRODUCTION

Glucocorticoids (GCs) have been routinely used in clinical practice to prevent diseases of prematurity such as respiratory distress syndrome. GCs treatment may reduce the incidence of necrotizing enterocolitis[1-3], in which a typical immaturity of the intestine[4-6] indicated that GCs may enhance intestinal maturation. Although this regulation seems to be mediated by the GCs receptor pathway, the precise regulatory mechanisms have not yet been documented.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. During late embryogenesis, the mouse colon develops from a pseudostratified, undifferentiated endoderm to a single-layered columnar epithelium accompanying with mesenchymal maturation. Mammalian α-fetoprotein (AFP) is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content. Recent reports showed that the AFP was expressed and produced in mesenchymal cells and was considered as an important growth factor with a specific function in gastrointestinal development, including pancreas and colon[8-10]. In contrast, AFP enhancer segment contains a sequence resembling the steroid hormone response element and the enhancer activity was mediated by dexamethasone in a dose-dependent manner[11]. Thus, AFP seems to play a role in GCs-induced precocious gastrointestinal maturation.

Numerous investigators have reported the ability of GCs to stimulate intestinal maturation in postnatal rodents[12-14]. However, little is known about the involvement of GCs in rat colon during development.

MATERIALS AND METHODS

Chemicals

Corticosterone acetate (CA), gel mount aqueous mounting medium (G0918), and mifepristone were purchased from Sigma Chemical (St. Louis, USA). TRIZOL reagent was purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). Takara RNA polymerase chain reaction (PCR) 3.0 Kit was from Takara (Dalian, China). Rabbit anti-goat IgG conjugates and goat polyclonal anti-AFP were from Santa Cruz Biotech (USA). Monoclonal antibody of anti-vimentin was from Chemicon International (Temecula, CA, USA). Protease inhibitor cocktail was from Roche (Mannheim, Germany). Nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA), BCA Protein Assay Kit and enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA) and CBL202 from Chemicon International, Inc. Temecula (CA, USA).

Animals

Pregnant female Sprague-Dawley rats in late gestation were purchased from Nanjing Medical University Animal Centre. After arrival, cages were checked twice daily for pups. The day of birth was designated as day 0, and experiments were conducted at various ages thereafter. Litter size was restricted to 12 pups per dam that was performed at day 2 postpartum. All animals were kept in a temperature-controlled room (22 ± 1℃) and maintained on a 12-h light-dark cycle. To prevent the stress of removing one animal from a cage on sequential days, all the animals in a cage were killed on a single day. Young rats were never separated from their mothers except during the period of fasting. During this period, the pups were placed in cages artificially warmed by electric light bulbs. Before studies, all rats were fasted for various times. To prevent mortality from prolonged fasting, the 8- and 10-d-old rats were fasted for 18 h, and 14-d-old rats were fasted for 24 h. Experiments were designed using littermate controls disregarding the sex of the pups. All animals were killed between 8 and 10 am[15]. CA was given at a dose of 100 μg/g body weight by intraperitoneal injection to normal pups on days 7, 9, and 11 to induce hypercorticoidism. Control animals were given identical volumes of normal saline[16]. To observe the effect of GCs on suckling rats, another experiment was designed as follows: 100 μg/g body weight of CA was given once to pairs of 7-d-old pups (HC group) by intraperitoneal injection. Control pups (C group) were injected with identical volumes of normal saline at the same day. Pups were treated with 50 μg/g body weight mifepristone (17β-hydroxy-11β-4-dimethyl-aminophenyl-17α-propynylestra-4,9-diene-3-one, RU38486), a glucocorticoid cytoplasm receptor antagonist alone (M group), or 100 μg/g hydrocortisone plus 50 μg/g mifepristone (H+M group)[17].

Colons from control and hypercorticoidism rats at 8, 10 and 14 d of age were used in this study and five rats were used in each age stage. Samples were fixed in 4% paraformaldehyde overnight at 4℃ followed by a standard protocol of dehydration and paraffin embedding. Five-micrometer sections were prepared for morphological and fluorescence immunohistochemical studies. Total RNA and lysate were extracted from tissues at each time point for the reverse transcriptase PCR (RT-PCR) and Western blotting analysis. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee.

Morphology

Routine hematoxylin and eosin-stained sections for light microscopic (LM) evaluation were used to study the hormonal effects in the morphological development of colon. Slides were viewed under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Morphologic measurement made by the same technician was blinded to the different treatment groups. Under LM, the crypt depth and villous height of the villous zone were mea-

sured using an image analysis system (NYD100). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of five animals in each group.

Serial levels of AFP

Blood samples of rats were centrifuged at 2000 × *g* for 20 min, and sera were stored at -20℃ until analyzed. AFP levels in the rat serum were measured by the routine standard radioactive method used in the Nanjing Clinical Nuclear Medicine Center (Nanjing, China).

RNA expression of AFP

Total RNA was isolated from tissues by Trizol according to the protocol supplied by the manufacturers. cDNA was synthesized using Takara RNA PCR 3.0 Kit in a total volume of 10 μL, containing 0.5 μL avian myeloblastosis virus RT, 0.5 μL random 9 primer, 2 μL 25 mmol/L $MgCL_2$, 1 μL 10 × RT buffer, 1 μL dNTP mixture (each 10 mmol/L), 0.25 μL RNase inhibitor, 1 μL RNA, and 3.75 μL dH_2O. Conditions for RT were: 30℃ for 10 min, 42℃ for 25 min, 99℃ for 5 min, and 5℃ for 5 min. PCR was performed in 50 μL reactions containing 2.5 ng cDNA, 1 μL each primer pair, and 25 μL Premix *Taq* in the Takara RNA PCR kit. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each paired primers. The following AFP primer pairs were used: 5'-GCTGAACCCAGAGTACTGCAC-3' (forward), and 5'-GACACGTCGTAGATGAACGTG-3' (reverse). Amplification reactions were carried out for 30 cycles at 94℃ for 30 s, 58.4℃ for 30 s, and at 72℃ for 1 min.

The amplified products were 443 bp and analyzed on 1% agarose gels and visualized by ethidium bromide staining. Omitting RT, cDNA or DNA polymerase were adopted in the controls, and showed no reaction bands. The data were normalized by actin.

Protein expression of AFP

The tissues were homogenized in a sample buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail. An equal amount of protein samples were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferred to nitrocellulose membranes and blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween 20 overnight at 4℃, polyclonal antibody for AFP and the corresponding secondary antibody were applied. Blots were visualized with enhanced chemiluminescence reagents and exposed to X-Omat BT film. Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to band intensities at e18.5. The β-actin was used as an internal control and the primary antibody was omitted for negative controls.

Regional and cellular localization of AFP

Double-immunofluorescent staining of AFP and vimentin, a specific marker of mesenchymal cell, were used to determine the regional and cellular localization of AFP in rat colons. Staining was performed according to the standard procedures. Briefly, the sections were deparaffinized in xylene, cleared with graded ethanol in phosphate buffered saline (PBS), and then placed in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100℃ for antigen retrieval. The sections were applied to goat anti-AFP polyclonal antibody overnight at 4℃ and then linked with FITC-labeled rabbit anti goat-IgG. After washing by Tris buffered saline (TBS), mouse anti-vimentin monoclonal antibody and rhodamine-labeled anti-mouse IgG were applied. Sections were placed in Gel Mount aqueous mounting medium with a cover glass and were examined under an Olympus BX51 microscope. Controls were treated by omitting the primary or secondary antibodies. No staining was observed under the negative control conditions. Images were taken at a magnification of × 200. An image analysis system (NYD100) was used for quantitative analysis of cell density (cell number/view field) of the AFP-positive cells in the rat colon. Four sections from four rats in each group were used. AFP-positive cells were counted in five randomly selected view fields per section at a magnification of × 400. At least 20 fields in each group were analyzed.

Statistical analysis

All experiments were done in triplicate. The experimental data was analyzed using PDQuest 7.0 software (Bio-Rad Laboratories, Hercules, CA, USA) and one-way analysis of variance and paired *t* test were used. Data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

Injecting corticosterone into the suckling animals for 1, 2, and 3 d showed no effect on the animal's intestinal and body weights (data not shown). The crypt depth and villous height after corticosterone treatment were higher in the colon of 8- and 10-d-old rats with hypercorticoidism than in the control animals and did not influence those in the 14-d-old rats with hypercorticoidism (Table 1).

Expression of AFP

The serum AFP levels in the rats were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). In this study, we analyzed the effect of corticosterone on the level of AFP mRNA by RT-PCR. The AFP mRNA concentration increased significantly after corticosterone treatment, by 2.5-folds in the 8-d-old and 2.5-folds in the 10-d-old rats compared with those in the control animals (Figure 1). The transcriptional regulation of AFP expres-

Table 1 Morphological changes in suckling rat colon treated with corticosterone acetate

	Villous height (μm)			Crypt depth (μm)		
	8 d	10 d	14 d	8 d	10 d	14 d
Controls	124.6 ± 41.4	165.9 ± 34.4	254.3 ± 57.7	26.9 ± 10.3	40.4 ± 14.7	53.0 ± 23.5
CA	151.1 ± 35.1[a]	196.9 ± 29.6[a]	248.5 ± 42.3	39.1 ± 5.7[a]	50.3 ± 13.6[a]	60.5 ± 15.6

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

Table 2 Changes in number of α-fetoprotein-positive cells in suckling rat colon treated with corticosterone acetate (numbers/view field, mean ± SD, $n = 20$)

	8 d	10 d	14 d
Controls	21.6 ± 4.7	14.1 ± 4.3	12.3 ± 5.2
CA	38.2 ± 5.2[a]	23.8 ± 2.5[a]	11.5 ± 2.3

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

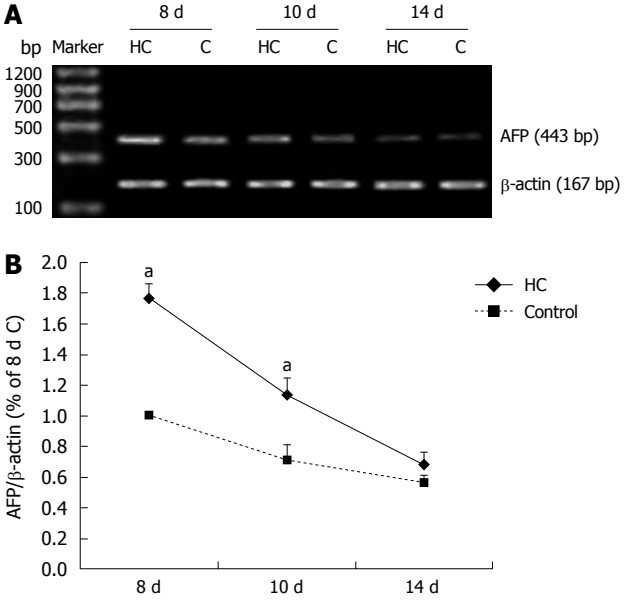

Figure 1 Expression of α-fetoprotein mRNA by reverse transcriptase polymerase chain reaction analysis in colons of suckling rats treated with or without hydrocortisone. Molecular weight markers are indicated on the left. A: The α-fetoprotein (AFP) mRNA levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

sion of corticosterone was confirmed by the Western blotting analysis (Figure 2).

Regional and cellular localization of AFP

The AFP positive cells were scattered on the epithelium in either corticosterone treated or untreated rats. The cell density of AFP-positive cells was higher in 8- and 10-d-old corticosterone-treated rats than in control animals (Table 2). Double-immunofluorescent staining for the vimentin and AFP showed that there was complete overlap between the AFP-positive cells and the antibody staining for vimentin in all the tested animals (Figure 3).

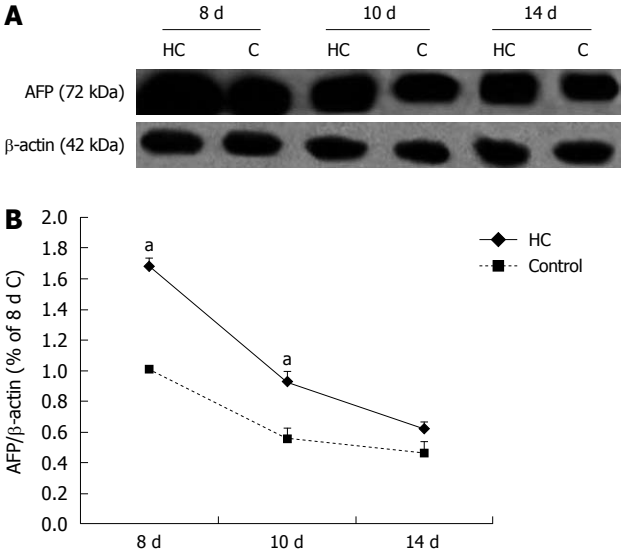

Figure 2 Expression of α-fetoprotein protein by Western blotting analysis in colons of suckling rats treated with or without hydrocortisone as indicated in lanes, respectively. Western blotting analysis using α-fetoprotein (AFP) (C-19), revealed a 72 kDa isoform. A: AFP levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

Effect of mifepristone

The effect of increased crypt depth and villous height in the colon of 8-d-old rats with hypercorticoidism was blocked by mifepristone (Figure 4). The increased expression of AFP was also inhibited in the rats treated with mifepristone (Figure 5).

DISCUSSION

Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. The aim of this study was to assess the role of exogenous GCs in the maturation of the colon. Accordingly, we used the rats in the first postnatal week whose circulating concentrations of natural GCs were very low (< 0.5 μg/mL)[18].

Exogenous administration of GCs causes precocious maturation of the intestine. In this study, GCs did result in an increase in villous height and crypt depth of the colon (Table 1) in 8- and 10-d-old rats compared with the age-matched controls. This effect was completely blocked

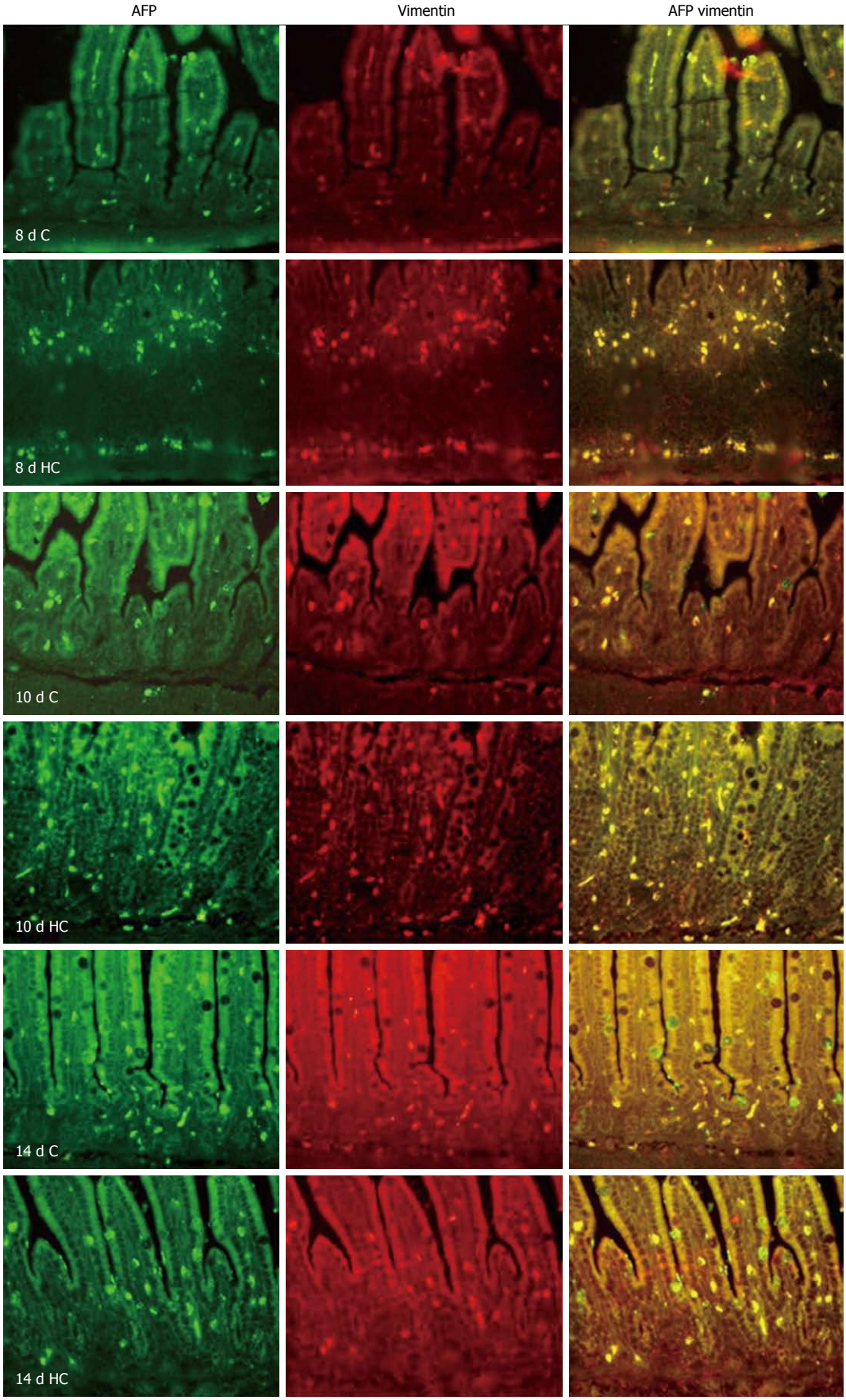

Figure 3 Immunofluorescence localization of α-fetoprotein and vimentin in the suckling rats treated with or without hydrocortisone. Labeling by the α-fetoprotein (AFP) antibody was detected with a fluorescein isothiocyanate (green) labeled secondary antibody. Labeling of vimentin was detected with a rhodamine-(red)-labeled secondary antibody on the same section. The overlap of AFP (green) and vimentin (red) labeling displayed orange color. Double-staining revealed complete colocalization of AFP and vimentin in the same cells of rat colons treated with or without hydrocortisone. All of the primary magnifications are × 200.

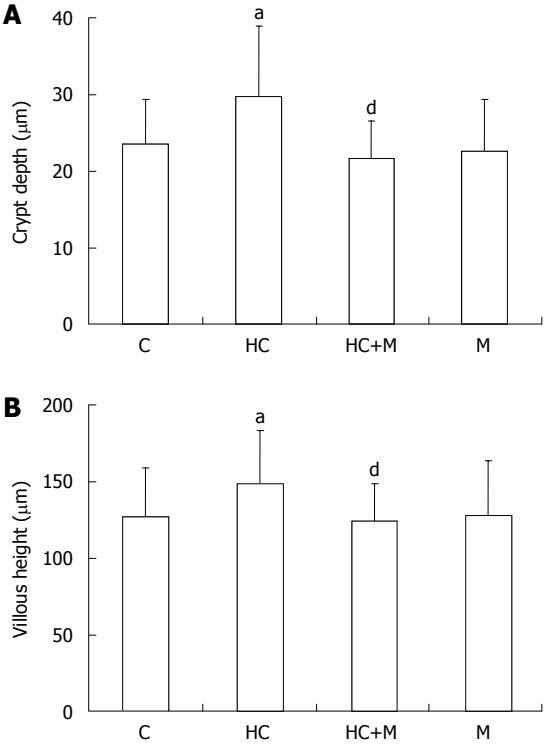

Figure 4 Morphological changes in colons of suckling rats treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. Villous height (A) and crypt depth (B) were determined. Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. $^{a}P < 0.05$ *vs* control animals; $^{d}P < 0.01$ *vs* hydrocortisone-treated animals.

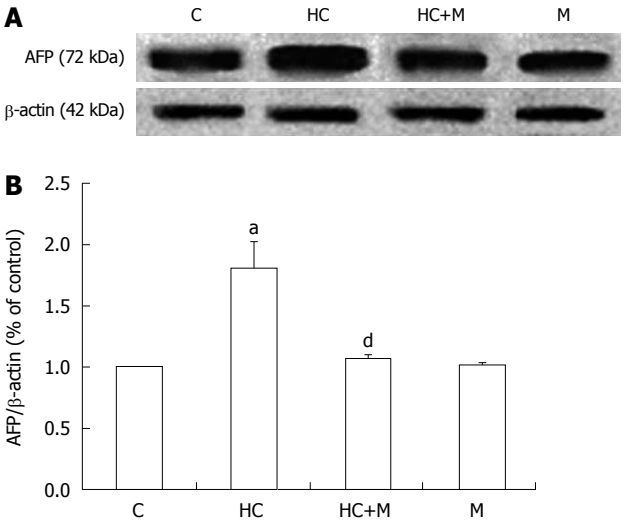

Figure 5 α-fetoprotein expressions in 8-d-old rat colons treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. α-fetoprotein (AFP) expression was determined by the Western blotting using AFP polyclonal antibody (A), and results indicated are in percentage above the β-actin value and are representative of three independent experiments (B). Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. $^{a}P < 0.05$ *vs* control animals; $^{d}P < 0.01$ *vs* hydrocortisone-treated animals.

by the mifepristone, a glucocorticoid cytoplasm receptor antagonist (Figure 4). It is possible that GCs had a precocious effect on the development of rat colon in the first postnatal week, and in the rat small intestine as well[19,20].

In contrast, the GCs had no effect on the colon in 14-d-old rats. It has been suggested that immature gastrointestinal tract seems to have more sensitive responsiveness to the regulation of GCs[21]. It is possible that prepartum cellular differentiation progresses until day 10 in rats after birth[22]. On the other hand, cytoplasm receptors of GCs are activated after the binding of GCs that allows their translocation to the nucleus. Of note is the pattern of ontogeny changes in the concentration of GCs receptors in the small intestine as cytoplasm GCs receptors are present in the intestine at all ages, but at higher concentrations during the two postnatal weeks than in older rats[23]. Moreover, GCs receptors themselves have been shown to down-regulate by GCs through GCs enhanced expression of a factor (repressor), which binds the regulatory sequence in the GCs receptor gene promoter[24]. This might serve as a negative feedback control in the GCs response systems under developmental conditions. In 14-d-old rats, the plasma corticosterone of GCs was raised to 5 μg/mL[18], and a loss of responsiveness to GCs for colon development might occur in these rats. No effect of exogenous GCs administration could be also explained by a fall in GCs receptors in 14-d-old rats with a natural rise in corticosterone level, although this was not tested in this study.

AFP is known to be associated with the successful completion of term pregnancies in mammals and even minute amounts of AFP may still be necessary during human pregnancy[25]. The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has been demonstrated in a multitude of cell types, including placental, ovarian, uterine, and lymphoid, epidermal, endothelial, testicular, breast, and liver[26-30]. In our previous studies, we indicated that AFP was expressed and produced in mesenchymal cells, which might act as a potent paracrine regulator of colonic cell proliferation and organ maturation[10]. In this study, exogenous administration of GCs demonstrated an increased expression of AFP, this effect was also inhibited by mifepristone (Figures 2 and 3). Double-immunofluorescent staining for the vimentin, a mesenchymal cell marker, and AFP showed that the AFP was localized in the mesenchymal cells both in GCs-treated and control colons, similar to our previous study[10].

In present study, the AFP levels in the rat serum were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). High cortisone concentration in serum prompts the acceleration of colonic ontogenesis, and is not accompanied by an increased level of serial AFP. In the current study, we did observe an increased expression of AFP in both RNA and protein levels in the GCs-treated premature colon of rats. Therefore, mesenchymal cell-derived AFP acted as a potent paracrine regulator in rat developmental colon.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. The epithelial-mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults[31,32]. There is no report that GCs could regulate the growth colonic mucosa directly. Our *in vitro* study also showed a negative result for GCs stimulating the proliferation of colonic epidermis (data not shown). The exogenous GCs were blocked, and the effect of accelerating maturation and the increased expression of AFP were also inhibited. Therefore, mesenchymal cell-derived AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. Our present study may help us discern whether the epithelial-mesenchymal interactions and the effect of GCs enhance intestinal maturation in the gastrointestinal tract.

The involvement of GCs in AFP expression in the gut still remains an open question. Studies are now in progress in our laboratory using animal and tissue culture models to test it.

In summary, our present study for the first time demonstrated that GCs promote the development of rat colon. AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

COMMENTS

Background

Glucocorticoids (GCs) are considered to play an important role in the maturation of the gastrointestinal (GI) tract. However, the mechanism of GCs on GI system development has not been fully elucidated.

Research frontiers

Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. This study assessed the role of exogenous GCs in the maturation of the colon in rats.

Innovations and breakthroughs

This study for the first time demonstrated that GCs promote the development of rat colon. α-fetoprotein (AFP) appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

Applications

This animal model is a useful tool for the study of mechanism of gastrointestinal mucosal proliferation and differentiation *in vivo*.

Terminology

Corticosterone acetate is a chemical compound of GCs. Mammalian AFP is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content.

Peer review

This is an excellent study.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2941

World J Gastroenterol 2011 June 28; 17(24): 2941-2944
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Is hyperhomocysteinemia relevant in patients with celiac disease?

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Received: August 10, 2010 Revised: November 13, 2010
Accepted: November 20, 2010
Published online: June 28, 2011

Abstract

AIM: To investigate whether this might be related to the presence of hyperhomocysteinemia.

METHODS: From January 1998 to December 2008, we evaluated the presence of hyperhomocysteinemia in a series of 165 adult celiac disease (CD) patients (138 females and 27 males, mean age 43 years).

RESULTS: Hyperhomocysteinemia was evident in 32 patients (19.3%), although most of them had moderate levels (mean value 25 mcg/ml; range 15-30). Only one patient had a history of myocardial infarction (heterozygosis for N5-N10-metil tetrahydrofolate reductase mutation).

CONCLUSION: The systematic assessment of hyperhomocysteinemia seems, at present, unjustified in CD patients.

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Key words: Celiac disease; Endoscopy; Histology; Hyperhomocysteinemia

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Casella G, Bassotti G, Villanacci V, Di Bella C, Pagni F, Corti GL, Sabatino G, Piatti M, Baldini V. Is hyperhomocysteinemia relevant in patients with celiac disease? *World J Gastroenterol* 2011; 17(24): 2941-2944 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2941.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2941

INTRODUCTION

Hyperhomocysteinemia, considered as an important risk factor in venous thrombosis[1-3], has a prevalence in the general population of 5%-7%[4], and causes damage of the vascular endothelium by disrupting the release of nitric oxide, an important vasodilator factor[5], followed by

platelet activation and thrombus formation[4].

Celiac disease (CD) is a gluten-sensitive enteropathy due to intolerance to dietary wheat gliadin and related proteins in genetically predisposed individuals[6]. The malabsorption of folates and vitamins (the deficiency of which may be a cause of hyperhomocysteinemia) is frequent in CD patients, either in the classic or oligosymptomatic type[7], and several cases of thrombosis have been reported in patients with CD before establishing a diagnosis of gluten-related duodenal mucosal damage[8,9]. Thus, we investigated the presence of hyperhomocysteinemia in a series of patients with CD, to see whether it might be increased and represent a marker of increased venous thrombosis in these patients.

MATERIALS AND METHODS

Patients

In the period January 1998-December 2008, 165 patients with CD (27 men, 138 women, mean age 43 years) were studied. Inclusion criteria were: positivity for anti-endomysial IgA and anti-transglutaminase IgA antibodies (Eurospital, Trieste, Italy) and duodenal histology suggestive for CD.

Histological assessment

Four samples were obtained by endoscopy forceps from the proximal and distal parts of the duodenum. The biopsies, correctly oriented on acetate cellulose filters (Bio-Optica, Milano, Italy), were fixed in 10% buffered formalin, processed and included in paraffin. After obtaining 5 μm thick sections, these were stained with Hematoxylin-Eosin; some sections were also processed for immunohistochemistry and stained with an anti-CD3 monoclonal antibody (Dako, Denmark) to identify intraepithelial lymphocytes (IEL). IEL density was expressed as the number of IEL/100 epithelial cells, with a density value of > 25 cells considered as pathological. Histological classification was based on the Marsh-Oberhuber criteria[10] and a new, recently proposed simpler classification[11,12] (Table 1).

Laboratory assessment

Serum homocysteinemia, vitamin B12 and folic acid levels were measured in all patients. In case of hyperhomocysteinemia, mutations in N5-N10-metil tetrahydrofolate reductase (MTHFR), cystathionine beta synthetase (CBS) and the prothrombin gene were searched for. DNA was extracted from whole blood collected in tubes containing K3-EDTA using a commercial kit (Genomic DNA Isolation kit, Puregene -Gentra System). DNA analysis for MTHFR gene mutation (C677T) was performed by a PCR-RFLP method, as previously described[13]. A fragment of 232 base pairs was then amplified by polymerase chain reaction. The fragment was digested by *Hin*f I restriction enzyme, and subsequent electrophoresis on ethidium bromide stained 3% agarose gel was performed.

The concentration of total homocysteine in plasma (K3-EDTA tubes) was determined by high performance liquid chromatography, as previously described[14]. Basal hyperhomocysteinemia (normal value 5-15 μmol/L) was classified as moderate (16-30 μmol/L), intermediate (31-100 μmol/L) and severe (> 100 μmol/L) according to Hankey *et al*[15]. In all patients, the presence of any thrombotic episode was also evaluated.

The study was approved by the Institutional Review Board of the Desio Hospital.

Table 1 The Marsh-Oberhuber classification of duodenal histological lesions in celiac disease, compared to the "simplified classification"[11,12]

Histologic type	IEL	Glandular crypts	Villi	Simplified
0	Normal (< 25/100 epithelial cells)	Normal	Normal	Normal
1	Increased	Normal	Normal	Grade A
2	Increased	Hyperplastic	Normal	Grade A
3a	Increased	Hyperplastic	Mild atrophy	Grade B1
3b	Increased	Hyperplastic	Moderate atrophy	Grade B1
3c	Increased	Hyperplastic	Severe (total) atrophy	Grade B2

IEL: Intra-epithelial lymphocytes.

RESULTS

Histological findings

Most CD patients (24/32, 75.0%) showed mild to severe villous atrophy, with the latter being present in 41.0% of patients (Table 2).

Laboratory findings

Overall, hyperhomocysteinemia was detected in 32 (19.4%) CD patients (24 women, 8 men); average symptoms' onset was 7 (range 1-40) years. Table 3 shows the serologic findings of these patients. Most patients (29/32, 91.0%) had moderate hyperhomocysteinemia, two (6.0%) intermediate and one (3.0%) severe increase of this value. Mutation of MTHFR was found in 13 (41.0%) patients, 7 homozygotes and 6 heterozygotes; one patient displayed heterozygotic mutation of the prothrombin gene. No CBS mutations were found.

Serum B12 vitamin levels were low in 5 (15.6%) patients and serum folate levels were low in 6 (19.0%) patients. No correlation (Spearman's test) was found between serum homocysteine and age (r = 0.10, P = 0.58), gender (r = 0.66, P = 0.07), onset of symptoms (r = -0.06, P = 0.75), vitamin B12 (r = -026, P = 0.14), folic acid (r = 0.05, P = 0.75), and histological grading (r = -0.01, P = 0.9). Moreover, no correlation was also found between histological grading, vitamin B12 (r = -0.10, P = 0.56) and folic acid (r = -0.2, P = 0.3) values.

Clinical findings

Concerning vascular pathology, one patient with heterozygosis for MTHFR mutation and moderate hyperhomocysteinemia had myocardial infarction, whereas the single

Table 2 Demographic, histological findings and associated diseases of 32 celiac disease patients with hyperhomocysteinemia

No.	Sex/age (yr)	Histology (Marsh/simplified classification)	Associated diseases
1	F/37	Marsh 2 (Grade A)	IgA deficit
2	F/38	Marsh 3a (Grade B1)	
3	M/34	Marsh 3a (Grade B1)	
4	F/32	Marsh 3c (Grade B2)	
5	F/20	Marsh 3c (Grade B2)	
6	F/47	Marsh 3b (Grade B1)	
7	F/64	Marsh 1 (Grade A)	
8	F/41	Marsh 3c (Grade B2)	Epilepsy
9	F/22	Marsh 2 (Grade A)	IgA deficit
10	F/61	Marsh 3a (Grade B1)	
11	M/44	Marsh 3c (Grade B2)	NASH
12	F/35	Marsh 3c (Grade B2)	type 1 diabetes
13	F/39	Marsh 3c (Grade B2)	
14	F/40	Marsh 3c (Grade B2)	
15	F/56	Marsh 3c (Grade B2)	PBC
16	F/35	Marsh 3c (Grade B2)	
17	F/42	Marsh 2 (Grade A)	
18	M/33	Marsh 3b (Grade B1)	Sarcoidosis
19	F/31	Marsh 3a (Grade B1)	
20	F/42	Marsh 3a (Grade B1)	
21	M/28	Marsh 3a (Grade B1)	
22	F/41	Marsh 3c (Grade B2)	
23	F/45	Marsh 3c (Grade B2)	
24	F/21	Marsh 3a (Grade B1)	
25	F/29	Marsh 3b (Grade B1)	
26	F/55	Marsh 3a (Grade B1)	Osteoporosis
27	F/78	Marsh 3c (Grade B2)	
28	M/47	Marsh 3c (Grade B2)	
29	M/63	Marsh 3b (Grade B1)	Psoriasis, myocardial infarction
30	M/18	Marsh 3a (Grade B1)	
31	M/40	Marsh 1 (Grade A)	
32	F/32	Marsh 3a (Grade B1)	Sarcoidosis

IgA: Immunoglobulin A; PBC: Primary biliary cirrhosis.

Table 3 Serologic findings of 32 celiac disease patients with hyperhomocysteinemia

No.	Serum homocysteine (NV 5-15 μmol/L)	Serum B12 vitamin (NV 190-66 pg/mL)	Serum folic acid (NV 2-14 ng/mL)	Genetic mutation
1	14	365	10	
2	13.5	267	4.7	
3	15	369	6	
4	20	356	1	
5	13	174	5	MTHFR (het)
6	21	311	1.5	
7	15	354	12	
8	44	293	5.2	MTHFR (hom)
9	15	493	6	MTHFR (hom)
10	17	333	1	MTHFR (het)
11	19	383	3	
12	17	198	2	
13	15	400	3	MTHFR (het)
14	27	720	4	MTHFR (hom)
15	13	699	3	MTHFR (hom)
16	21	246	2.4	
17	18	265	5.1	
18	19	282	1	Prothr (het)
19	20	457	3	MTHFR (hom)
20	14	555	0.5	
21	13	291	2	
22	23	280	6	
23	14	329	1	MTHFR (het)
24	17	684	10	
25	19	566	5.6	
26	20	188	2	
27	20.5	154	3	
28	20.5	216	2	MTHFR(het)
29	16	164	13	MTHFR (het)
30	25	555	8	
31	149	150	2	MTHFR (hom)
32	31	385	2	MTHFR (hom)

het: Heterozygosis; hom: Homozygosis; MTHFR: N5-N10-metil tetrahydrofolate reductase.

patient with severe hyperhomocysteinemia underwent coronary angiography for atypical chest pain, but no evidence of vessel pathology was found. No patient in this series had episodes of venous or arterial thrombosis, or any stroke episodes.

DISCUSSION

Our findings show that hyperhomocysteinemia is relatively frequent in patients with CD, being present in about 20% of the patients in our series. Hyperhomocysteinemia might represent a link between undiagnosed gluten-sensitive enteropathy and some of its complications[16]. Interestingly, these results were similar to those obtained in an overlapping geographic area, which showed the presence of hyperhomocysteinemia in about 20% of newly diagnosed CD patients compared to about 6% of controls[17].

Hyperhomocysteinemia may be due to genetic factors, with CBS deficiency being considered the most common genetic cause[5,17], or from acquired folate and vitamin B12 deficiencies[18,19]. A homozygous deficiency of MTHFR, the vitamin B12 dependent enzyme for the remethylation of homocysteine to methionine, may cause hyperhomocysteinemia and it has a worse prognosis than CBS deficiency for the absence of an effective therapy[20]. Moreover, treatment with a gluten-free diet and folic acid in CD patients with MTHFR variants does not consistently improve hyperhomocysteinemia[21].

Thus, CD (in which malabsorption of folate and vitamin B12 is common[22]) might lead to increased cardiovascular risks due to an increase of secondary (acquired) hyperhomocysteinemia, further aggravated by the possible presence of genetic abnormalities responsible for hyperhomocysteinemia. However, notwithstanding the relative frequency of hyperhomocysteinemia in our CD patients, this was almost always of moderate entity, with only one patient displaying high levels. Interestingly, the only patient to have a cardiovascular event (myocardial infarction) had relatively low levels of hyperhomocysteinemia and presented heterozygous mutations of MTHFR. No CBS mutations were found in our series. Only one mutation of the prothrombin gene was found, and this is in line with the paucity of reports of such mutations in CD patients[23].

In conclusion, at present it seems unnecessary to systematically investigate CD for the presence of hyperhomocysteinemia; conversely, a serological screening for CD in patients with hyperhomocysteinemia, cardiovascular events and vitamin deficiency could be considered, especially because adult CD patients may display only a few to no intestinal symptoms[24,25], and the onset of the disease may rarely be due to a thrombotic event[26-28].

COMMENTS

Background

Venous thrombosis has been reported in patients with celiac disease (CD). Since this might be related to hyperhomocysteinemia, a risk factor for vascular disease, we investigated the prevalence of hyperhomocysteinemia in a series of adult celiac patients.

Research frontiers

An increased prevalence of hyperhomocysteinemia in CD might lead to increased cardiovascular risk.

Innovations and breakthroughs

To date, most data on this topic originates from single reports, and only one other study investigated systematically celiac patients.

Applications

It appears that, given the low prevalence of hyperhomocysteinemia in celiac patients, it is unnecessary to screen systematically patients; this is useful information in terms of sanitary expenses.

Peer review

The authors evaluated in a cohort of 165 CD patients the presence of hyperhomocysteinemia during a period of time of 10 years. They showed that seems unnecessary to investigate systematically CD for the presence of hyperhomocysteinemia. Their work could contribute to the epidemiologic information of the CD in the Italian population.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2945

World J Gastroenterol 2011 June 28; 17(24): 2945-2952
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients

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Received: August 28, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: June 28, 2011

Abstract

AIM: To analyze the association between the emergence of tyrosine-methionine-asparatate-asparatate (YMDD) mutants (reverse transcription; rtM204I/V) and deterioration of liver function during long-term lamivudine treatment of Japanese patients with chronic hepatitis B virus (HBV) infection.

METHODS: The data of 61 consecutive Japanese patients with chronic hepatitis B who underwent continuous lamivudine treatment for more than 24 mo and had a virological response were analyzed. Analysis of YMDD mutants was done by real-time polymerase chain reaction with LightCycler probe hybridization assay for up to 90 mo (mean, 50.8 mo; range, 24-90 mo).

RESULTS: A mixed mutant-type (YMDD + tyrosine-isoleucine-asparatate-asparatate: YIDD or tyrosine-valine-asparatate-asparatate: YVDD) or a mutant-type (YIDD or YVDD) were found in 57.4% of 61 patients at 1 year, 78.7% of 61 patients at 2 years, 79.6% of 49 patients at 3 years, 70.5% of 34 patients at 4 years, 68.4% of 19 patients at 5 years, 57.1% of 14 patients at 6 years, and 33.3% of 6 patients at 7 years. Of the 61 patients, 56 (92%) had mixed mutant- or a mutant-type. Only 5 (8%) had no mutants at each observation point. Virological breakthrough was found in 26 (46.4%) of 56 patients with YMDD mutants, 20 of whom had a hepatitis flare-up: the remaining 30 (53.6%) had neither a virological breakthrough nor a flare-up. All 20 patients who developed a hepatitis flare-up had a biochemical and virological response after adefovir was added to the lamivudine treatment.

CONCLUSION: Our results suggest that it is possible to continue lamivudine treatment, even after the emergence of YMDD mutants, up to the time that the patients develop a hepatitis flare-up.

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Key words: Tyrosine-methionine-asparatate-asparatate mutant; Hepatitis B virus; Lamivudine; Drug resistance

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Murata M, Furusyo N, Unno M, Ogawa E, Toyoda K, Taniai H, Ohnishi H, Hayashi J. Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients. *World J Gastroenterol* 2011; 17(24): 2945-2952 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2945.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2945

INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects more than 350 million people worldwide, with 75% living in the Asia-Pacific region[1,2]. Although Japan was historically endemic for HBV infection, our previous studies have shown that the prevalence of hepatitis B surface antigen (HBsAg) carriage in Okinawa, Japan markedly decreased from 12.4% in 1970 to 4.2% in 1996[3,4]. However, chronic HBV infection continues to be a major health problem because it leads to the development of liver cirrhosis, hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Because HBV replication is associated with liver injury, therapy for patients with chronic HBV infection aims to stop or reduce disease progression and to prevent the development of hepatic decompensation through the sustained suppression of HBV replication[5]. For this purpose, interferon or oral antiviral nucleos(t)ide analogues, such as lamivudine, adefovir, entecavir, telbivudine and tenofovir have been approved for the treatment of patients with chronic HBV infection. Previous studies have shown that HBV genotype influences liver disease progression[6,7], and our epidemiological study of the Japanese HBV genotype distribution showed that most patients were infected with genotype C[8]. Because genotype C has been reported to be associated with severe liver damage and to be resistant to non-pegylated interferon[8-10], Japanese patients with chronic hepatitis B are often given nucleos(t)ide analog treatment. Nucleos(t)ide analogs have fast and potent inhibitory effects on HBV polymerase and reverse transcriptase activity, are safe and easy to use, and can induce HBV DNA suppression, alanine aminotransferase (ALT) normalization and improvement of liver histology[11-14].

Lamivudine is the first oral nucleoside analogue to be approved for the treatment of chronic hepatitis B patients, and it has been shown to suppress HBV replication by interfering with HBV DNA polymerase and disease activity, to reduce the incidence of HCC, and to improve survival[15,16]. A study that included a large number of Japanese chronic hepatitis B patients showed lamivudine to have good virological and biochemical efficacy in long-term treatment[17].

Although there is much evidence supporting the effectiveness of lamivudine for chronic hepatitis B patients, the clinical benefit of lamivudine treatment has been eroded, in the case of long-term treatment, by the emergence of lamivudine-resistant HBV mutants with mutation of the reverse transcriptase domain of the polymerase gene. The emergence of lamivudine-resistant mutants is mainly based on point mutation from methionine to valine/isoleucine at rt204 (rt204V/I) in the tyrosine-methionine-asparatate-asparatate (YMDD) motif[18]. The emergence of lamivudine-resistant HBV has been linked to virological breakthrough, sometimes followed by biochemical breakthrough, and to flare-ups of hepatitis[19].

The detection of YMDD mutants has mainly been done by methods such as direct DNA sequencing or hybridization[20,21], but these methods are labor-intensive and time-consuming. Fluorometric real-time polymerase chain reaction (PCR) with the LightCycler probe hybridization assay is reported to be an easy, rapid and accurate method for the detection of YMDD mutants[22,23]. Few studies have sequentially assessed the emergence of YMDD mutants during long-term lamivudine treatment in Japan. The aim of the present study was to analyze the association between the emergence of YMDD mutants and deterioration of liver function during long-term lamivudine treatment of Japanese chronic hepatitis B patients by use of the LightCycler probe hybridization assay.

MATERIALS AND METHODS

Patients

The study included 61 consecutive Japanese patients with chronic hepatitis B who underwent continuous lamivudine treatment for more than 24 mo, and had a virological response (defined as a decline of more than 4.0 log copies/mL in HBV DNA level during treatment). The patients started lamivudine treatment between February 2001 and May 2007 at the Department of General Internal Medicine, Kyushu-University Hospital in Fukuoka, Japan. Before the start of lamivudine treatment, all patients had HBsAg and detectable levels of HBV DNA by PCR assay. The diagnosis of chronic hepatitis and cirrhosis was based on a liver biopsy for most patients, but if unavailable it was based on clinical laboratory and ultrasonography data. None of the patients tested positive for antibody to hepatitis C virus or human immunodeficiency virus type 1, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

All patients received lamivudine (Zeffix, Glaxo Smith Kline, UK) in a single oral daily dose of 100 mg. Observation was for up to 90 mo (mean, 50.8 mo; range, 24-90 mo) after the start of lamivudine administration, and the emergence of YMDD mutants during treatment was identified using the LightCycler probe hybridization assay. Serum ALT, hepatitis B e antigen (HBeAg), anti-HBe, and HBV DNA were measured every 1-2 mo. Sera were tested for mutation of the HBV polymerase gene every 6-12 mo during treatment.

Definitions of "virological breakthrough" and "flare-up" of hepatitis

Virological breakthrough was defined as an increase in serum HBV DNA of more than 1 log copies/mL from the nadir of the initial response[19]. A flare-up of hepatitis was defined as an increase in ALT level to more than 3 times the upper limit of normal.

Routine laboratory tests and viral markers

Biochemical tests were performed using standard procedures before and at least once monthly during treatment. HBsAg, HBeAg and anti-HBe were determined by a chemiluminescence enzyme immunoassay (Abbott Japan Co., Tokyo, Japan). HBV genotype analysis was performed by

a previously reported method[24]. Serum HBV DNA level was measured by a PCR-based method (Roche Amplicor HBV Monitor; Roche Diagnostics, Mannheim, Germany). The detection range of the assay was between 2.6 (corresponding to 400 copies/mL) and 8.7 log copies/mL.

Serum samples and extraction of HBV DNA

Serum samples were obtained from all patients before and during lamivudine treatment and stored at -20℃ until use. HBV DNA was extracted from serum using the QIAamp DNA mini kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions.

Monitoring the emergence of lamivudine-resistant mutants by the LightCycler probe hybridization assay

Lamivudine-resistant mutation was detected by rapid PCR amplification across the YMDD-encoding gene locus and analysis of the hybridization kinetics of an integrated probe to infer its sequence was done using the LightCycler (Roche Diagnostics) according to the method reported by Whalley *et al*[22].

Briefly, HBV DNA was extracted from serum, and a 399 bp region of the polymerase gene was amplified by hemi-nested PCR assay. The amplified PCR product was denatured and hybridized to the Bi-probe system, which uses Cy5-labeled probes in conjunction with SYBR Green I (Bio/Gene, Kimbolton, United Kingdom). The LightCycler was used for amplification of PCR clones and to determine the melting characteristics of the probe-amplification hybrid. A melting curve analysis of the data was performed using the LightCycler analysis software v3.5 (Roche Diagnostics). Melting curves were converted to melting peaks by plotting the negative derivative of fluorescence with respect to temperature (-dF/dT). This analysis gave the melting temperature (*Tm*) at which 50% of the hybridizing probe was annealed to the PCR product. Because the presence of a single-base mismatch results in a shift in the melting temperature to a temperature lower than that for the probe-specific sequence, analysis of the probe melting curves allows differentiation of the wild-type YMDD from the YMDD mutants including YIDD, YVDD, and the YMDD/YIDD and YMDD/YVDD mixed types. The detection limit of this assay was about 10%-20% of the total virus pool (data not shown).

Statistical analysis

The distribution of continuous variables was analyzed by the Mann-Whitney *U* test. Differences in proportions were tested by Fisher's exact test. A two-tailed *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed with the SPSS statistical package (version 11.0, SPSS, Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics

Of the 61 patients, 45 were male with a median age of 50 years (range, 28-65 years) and 16 female with a median age of 49 years (range, 38-69 years). All patients were infected with genotype C, 36 (59%) were negative for HBeAg, 15 (25%) were cirrhotic, and the median HBV DNA level was 6.5 log copies/mL (range, 2.7-8.7 log copies/mL).

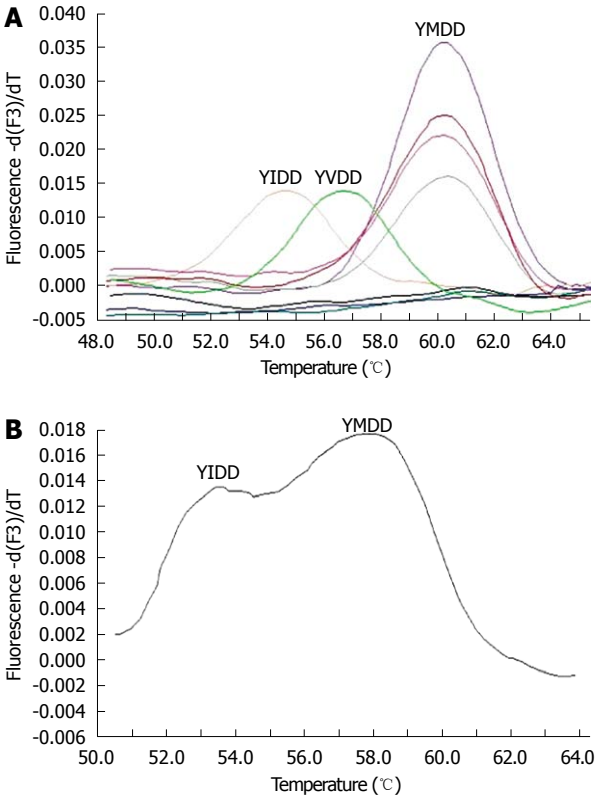

Figure 1 Melting curve analysis using the LightCycler probe hybridization assay. The melting curves were converted to melting peaks by plotting the negative derivative of fluorescence with respect to temperature [-d(F3)/dT]. The melting temperature (*Tm*) for each of the YMDD mutants is indicated with a vertical line. A: Representative results for differentiating HBV YMDD mutants. YMDD, YVDD and YIDD mutants showed distinct *Tm* values; B: The shoulder curve indicated the mixed mutant-type. Representative results show YMDD + YIDD mutants.

Melting curve analysis for the detection of HBV YMDD mutants

YMDD mutants were analyzed by melting curve analysis. The melting peaks of the wild-type and mutant HBV strains were typically observed at different temperatures, as shown in Figure 1A. The melting temperatures of the YIDD and YVDD mutants were approximately 9℃ and 2.5℃ lower than that of the wild-type, respectively. Because the melting curve showed a double peak in the case of YIDD or YVDD mutant mixed with the wild-type YMDD, as shown in Figure 1B, this type of melting curve was considered to be the mixed mutant-type.

Seven-year change in the wild, mixed mutant and mutant-types

The mixed mutant- or mutant-type was found in 57.4% of 61 patients at 1 year, 78.7% of 61 patients at 2 years,

Table 1 Comparison of patients with and without the emergence of YMDD mutants during treatment, median (range)

Characteristics	Mutant- or mixed mutant-type (n = 56)	Not detected or wild-type (n = 5)	P-value
No. of men (%)	42 (75.0)	3 (80.0)	NS
Age (yr)	50 (28-69)	52 (34-55)	NS
ALT level (U/L)	79 (15-1593)	63 (44-108)	NS
Albumin (g/dL)	4.1 (2.9-5.0)	4.3 (4.0-4.3)	NS
Platelet count (× 10^4/mL)	13.1 (3.3-43.3)	17.8 (15.2-26.0)	NS
HBeAg positivity (%)	23 (41.1)	2 (40.0)	NS
HBV-DNA level (log copies/mL)	6.5 (2.7-8.7)	7.0 (5.4-8.7)	NS
Cirrhosis (%)	22 (39.3)	1 (20.0)	NS
History of HCC (%)	8 (14.3)	1 (20.0)	NS
Virological breakthrough (%)	26 (46.4)	0	< 0.0001
Patients with hepatitis flare-ups (%)	20 (35.7)	0	< 0.0001

Between group comparison was done of whether or not YMDD mutant was detected at each observation point. ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; NS: Not significant.

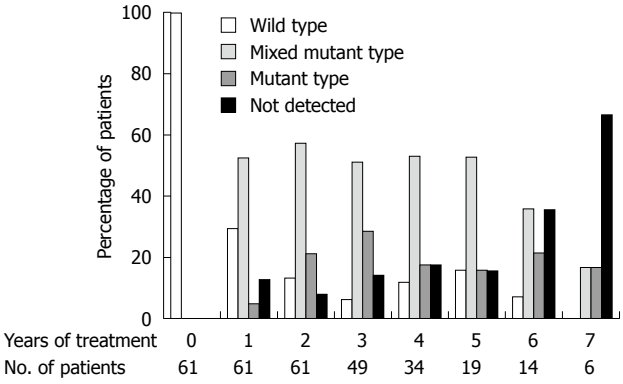

Figure 2 Change in the proportion of YMDD wild, mixed mutant, and mutant-types during lamivudine treatment.

79.6% of 49 patients at 3 years, 70.5% of 34 patients at 4 years, 68.4% of 19 patients at 5 years, 57.1% of 14 patients at 6 years, 33.3% of 6 patients at 7 years, as shown in Figure 2.

Comparison of patients with and without the emergence of YMDD mutants during treatment

Of the 61 patients, 56 (92%) had the mixed mutant- or mutant-type: Only 5 (8%) had none of the tested mutations during the observation period. Virological breakthrough or a flare-up of hepatitis was observed in 26 (46.4%) and 20 (35.7%), respectively, of the 56 patients with YMDD mutants. None of the 5 patients without YMDD mutants had virological breakthrough or a flare-up of hepatitis. Virological breakthrough or a flare-up of hepatitis was experienced significantly more often by patients with than without YMDD mutants ($P < 0.0001$).

No significant differences in sex, age, pretreatment ALT level, serum albumin, platelet count, frequency of HBeAg positivity, pretreatment HBV DNA level, presence of cirrhosis, or history of HCC were observed between these groups. Of the 56 patients with YMDD mutants, 30 (53.6%) had no virological breakthrough. However, no significant differences in sex, age, pretreatment ALT level, serum albumin, platelet count, frequency of HBeAg positivity, pretreatment HBV DNA level, presence of cirrhosis, or history of HCC were observed for the patients with mutants, with or without virological breakthrough (data not shown) (Table 1).

Table 2 Comparison of the emergence of YMDD mutants during treatment by hepatitis B e antigen status of patients, median (range)

Characteristics	HBeAg negative (n = 36)	HBeAg positive (n = 25)	P-value
No. of men (%)	28 (78)	17 (68)	NS
Age (yr)	50 (29-69)	50 (28-60)	NS
HBV DNA level (log copies/mL)	5.8 (2.7-7.6)	7.6 (4.1-8.7)	< 0.0001
Patients with YMDD mutants (%)	33 (92)	23 (92)	NS
Virological breakthrough (%)	12 (33)	14 (56)	NS
Patients with flare-ups of hepatitis (%)	9 (25)	11 (44)	NS

HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; NS: Not significant.

Of the 61 patients, 36 (59%) were HBeAg negative. Although about 90% of patients with or without HBeAg had YMDD mutants, HBeAg negative patients had a tendency to have a lower frequency of virological breakthrough and hepatitis flare-ups than HBeAg positive patients. However, no significant between group differences were found in sex, age, number of patients with YMDD mutants, the frequency of virological breakthrough and hepatitis flare-ups; only pretreatment HBV DNA level showed a significant difference (Table 2).

Characteristics of the patients with hepatitis flare-up

Of the 61 patients, a flare-up of hepatitis was experienced by 20 (32.8%), 15 (75%) males, 5 (25%) females, median age 56 years (range, 44-65 years), 11 (55%) with cirrhosis, and 14 (70%) with HBeAg. All patients who developed flare-ups of hepatitis following an increase in the HBV DNA level had YMDD mutation. The peak HBV DNA level (median 6.7 log copies/mL; range, 5.7-8.0 log copies/mL) at the time of a flare-up was significantly lower than at pretreatment (median 7.6 log copies/mL; range, 6.0-8.7 log copies/mL) ($P < 0.05$) (Table 3).

Clinical course of patients with hepatitis flare-up

All 20 patients who had a flare-up were prescribed 10 mg of adefovir dipivoxil daily, in addition to lamivudine treatment, and all had a biochemical and virological response.

DISCUSSION

Early detection and monitoring of HBV genotypic resis-

Table 3 Characteristics of the patients with flare-ups of hepatitis

Patient	Age (yr)	Sex	Cirrhosis	HBeAg	Change of HBV DNA level after treatment (log copies/mL)			Mutant type
					Pre-treatment	Nadir	Virological breakthrough with hepatitis flare-ups	
1	54	F	+	+	8.7	4.7	7.6	YIDD/YVDD
2	56	M	+	+	8.1	4.1	7.9	Mixed
3	59	M	+	-	6.6	< 2.6	6.8	YIDD
4	60	M	+	+	7.9	< 2.6	8.0	Mixed
5	65	M	+	+	6.5	< 2.6	6.7	Mixed
6	49	M	+	+	7.9	3.9	7.0	YIDD
7	58	M	-	+	8.7	3.9	7.2	YVDD
8	51	M	-	+	7.8	3.8	7.6	YIDD/YVDD
9	58	M	-	+	8.7	4.3	7.9	Mixed
10	61	M	+	-	6.0	< 2.6	6.6	Mixed
11	51	F	-	+	7.2	< 2.6	5.9	YIDD
12	62	F	-	+	7.7	< 2.6	6.6	Mixed
13	44	M	+	+	6.7	< 2.6	5.9	YVDD
14	56	M	-	+	8.7	5.0	7.0	YVDD
15	50	F	-	+	7.6	5.6	6.2	YIDD
16	47	M	+	+	7.9	3.9	6.4	YIDD
17	59	F	+	-	6.9	< 2.6	6.1	YVDD
18	45	M	-	-	6.9	< 2.6	6.1	Mixed
19	61	M	-	-	6.7	< 2.6	5.9	Mixed
20	56	M	+	-	6.6	< 2.6	5.7	YVDD

HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

tance in patients with chronic hepatitis B using nucleoside analogues allows clinicians to avoid virological breakthroughs followed by flare-ups of hepatitis. In this retrospective study, we found that the LightCycler probe hybridization assay was useful for monitoring the emergence of YMDD mutants. Furthermore, our results provide important data on the YMDD mutation of Japanese chronic hepatitis B patients undergoing long-term lamivudine treatment.

Continuous lamivudine treatment is associated with an increased percentage of patients with YMDD mutants. Lamivudine-genotypic resistance was reported in 24% of patients after 1 year of treatment, 41% after 2 years, 53% after 3 years and 70% after 4 years by PCR using a restriction fragment-length polymorphism assay (PCR-RFLP)[25]. Similarly, the present study showed an increased percentage of patients with YMDD mutants within 3 years of treatment, although the percentage was higher than that of the previous report, probably a reflection of different sensitivities of the assay used for the detection of YMDD mutants. Direct DNA sequencing, PCR-RFLP, and reverse hybridization line probe assay are common methods of detecting lamivudine-genotypic resistance[20]. The detection limits of these assays are about 20%, 5%, and down to 10% of the total viral pool, respectively[21]. These methods are labor-intensive, time-consuming, and have the risk of contamination because they require s separate set of endonuclease reactions for each of the mutants, or a specific probe for each mutant. The LightCycler probe hybridization assay can detect YMDD mutants within 1 h and the risk of carryover contamination is minimal because PCR is performed in a closed glass capillary. The detection limit of this assay is about 10%-20% of the total virus pool, and minor subpopulations can be detected (those constituting about 20% of the total population). Our results may more accurately reflect the actual rates of YMDD mutants than were found in previous reports. Because of the quasi-species nature of HBV, YMDD mutants have been detected in patients with chronic hepatitis B who never received lamivudine treatment using a more sensitive method than our method[26-28]. Unfortunately, the present study could not determine if preexisting mutants prevailed after the initiation of lamivudine treatment because this assay could not detect YMDD mutants before treatment.

The present study showed that virological breakthrough and flare-ups of hepatitis occurred after the emergence of YMDD mutants, as reported previously[29]. Therefore, monitoring ALT and HBV DNA levels after the emergence of YMDD mutants is clinically important for the management of patients treated with lamivudine. It has been reported that a short latency to the emergence of YMDD mutants, mixed type YMDD mutant (YIDD + YVDD type), and a low ALT level in patients with YMDD mutants were associated with virological breakthrough or flare-up of hepatitis[30]. In the present study, 20 (35.4%) of 56 patients with YMDD mutants developed flare-ups after virological breakthrough during the treatment. As reported in previous studies[17,31], our results also showed that a flare-up of hepatitis was frequently observed in patients with cirrhosis, or HBeAg positive patients, which may be related to the more active liver disease of HBeAg positive patients. However, our results showed no virological breakthrough by about half of our patients with YMDD mutants during long-term lamivudine treatment. It is important to consider the prognosis

of the patients who continued lamivudine treatment after the emergence of YMDD mutants. It has been reported that there was no benefit for patients who continued lamivudine treatment after the emergence of YMDD mutants compared with patients who discontinued treatment, based on a comparison of the rates of flare-ups of hepatitis, hepatic decompensation, and HBe seroconversion over a 12-mo period[32]. Another report, however, showed a benefit of long-term lamivudine treatment, for 8 years, in Asian patients with YMDD mutants without advanced disease, who had a lower risk of development of cirrhosis and HCC, a greater reduction of HBV DNA level, and a similar rate of flare-ups of hepatitis compared with untreated patients[33]. Our data from the present study suggests that it is possible to continue lamivudine treatment even after the emergence of YMDD mutants if clinicians note the above risk factors associated with virological breakthrough or flare-ups of hepatitis.

The present study showed that about half of patients with YMDD mutants did not encounter virological breakthrough during long-term lamivudine treatment. It has previously been shown that YMDD mutants (rtM204V or rtM204I) have preexisting polymorphisms in HBV-infected patients because of the quasi-species nature of HBV in infected individuals, and that these mutants appeared randomly in viral populations, which had a replication disadvantage to the YMDD wild-type in the absence of lamivudine[34]. A previous study showed that HBV mutants with mutations in the YMDD motif in patients before treatment would not be selected by lamivudine or induce breakthrough hepatitis[27]. Furthermore, the rtM204V mutant in domain C frequently accompanies rtL180M mutants in domain B[26]. *In vitro* studies showed that rtM204I alone had lower replication competency than rtL180M/rtM204V[35]. These data suggest that the gain of replication capacity of YMDD mutants during lamivudine treatment may be associated with multiple factors, including intrinsic replicative advantages potentially conferred by mutations accumulating outside domain C, the fluctuating environment in which these mutants replicate, and the host immune response.

In the present study, 5 (8%) of 61 patients had no emergence of YMDD mutation during the treatment. Hashimoto *et al*[30] reported that factors associated with YMDD mutants not appearing during 5-year lamivudine therapy for patients with chronic HBV infection are HBeAg negativity, lack of cirrhosis, and high γ GTP level. We were not able to confirm their results because there were too few patients free of YMDD mutants to draw a significant conclusion.

Adding adefovir dipivoxil, which is without cross-resistance to lamivudine, is effective for achieving a virological and biochemical response in patients with lamivudine-resistance[36,37]. The American Association for the study of Liver Disease guidelines on HBV recommend the addiction of a second drug in the event of lamivudine resistance[38,39]. It has been reported for patients with lamivudine resistance that the virological and biochemical response rates were similar between a group being switched to adefovir monotherapy and a group for which adefovir was added to lamivudine treatment, but adefovir resistance more frequently occurred in the patients who had combined adefovir and lamivudine treatment[37,40]. Therefore, the add-on treatment is thought to be superior to switching treatment with regard to the prevention of subsequent multi-drug resistance. The above data supported our result of a biochemical and virological response by all patients who had adefovir added to lamivudine treatment after a flare-up of hepatitis.

In conclusion, no virological breakthrough was observed in about half of the patients with YMDD mutants during long-term lamivudine treatment. Patients who developed flare-ups of hepatitis were successful in achieving a virological and biochemical response by addition of adefovir to lamivudine treatment. These data suggest that it is possible to continue lamivudine treatment even after emergence of YMDD mutants, at least until the patients develop a flare-up of hepatitis.

COMMENTS

Background

Although there is much evidence of the effectiveness of lamivudine in chronic hepatitis B patients, the number of patients with tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation, which is linked to virological breakthrough, sometimes followed by a flare-up of hepatitis, is higher with prolonged lamivudine treatment. There is little information about the association between YMDD mutants and deterioration of liver function during long-term lamivudine treatment.

Research frontiers

The detection of YMDD mutants has mainly been by methods such as direct DNA sequencing, polymerase chain reaction (PCR)-restriction fragment length polymorphism, or reverse hybridization line probe assay, but these methods are labor-intensive, time-consuming, and have the risk of contamination. In this study, the authors demonstrated that fluorometric real-time PCR with the LightCycler probe hybridization assay was an easy, rapid and accurate method for the detection of YMDD mutants.

Innovations and breakthroughs

Regardless of hepatitis B e antigen positivity, the present study showed, by use of the LightCycler probe hybridization assay, that about half of the patients with YMDD mutants did not encounter virological breakthrough during long-term lamivudine treatment. Furthermore, all patients who developed flare-ups of hepatitis had a biochemical and virological response after adefovir was added to the lamivudine treatment.

Applications

The results suggest that it is possible to continue lamivudine treatment, even after the emergence of YMDD mutants, up to the time that the patients develop flare-ups of hepatitis.

Peer review

In this study, Murata *et al* retrospectively analyzed 61 HBV patients for up to 90 mo to find out the association between lamivudine resistance from its emergence with the hepatic deterioration. The positive finding of this study is the indication of continuation of lamivudine therapy in patients of genotype C having YMDD mutation until the stage of hepatic flare-up.The potential of Light Cycler probe hybridization for detection and monitoring of such mutants has also been elucidated. This is an important issue because lamivudine resistance is associated with progressive liver disease.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2953

World J Gastroenterol 2011 June 28; 17(24): 2953-2957
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism

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Received: October 7, 2010 Revised: December 5, 2010
Accepted: December 12, 2010
Published online: June 28, 2011

Abstract

AIM: To study the safety and feasibility of total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

METHODS: Fifteen consecutive patients with hypersplenism due to cirrhosis were enrolled in this study from January 2006 to June 2010. All patients underwent total embolization of the main splenic artery. Clinical symptoms, white blood cell (WBC) and platelet (PLT) counts, splenic volume, and complications of the patients were recorded. The patients were followed up for 1 and 6 mo, and 1, 2, 3 years, respectively, after operation.

RESULTS: Total embolization of the main splenic artery was technically successful in all patients. Minor complications occurred in 13 patients after the procedure, but no major complications were found. The WBC and PLT counts were significantly higher and the residual splenic volume was significantly lower 1 and 6 mo, and 1, 2, 3 years after the procedure than before the procedure ($P < 0.01$). Moreover, the residual splenic volume increased very slowly with the time after embolization. All patients were alive during the follow-up period.

CONCLUSION: Total embolization of the main splenic artery is a safe and feasible procedure and may serve as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

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Key words: Liver cirrhosis; Hypersplenism; Coil embolization; Splenic artery

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He XH, Li WT, Peng WJ, Li GD, Wang SP, Xu LC. Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism. *World J Gastroenterol* 2011; 17(24): 2953-2957 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2953.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2953

INTRODUCTION

Partial splenic embolization (PSE) is a non-surgical procedure for hypersplenism resulting from hepatic disease and can thus avoid the disadvantages of splenectomy[1]. It has been shown that PSE can increase peripheral blood cell counts[2-5]. However, PSE often results in a number of complications, including daily intermittent fever, abdominal pain, nausea, vomiting, postemboliza-

tion syndrome, splenic abscess and rupture, pneumonia, refractory ascites, pleural effusion, and gastrointestinal bleeding[2-8].

Total embolization of the splenic artery is a safe and effective procedure for splenic artery aneurysms[9-13]. Moreover, stainless steel spring coils are used to embolize the main branch of splenic artery to increase the platelet (PLT) count before splenectomy[14]. To date, no report is available on the treatment of hypersplenism with total embolization of the main splenic artery. The present study was to study the safety and feasibility of total embolization of the main splenic artery for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

MATERIALS AND METHODS

Patients

Fifteen consecutive patients (10 males and 5 females with a mean age of 50.07 ± 8.98 years, ranging 38-60 years) with hypersplenism due to cirrhosis were enrolled in this study from January 2006 to June 2010 and subsequently underwent computed tomography (CT) follow-up at our hospital. The causes of cirrhosis were hepatitis B virus (HBV) infection in 13 patients and hepatitis C virus (HCV) infection in 2 patients. The patients were diagnosed as hypersplenism based on their history, clinical laboratory tests, ultrasonography and CT. The protocol was approved by The Ethics Committee of Fudan University and the patients provided their written informed consent.

The inclusion criteria were those with hypersplenism, HBV/HCV-related active cirrhosis, neutropenia (neutrophil count $\leqslant 2.0 \times 10^9$ cells/L), thrombocytopenia (PLT count $\leqslant 50 \times 10^9$ cells/L) or both, and follow-up time > 2 years. Those with severe jaundice [serum total bilirubin (TB) level $\geqslant$ 81.4 μmol/L] or spontaneous bacterial peritonitis were excluded from the study.

Hypersplenism was classified as Child-Pugh class A in 10 patients, class B in 3 patients, and class C in 2 patients. The demographics of these patients are summarized in Table 1.

Endovascular techniques

Metallic coils and gelfoam were used as embolization materials, either alone or in combination. In general, the embolization coils used in this series were standard 0.089-cm (0.035-in.) fibered coils, microcoils (Tornado; Cook Inc., Bloomington, IN, USA).

Embolization was performed in all patients *via* the femoral artery. Selective angiography of the celiac trunk, splenic artery, and superior mesenteric artery was performed *via* the right femoral artery with a 5-Fr diagnostic catheter (Cook). Patency of the collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery on a celiac arteriogram was monitored to avoid total splenic infarction. Total embolization of the main splenic artery was performed after confirmation of these connections.

All embolization procedures were performed by 2 experienced interventional radiologists. Details of the coiling procedures have been described previously[9,11,15]. Selective splenic artery, celiac, and superior mesenteric artery angiograms were performed to confirm occlusion of the main splenic artery and patency of the collateral arteries after embolization. Preoperative antibiotic prophylaxis was used routinely for 3 d. Following embolization, patients were monitored and antibiotics were continued after the procedure for several days to avoid infectious complications.

Follow-up protocol and postoperative outcome evaluation

All patients were followed up at our outpatient clinic. Peripheral blood cell parameters, including white blood cell (WBC), PLT, and red blood cell counts, were monitored at different time points prior to the procedure and on days 3, 14, and 30 after the procedure, then at 6-mo intervals during the 3-year follow-up period. To determine the effect of embolization on liver function, serum levels of aspartate aminotransferase, alanine aminotransferase, TB, and albumin were measured and prothrombin time was calculated at the same follow-up time points as above before and after the procedure. The procedure-related frequency and type of complications were recorded.

Abdominal CT scans were routinely performed before and 2 wk after the procedure, and then every 6 mo during the follow-up. Based on enhanced CT images, the pretreatment splenic volume and the post-embolization residual splenic volume were measured and compared on a workstation (Siemens Syngo MMWP VEZIA) using the volumetric analysis software. The infracted splenic volume (mL) was measured by subtracting the noninfarcted splenic volume from the pretreatment splenic volume. The splenic infarction rate was calculated by dividing the infarcted splenic volume by the pretreatment splenic volume (× 100%).

The procedure-related complications were divided into major and minor ones. Major complications associated with the procedure, including splenic abscess, splenic rupture, pneumonia, refractory ascites or pleural effusion, gastrointestinal bleeding, rupture of varices, and hepatic failure, were defined as complicated disease requiring surgical intervention or prolonged postoperative hospital stay time of more than 30 d. Minor complications, including abdominal pain, fever, vomiting, abdominal fullness, and appetite loss, were defined as those that lead to no consequential events and can be tolerated by the patients.

Statistical analysis

All data were expressed as mean ± SD. Changes in WBC and PLT counts after PSE were evaluated by paired *t* test. The variables between the 2 groups were compared by Mann-Whitney test, χ^2-test or Fisher's exact test when ap-

Table 1 Outcomes of total embolization of the main splenic artery in 15 patients

Pa./age (yr)/sex	Virus	Child-Pugh	WBC count (× 10^9/L)						Platelets (× 10^9/L)						Splenic volumn (cm^3)						Compli-cation	Follow-up (mo)	Out-comes
			Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr	Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr	Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr			
1/48/F	B	A	1.3	7.8	6.5	5.3	4.9	5.1	35	181	142	136	132	128	829	367	265	265	312	312	AP, F, V	52	Alive
2/52/M	B	B	1.8	9.8	5.3	5.6	5.6	4.4	24	191	156	148	145	139	768	258	326	326	326	367	F, V	48	Alive
3/60/M	B	A	1.6	8.6	6.5	6.3	4.2	6.4	33	233	185	157	132	142	869	369	328	328	328	305	AP, F	47	Alive
4/51/M	B	A	1.8	8.3	5.4	7.2	6.2	4.6	45	173	158	156	148	132	815	289	289	287	250	285	F, V	45	Alive
5/34/F	B	A	1.1	7.8	7.2	6.8	5.2	4.5	48	165	161	152	153	148	724	366	242	235	235	235	AP, V	41	Alive
6/49/M	B	C	1.4	8.6	4.8	4.6	4.4	4.8	43	144	43	139	132	123	698	278	278	278	278	278	AP, V	40	Alive
7/50/F	B	A	1.5	8.5	5.8	4.8	4.6	4.2	25	156	145	146	134	129	758	325	325	312	314	314		37	Alive
8/59/M	C	B	1.1	7.8	6.3	6.7	7.3	4.8	36	143	145	132	128	132	846	319	319	310	310	310	AP, F, V	36	Alive
9/28/M	B	A	1.5	9.6	6.0	5.1	5.2	5.3	48	213	167	164	156	147	687	247	247	247	249	276	AP, F,	36	Alive
10/55/M	B	A	0.8	7.3	6.9	5.6	5.4	4.5	32	121	125	131	128	124	784	305	305	298	320	320	AP, F, V	42	Alive
11/58/F	B	C	1.7	6.8	8.5	6.8	3.9	4.2	26	163	184	146	139	132	755	362	362	362	362	345	AP, F	38	Alive
12/46/M	B	A	0.9	5.3	4.8	3.8	4.5		48	146	135	141	136		732	247	285	285	285		AP, F, V	28	Alive
13/58/F	C	A	1.2	8.2	3.8	4.8	5.0		32	153	138	146	134		848	328	328	318	315		AP, F	30	Alive
14/55/M	B	B	1.4	8.3	5.2	4.5	4.4		35	164	176	163	165		683	361	356	346	327			26	Alive
15/48/M	B	A	1.6	8.8	6.3	6.2	6.4		38	241	220	168	137		752	298	305	315	315		AP, F	24	Alive

Pre-EM: Pre-embolization; AP: Abdominal pain; F: Fever; V: Vomiting; WBC: White blood cell.

propriate. All statistical analyses were performed using the SPSS package, version 13.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Primary procedure results

Total embolization of the main splenic artery was technically successful in all patients, with no procedure-related complications. The mean postoperative hospital stay time was 8.40 ± 2.53 d (range, 5-15 d) after the procedure and the 30 d mortality rate was zero.

Minor complications occurred in 13 patients with no major complications found after the procedure. The most frequent side effects were abdominal pain, fever, and nausea. Prolonged fever, lasting over 15 d after the procedure, developed in 1 case. These side effects were controlled after conservative therapy.

Changes in peripheral blood cell counts after embolization

The outcomes of total embolization of the main splenic artery in 15 patients are shown in Table 1. All patients were assessed 1 and 6 mo, and 1, 2, 3 years after the procedure. The patients were followed up for 38.0 ± 8.32 mo (range, 24-52 mo). The mean WBC count increased from 1.4 (0.3) × 10^9/L before the procedure to 8.1 (1.1) × 10^9/L, 6.0 (1.2) × 10^9/L, 5.6 (1.0) × 10^9/L, 5.1 (0.9) × 10^9/L, and 4.8 (0.9) × 10^9/L, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.01$).

The mean PLT count increased from 36.5 (8.3) × 10^9/L before the procedure to 172 (34.1) × 10^9/L, 152 (38.7) × 10^9/L, 148 (11.6) × 10^9/L, 140 (11.1) × 10^9/L, and 134 (8.6) × 10^9/L, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.01$).

Changes in splenic volume after embolization

The mean splenic volume decreased from 769.87 (60.51) cm^3 before the procedure to 314.60 (44.52) cm^3, 304.0 (36.10) cm^3, 300.80 (35.20) cm^3, 301.73 (35.17) cm^3, and 306.00 (32.02) cm^3, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.05$). During the follow-up, the residual splenic volume in these patients increased a very slowly. The mean infarction rate of the spleen was 60% (range, 59%-61%) 3 years after the procedure. No death occurred during the follow-up.

DISCUSSION

The results of his study show that total embolization of the main splenic artery with coils is a safe and feasible procedure for hypersplenism due to liver cirrhosis. The peripheral blood cell parameters including WBC and PLT counts increased significantly during the follow-up and the residual splenic volume increased very slowly after embolization.

Hypersplenism is a well-known complication of portal hypertension due to cirrhosis, which can result in thrombocytopenia and/or leukocytopenia. Splenectomy can eliminate hypersplenism-induced blood cell destruction, but the incidence of severe complications after splenectomy is 9.6%-26.6% whether laparoscopy or open splenectomy is performed[16-18]. In addition, splenectomy is often associated with an increased long-term risk of septic events[16-18].

Although PSE is an effective alternative to splenectomy to increase the peripheral blood cell counts[1-5], severe complications of PSE, such as splenic abscess, splenic rupture, pneumonia, refractory ascites or pleural effusion, and gastrointestinal bleeding[6-8], greatly limit its use. Furthermore, the complications of PSE are correlated with the infracted splenic volume. In addition, when 50% or less than 50% of the spleen is embolized, hypersplenism would relapse shortly after PSE[2,8]. Therefore, to ensure a sustained increase in PLT and leukocyte counts,

the splenic infarction rate should be greater than 50%[8], which, however, inevitably results in severe complications. To increase the PLT and leukocyte counts and reduce the rate of severe complications, total embolization of the main splenic artery was performed for hypersplenism due to liver cirrhosis in the present study.

The key procedure for reducing the severe complications and ensuring the sustained increase in PLT and leukocyte counts is to confirm the patency of collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery. If these connections are absent or incomplete, total embolization of the main splenic artery should not be performed because the procedure may result in more severe complications.

When the main splenic artery is completely embolized, the main blood flow supplying the spleen is stopped, but the collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery may provide a small amount of blood for the spleen to avoid complete infarction of the spleen. Thus, most of the spleen should be embolized with reservation of a partial normal spleen. Thus, the PLT and leukocyte counts increase after the procedure, and the occurrence of severe complications can be circumvented. In this study, the safety and feasibility of total embolization for hypersplenism of the main splenic artery were studied.

As compared with PSE, total embolization of the main splenic artery has the following advantages, including a low risk of procedure-related complications, persistent maintenance of normal WBC and PLT counts, and a very slow increase in residual splenic volume.

Although these results are encouraging, this study had the following limitations. First, it was not a comparative study and the number of patients was small with no control group. Future randomized multicenter trials comparing PSE with total embolization are needed to determine their long-term clinical efficacy and risk of complications. Second, total embolization could not be performed in patients with no or incomplete collateral arteries.

In conclusion, total embolization of the main splenic artery is a safe and feasible procedure for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis and may serve as a supplemental treatment modality for it. Further clinical trials and expanded follow-up studies are needed to confirm its safety.

COMMENTS

Background

Partial splenic embolization (PSE) is a non-surgical procedure for hypersplenism resulting from hepatic disease, thus avoiding the disadvantages of splenectomy. However, after PSE, patients often experience complications, including daily intermittent fever, abdominal pain, nausea, vomiting, and postembolization syndrome.

Research frontiers

Total embolization of the splenic artery has been widely used in treatment of splenic artery aneurysms, but no report is available on treatment of hypersplenism with it. In this study, total embolization of the main splenic artery for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis was studied.

Innovations and breakthroughs

Total embolization of the main splenic artery was devised for the treatment of hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis. All procedures were performed under fluoroscopic control. This is the first study reporting the treatment of hypersplenism with total embolization of the main splenic artery.

Applications

Total embolization of the main splenic artery is a safe and feasible procedure and may serve as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis with a low complication rate and a good mid-term clinical efficacy.

Terminology

Hypersplenism is a well-known complication of portal hypertension due to cirrhosis, which can result in thrombocytopenia and leukocytopenia.

Peer review

The finding in this study is interesting. Further study is needed confirm its safety in a much larger series of patients.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2958

World J Gastroenterol 2011 June 28; 17(24): 2958-2964
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer

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Received: November 17, 2010 Revised: March 11, 2011
Accepted: March 18, 2011
Published online: June 28, 2011

Abstract

AIM: To evaluate bacterial cytosine deaminase (bCD) mutant D314A and 5-fluorocytosine (5-FC) for treatment of colon cancer in a mouse model.

METHODS: Recombinant lentivirus vectors that contained wild-type bCD gene (bCDwt), and bCD mutant D314A gene (bCD-D314A) with green fluorescence protein gene were constructed and used to infect human colon carcinoma LoVo cells, to generate stable transfected cells, LoVo/null, LoVo/bCDwt or LoVo/bCD-D314A. These were injected subcutaneously into Balb/c nude mice to establish xenograft models. Two weeks post-LoVo cell inoculation, PBS or 5-FC (500 mg/kg) was administered by intraperitoneal (i.p.) injection once daily for 14 d. On the day after LoVo cell injection, mice were monitored daily for tumor volume and survival.

RESULTS: Sequence analyses confirmed the construction of recombinant lentiviral plasmids that contained bCDwt or bCD-D314A. The lentiviral vector had high efficacy for gene delivery, and RT-PCR showed that bCDwt or bCD-D314A gene was transferred to LoVo cells. Among these treatment groups, gene delivery or 5-FC administration alone had no effect on tumor growth. However, bCDwt/5-FC or bCD-D314A/5-FC treatment inhibited tumor growth and prolonged survival of mice significantly ($P < 0.05$). Importantly, the tumor volume in the bCD-D314A/5-FC-treated group was lower than that in the bCDwt/5-FC group ($P < 0.05$), and bCD-D314A plus 5-FC significantly prolonged survival of mice in comparison with bCDwt plus 5-FC ($P < 0.05$).

CONCLUSION: The bCD mutant D314A enhanced significantly antitumor activity in human colon cancer xenograft models, which provides a promising approach for human colon carcinoma therapy.

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Key words: Suicide gene therapy; Bacterial cytosine deaminase; Mutant; D314A; 5-fluorocytosine; Colon cancer

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Deng LY, Wang JP, Gui ZF, Shen LZ. Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer. *World J Gastroenterol* 2011; 17(24): 2958-2964 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2958.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2958

INTRODUCTION

Colorectal cancer has one of the highest mortalities worldwide[1]. Conventional therapies consist of surgery, chemotherapy, radiotherapy, and biotherapy. Despite advances in the treatment of colorectal cancer, the prognosis for locally advanced or metastatic disease remains relatively

poor[2]. Therefore, it is crucial to develop novel therapeutic strategies, not only to completely cure cancer, but also to prevent its recurrence. Among these approaches, gene-directed enzyme-prodrug therapy (GDEPT) has received considerable attention[3,4].

Suicide gene therapy for cancer is an appealing alternative to standard methods of chemotherapy because most chemotherapeutic agents lack tumor specificity. Classic chemotherapeutic agents are often unable to distinguish tumor cells from normal dividing cells, which results in indiscriminate toxic effects. In contrast, suicide gene therapy allows for specific targeting of the tumor while preventing damage to normal cells. This is accomplished by introducing a gene that encodes a prodrug-activating enzyme into cancer cells. Although delivery systems have not been fully optimized, delivery of the gene is typically done using either a viral vector (retrovirus or adenovirus) or by other non-viral means[5-7]. Once the gene is delivered into the cancer cell, a non-toxic prodrug is administered. The enzyme converts the non-toxic prodrug into its active and lethal form that results in cancer cell death. *Escherichia coli* or bacterial cytosine deaminase (bCD) is responsible for the activation of the non-toxic prodrug 5-fluorocytosine (5-FC) to its toxic form, 5-fluorouracil (5-FU)[8]. The absence of an endogenous cytosine deaminase in mammalian cells provides for deamination of 5-FC only in cells that express bCD. This is followed by the conversion of 5-FU into its deoxyribonucleoside, fluorodeoxyuridine (FUdR) by thymidine phosphorylase. Upon phosphorylation of FUdR by endogenous thymidine kinase, thymidylate synthase is irreversibly inhibited by the product, 5FdUMP, thereby preventing dTTP formation and ultimately leading to inhibition of DNA synthesis.

One of the key advantages of the bCD/5-FC enzyme/prodrug system is the phenomenon known as the "bystander effect", which is defined as the killing of untransfected cells neighboring those cells transfected with the suicide gene[8]. This type of killing has been described extensively with regard to the herpes simplex virus-1 thymidine kinase and ganciclovir enzyme/prodrug system, and occurs primarily by the transfer of toxic antimetabolites through gap junctions[9,10]. Unlike phosphorylated ganciclovir, 5-FU is a small, uncharged molecule that can pass freely in and out of the cell by diffusion. Consequently, cell-cell contact is not required for the bystander effect with bCD/5-FC; an advantage for those cell types with limited gap junctions[11]. bCD has been used successfully in gene therapy for a variety of animal tumor models and is currently under investigation for the treatment of human cancers[12-15].

The limiting factors for successful suicide GDEPT are transfection efficiency and the ability of the enzyme to turn over the prodrug, which is an analog of its natural substrate. From a kinetic perspective, 5-FC is a poor substrate for bCD (Km = 3.3 mmol/L) compared with its native substrate, cytosine (Km = 0.2 mmol/L)[16]. Thus, high doses of this prodrug must be administered to achieve cell killing. The plasma levels of 5-FC required to obtain a significant amount of active metabolites may lead to adverse effects. This is observed with 5-FC, whereas deamination by CD of bacterial intestinal microflora into 5-FU is responsible for side effects[17]. Other studies have suggested that the CD from *Saccharomyces cerevisiae* (yCD) displays a kinetic advantage towards 5-FC over bCD[18]. However, yCD is considerably less thermostable than bCD; a characteristic that may make the bacterial enzyme a preferable catalytic system for gene therapy.

Fortunately, Mahan *et al*[16,19] have used random mutagenesis to create novel bCDs that demonstrate an increased preference for 5-FC over cytosine. Among these mutants isolated, the mutant D314A [substitution of an alanine (A) for the aspartic acid (D) at position 314 of bCD] enzyme demonstrates a dramatic decrease in cytosine activity (17-fold), as well as a slight increase in activity toward 5-FC (twofold), which indicates that mutant D314A prefers the prodrug over cytosine by almost 20-fold. Despite the thermolability of yCD, others have suggested that yCD is superior to bCD in gene therapy settings because of a 23-fold relative substrate preference for 5-FC that is displayed by yCD[18]. However, given the thermostability of bCD and the 19-fold relative substrate preference that the bCD mutant D314A displays towards 5-FC, bCD D314A may be a superior suicide gene to yCD. These results indicate that bCD mutant D314A is a superior candidate for suicide gene therapy. Recently, this mutant D314A has been demonstrated to enhance cytotoxicity of human glioma and pancreatic cancer cells, and to increase therapeutic efficacy against human glioma and human pancreatic tumor xenografts, especially combined with radiotherapy[20,21]. However, there have been only a few studies of bCD mutants in colorectal cancer.

Previously, we have used the bCD gene to treat colon cancer, and have found that the efficacy of wild-type bCD is not sufficient to abolish cancer cells *in vitro* or *in vivo*, therefore, combination therapy with other genes, such as interleukin-2 or interferon-γ, is needed to improve the cytotoxicity of bCD[7,22,23]. Recently, we have constructed bCD-D314A using site-directed mutagenesis[19], and have demonstrated that it has significantly increased cytotoxicity in human colon cancer cell line LoVo, compared with wild-type bCD (bCDwt) *in vitro*[24].

The aim of the present study was to investigate whether bCD-D314A suicide gene and 5-FC prodrug therapy produce increased therapeutic efficacy *in vivo* for human colon cancer in nude mice using lentiviral vectors. The results presented here indicated that mutant bCD-D314A was able to significantly enhance antitumor efficacy in human colon cancer xenograft models *in vivo* compared with bCDwt.

MATERIALS AND METHODS

Cells and cell culture

Human colon cancer cell line LoVo (CCL-229; ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). HEK 293T (human embryonic kidney 293T cell line containing SV40 large T antigen) (CRL-11268; ATCC) was cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% cosmic calf serum (Hy-

clone, Logan, UT, USA), 2 mmol/L L-glutamine (Sigma, St. Louis, MO, USA), 100 U/mL penicillin (Sigma) and 0.1 mg/mL streptomycin (Sigma). LoVo and HEK 293T cells were in a 5% CO_2-humidified atmosphere at 37℃.

Reagents, animals, plasmids and vectors

5-FC was obtained from Sigma. Restriction enzymes *Hin*d Ⅲ, *Kpn* I , *Nhe* I , *Eco*R I and *Dpn* I , T4 DNA ligase, pfu DNA polymerase, DNA marker DL2000 and PCR reagents were obtained from Takara (Otsu, Shiga, Japan). Primers were chemically synthesized by Shanghai Generay Co. Ltd. (Shanghai, China). The plasmid DNA extraction (Mini) kit was provided by Qiagen (Crawley, West Sussex, UK). TRIzol and Lipofectamine 2000™ transfection reagent were obtained from Invitrogen.

Female athymic Balb/c (nu/nu) nude mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and were housed under aseptic conditions in micro-isolator cages, which were approved by the local Institutional Animal Care and Use Committee.

The plasmid pcDNA3.1/bCDwt that contained whole-length wild-type bCD gene, and pcDNA3.1/bCD-D314A that contained the mutant *D314A* gene, were prepared and stored in our department. The pLJM1-GFP lentivirus vector with green fluorescence protein (GFP) gene was a generous gift from Prof. J Li (Nanjing Medical University).

Recombinant bCDwt or bCD-D314A plasmids construction and identification

The pcDNA3.1-bCDwt and pcDNA3.1-bCD-D314A plasmids were all double digested with *Hin*dⅢ and *Kpn* I . The products of enzyme digestion were connected to the lentiviral vector pLJM1-GFP, which was double digested with *Nhe* I and *Eco*R I , to produce pLJM1-bCDwt-GFP and pLJM1-bCD-D314A-GFP. These plasmids were then transformed into *E. coli* XL1-Blue. The colonies were selected for PCR identification. The sense sequence of bCD primers was 5'-CGCAAATGGGCGGTAGGCGTG-3', whereas the antisense sequence was 5'AATTCTCAACGTTTGTAATCGATGG-3'. These recombinant plasmids were extracted and sent to BGI Sequencing Company (Shanghai, China) for sequencing.

Recombinant lentivirus construction, cell infection and stable cell line generation

To produce recombinant lentiviruses that encoded bCD-wt, bCD-D314A or GFP gene, three types of plasmids (pLJM1-bCDwt-GFP, pLJM1-bCD-D314A-GFP and pLJM1-GFP) were transfected to 293T cells according to the instructions for Lipofectamine 2000™ (Invitrogen). The virus-containing supernatant was collected 48 h after transfection, concentrated by centrifugation (4000 r/mim, 4℃ for 5 min), and filtered with a 0.45-μm membrane filter. The virus titers were determined in 293T cells.

For LoVo cell infection, there were three groups: bCDwt-GFP, bCD-D314A-GFP, and GFP (null). LoVo cells were seeded at a density of 1×10^5 cells in a 60-mm plate and infected with different lentiviral vectors in the presence of 10 μg/mL polybrene (Millipore, Billerica, MA, USA). At 10-12 h post-infection, the growth medium was replaced. Forty-eight hours later, the GFP expression of transduced cells was observed under fluorescence microscopy. LoVo cells were infected twice in the same way. At 3 d after transfection, the FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA) was used for fluorescence-activated cell sorting and then stable transfected cells, LoVo/null, LoVo/bCDwt and LoVo/bCD-D314A were cultured in a 5% CO_2-humidified incubator at 37℃.

Detection of bCD gene in transfected LoVo cells with RT-PCR

Total RNA was extracted from transfected LoVo cells harvested from the different groups with TRIzol reagent. First-strand cDNA was synthesized by reverse transcription according to the instructions for M2MLV (Promega, Madison, WI, USA). The sense sequence of bCD primers used in RT-PCR was 5'TTATGTCGAATAACGCTTTACAAAC-3', whereas the antisense sequence was 5'TACCTCCACGTTTGTAATCGATGGC-3'. PCR was performed for 35 cycles (94℃ for 1 min, 60℃ for 1.5 min, 72℃ for 1.5 min) in an automated DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA).

Xenograft tumor model study

To compare antitumor effects of bCDwt- and bCD-D314A-mediated molecular chemotherapy *in vivo*, combination of 5-FC, pools of LoVo, LoVo/null, LoVo/bCDwt or LoVo/bCD-D314A cells [5×10^6 cells in 100 μL PBS (pH 7.3)] were injected subcutaneously (s.c.) into the right flanks of 5-6-wk-old female Balb/c nude mice ($n = 20$, respectively). Two weeks post-LoVo cell inoculation, PBS or 5-FC (500 mg/kg) was administered by i.p. injection once daily for 14 d. Starting at day 1, the tumor volume was monitored daily using caliper measurement, calculated using the formula: $\pi/6 \times (\text{width} \times \text{length}^2)$. On the day after LoVo cell injection, mice were monitored daily for survival.

Statistical analysis

The treatment groups were compared with respect to tumor size. To test for significant differences in tumor volume among treatment groups, one-way ANOVA was conducted. When ANOVA indicated that a significant difference existed ($P < 0.05$), multiple comparison procedures were used to determine where the differences lay. Kaplan-Meier survival curves were analyzed by the log-rank test, and specific pairwise multiple comparisons were made using the Holm-Sidak method. All comparisons were made using the 0.05 level of significance.

RESULTS

Identification of recombinant lentiviral plasmids

Sequencing results of recombinant lentiviral plasmids showed that pLJM1-bCDwt-GFP contained the wild-type bCD gene, and pLJM1-bCD-D314A-GFP contained the mutant D314A (Figure 1), which indicated that the two recombinant lentiviral plasmids were constructed successfully.

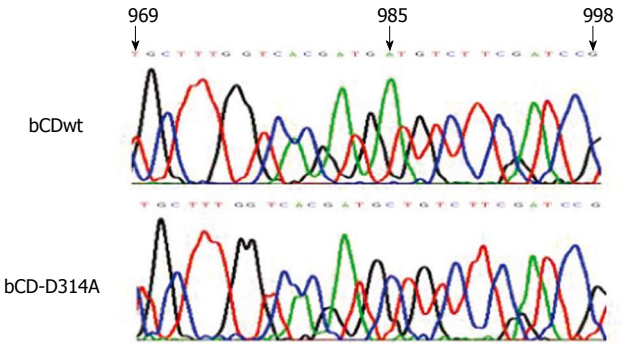

Figure 1 Sequencing results of recombinant plasmid pLJM1-bCDwt and pLJM1-bCD-D314A. The codon 985 of bCDwt, A, was mutated to C in bCD-D314A, which was the mutant D314A of bCD.

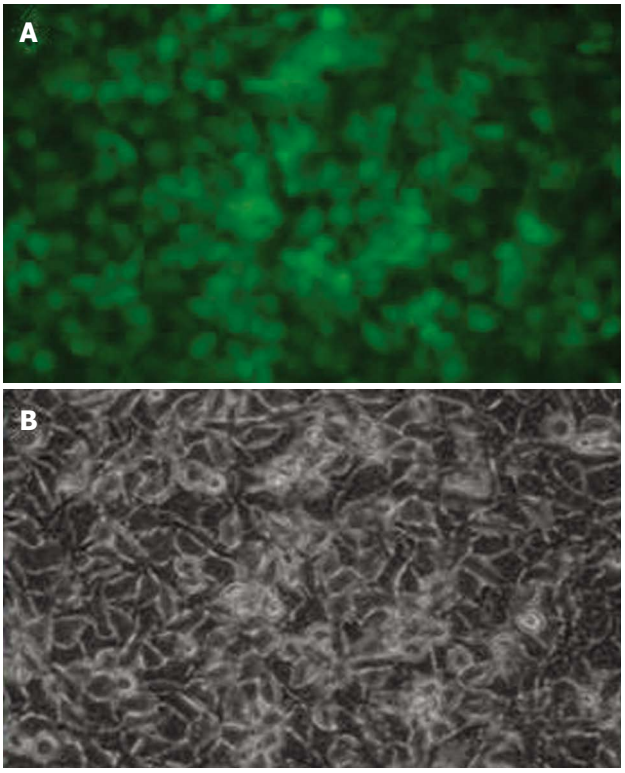

Figure 2 LoVo cell with fluorescence after transfection with lentiviral vector containing bCDwt-GFP gene (× 100). A: Green fluorescence field; B: Visible light field.

Efficacy of gene delivery by lentiviral vectors

Lentiviral vectors have improved efficiency to deliver genes. In this study, GFP was used as a reporter gene. Figure 2 shows that the efficacy of gene delivery by lentiviral vectors was satisfactory.

Identification of bCD gene in LoVo cells transfected with lentiviral vectors

LoVo cells transfected with different lentiviral vectors, LoVo/null, LoVo/bCDwt and LoVo/bCD-D314A, were subjected to RT-PCR to identify bCD gene expression. As shown in Figure 3, LoVo/bCDwt and LoVo/bCD-D314A cells had bCD gene expression, while bCD gene was not detected in LoVo/null cells.

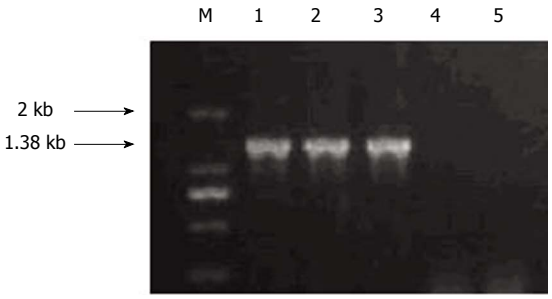

Figure 3 Identification of bCD gene in LoVo cells transfected with different lentiviral vectors. M: Marker; 1: Positive control (pcDNA3.1/bCDwt); 2: LoVo/bCDwt; 3: LoVo bCD-D314A; 4: LoVo/null; 5: Negative control (LoVo).

Antitumor effects of bCDwt and bCD-D314A in combination with 5-FC in human colon carcinoma xenograft model

To evaluate the potential of bCD-D314A gene therapy with 5-FC *in vivo*, LoVo cells with different stably gene delivery were injected s.c. into the right flank of athymic nude mice. Two weeks after cell inoculation, before treatment, the tumors in each group were palpable and the mean volumes in each group did not differ significantly among the treatment groups ($P > 0.05$), and within treatment variances (PBS *vs* 5-FC) were not significantly different ($P > 0.05$). The baseline mean tumor volume at 14 d after tumor cell injection was 412.63 ± 36.79 mm^3.

PBS or 5-FC (500 mg/kg) was administered i.p. once daily for 2 wk. Starting at day 1, mice were monitored for tumor volume and survival. Inhibition of tumor growth was initially noted in mice treated with LoVo/bCDwt or LoVo/bCD-D314A in combination with 5-FC compared with the other groups on day 20 ($P < 0.05$) (Figure 4). There were no significant differences in tumor growth between the other groups ($P > 0.05$), which indicated that gene delivery or 5-FC administration alone had no influence on tumor growth. From day 20 onwards, tumors in the mice treated with LoVo/bCD-D314A and 5-FC shrunk daily, whereas the tumors in mice treated with LoVo/bCDwt and 5-FC increased gradually. The difference in tumor volume between these two groups became increasingly marked ($P < 0.05$). At the same time, the tumors in the other groups kept growing (Figure 4).

As to the influence of bCD-D314A and bCDwt on survival, we showed that bCD-D314A/5-FC or bCDwt/5-FC treatment significantly prolonged survival of mice in comparison with the other groups. As shown in Figure 5, the median survival time of other groups was about 35 d and there was no difference among them ($P > 0.05$), whereas it was prolonged to 62 or 94 d in the bCDwt/5-FC or bCD-D314A/5-FC group, respectively ($P < 0.05$). Furthermore, bCD-D314A plus 5-FC significantly prolonged survival of mice in comparison with bCDwt plus 5-FC ($P < 0.05$).

DISCUSSION

Worldwide, more than one million individuals will develop colorectal cancer annually, and the disease-specific mortal-

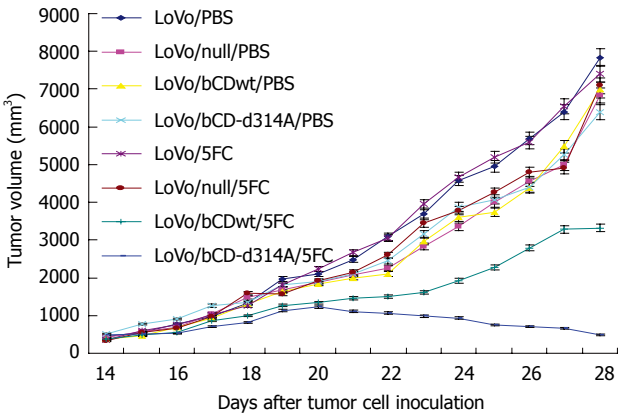

Figure 4 Growth of LoVo, LoVo/null, LoVo/bCDwt, and LoVo/bCD-D314A xenografts treated with PBS or 5-fluorocytosine. Treatment was started on 14 d after tumor cell inoculation. PBS or 5-fluorocytosine (5-FC) (500 mg/kg) was injected intraperitoneal once a day for 14 d. Data points represent the mean tumor volume of each group of animals. bCD: Bacterial cytosine deaminase; bCDwt: Wild-type bCD; bCD-D314A: bCD mutant D314A; LoVo/null: LoVo cell transfected with pLJM1-GFP; LoVo/bCDwt: LoVo cell transfected with pLJM1-bCDwt-GFP; LoVo/bCD-D314A: LoVo cell transfected with pLJM1-bCD-D314A-GFP.

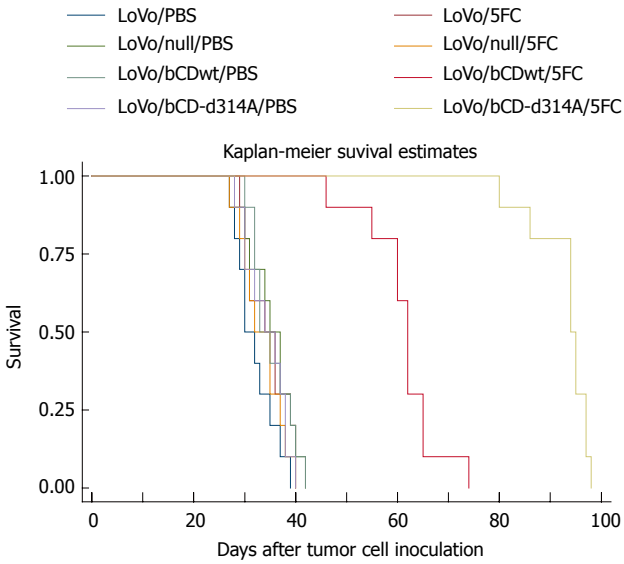

Figure 5 Efficacy of wild-type bCD gene or bCD mutant D314A gene suicide gene therapy in human colon carcinoma xenografts. LoVo human colon carcinoma cells with different gene transfection (5 × 10^6 cells/mouse) were injected into the right flank of athymic nude mice (10 mice/group). Two weeks after tumor cell injection, PBS or 5-fluorocytosine (5-FC) (500 mg/kg) was injected intraperitoneal once a day for 14 d. On the day after LoVo cell injection, mice were monitored daily for survival. bCD: Bacterial cytosine deaminase; bCDwt: wild-type bCD; bCD-D314A: bCD mutant D314A; LoVo/null: LoVo cell transfected with pLJM1-GFP; LoVo/bCDwt: LoVo cell transfected with pLJM1-bCDwt-GFP; LoVo/bCD-D314A: LoVo cell transfected with pLJM1-bCD-D314A-GFP.

ity rate is nearly 33% in the developed world[1]. Substantial progress has been made in understanding the molecular pathogenesis, diagnosis (hereditary and sporadic), and treatment of colorectal cancer during recent decades. Despite the use of 5-FU-based combination chemotherapy and active targeted drugs for treatment of metastatic colorectal cancer in the past decade, and improvement in overall survival for non-resectable disease, cure rates remain low[25]. There is a need for the development of new alternative therapeutic strategies. Gene therapy is a novel approach that might lead to improved treatments for colorectal cancer. Among these approaches, GDEPT using the bCD/5-FC system has been developed. The gene that encodes the CD that converts the prodrug 5-FC to 5-FU is delivered to the target tumor cells, which results in their death.

The most important characteristic of suicide gene therapy is its bystander effect. Although the viral or non-viral gene delivery systems currently available have poor efficacy for *in vivo* gene transfer, complete eradication of tumors has been seen in some experimental animal models, which is thought to depend on the bystander killing effect. In the bCD/5-FC system, the bystander effect is caused by the passive diffusion of 5-FU into the extracellular milieu and its diffusion into the adjacent cells, which requires no gap junctions[11]. The immune-related response also contributes to the bystander effect[26], which has been confirmed by our experimental results from the immunocompetent and immunodeficient mice[27]. Although this approach has been in development for several decades, new combinations with cancer therapies, such as selective conventional chemotherapy[28] and radiotherapy[29], are being tested.

Unlike conventional chemotherapy, suicide gene therapy renders specific killing of the tumor cells that express the suicide gene, but it may lead to systemic toxicity if these genes are delivered to normal cells. Thus, target specificity is of great importance to suicide gene therapy. The rationale behind suicide gene therapy is that, after targeted transfer of these genes into tumor cells, only tumor and neighboring cells will be rendered sensitive to their cytotoxic action. Specifically, targeted expression of the prodrug-activating enzyme avoids systemic toxicity, and results in high drug concentrations in the tumor mass and an improved therapeutic index compared with non-targeted gene delivery. To kill carcinoembryonic antigen (CEA)-positive colorectal carcinoma cells specifically using the bCD/5-FC system, we have constructed a new replication-deficient recombinant adenoviral vector that contains the bCD gene controlled by the CEA promoter, AdCEACD, and have evaluated its *in vitro* cytotoxic effects. We have shown that this vector can transfer bCD to CEA-positive tumor cells specifically by comparing the vector with cytomegalovirus (CMV) promoter, AdCMVCD[7]. However, the cytotoxic effects of bCD/5-FC decreased to some extent[22,23]. Although this loss of activity may be due to differences in transcriptional activation between the CEA and CMV promoters, the low affinity displayed by wild-type bCD towards 5-FC in comparison with cytosine is thought to be the principal factor that leads to the relatively poor turnover of 5-FC of wild-type bCD and limits the overall therapeutic response.

It has been shown previously that the bCD mutant, D314A, decreased efficiency for endogenous cytosine, which can compete with the prodrug for the active enzyme site, in combination with increased efficiency for 5-FC that resulted in a 19-fold relative substrate preference for 5-FC in comparison with bCDwt[16,19]. The bCD mutant D314A has been demonstrated to be an excellent candidate for

subsequent preclinical comparisons with wild-type bCD and yCD.

Recently, we have constructed the bCD mutant D314A using site-directed mutagenesis. The *in vitro* results have indicated that its killing and bystander effects on human colon cancer LoVo cells are enhanced significantly as compared with wild-type bCD[24]. Thus, the rationale for using the mutant bCD gene for colon carcinoma *in vivo* is that the bCD mutant D314A can more effectively convert 5-FC to 5-FU, and increase the antitumor activity and prolong survival.

In the present study, we investigated mutant bCD gene transfer with lentiviral vector for treatment of human colon cancer in xenograft models. Lentivirus-based vectors (lentivectors) have been developed with improved efficiency, specificity, and safety, and are being increasingly used in basic and applied research. Clinical trials of human gene therapy are currently underway using lentivectors in a wide range of human diseases[30]. In the present study, lentiviral vector was used to transfer suicide genes. These preliminary results confirmed the efficacy of lentiviral vector for suicide gene delivery.

After the LoVo cells stably transfected with bCDwt gene or mutant bCD-D314A gene were established, they were inoculated into athymic nude mice to produce xenograft tumor models. Afterwards, 5-FC was administered. As expected, a more potent cytotoxicity effect for colon cancer was obtained using bCD-D314A/5-FC treatment in comparison with bCDwt/5-FC. During 5-FC administration, the tumors treated with bCDwt/5-FC or bCD-D314A/5-FC grew slower than those in other treatment groups, which indicated that 5-FC or suicide gene transfer alone had no effect on colon cancer. The comparative study of bCD-D314A/5-FC and bCDwt/5-FC showed an increased antitumor effect, and decreased tumor growth was observed following bCD-D314A/5-FC gene therapy. Furthermore, survival analysis showed that bCD-D314A/5-FC therapy prolonged life significantly, which confirmed the enhanced antitumor activity of bCD mutant D314A.

Although the intratumoral or blood 5-FU concentration was not estimated after 5-FC administration in this study, the enhanced antitumor effect of bCD mutant D314A in combination with 5-FC was thought to be due to its ability to convert 5-FC to 5-FU more effectively, which is consistent with recently published data in other tumor models[20,21]. The blood and tumor levels of 5-FC and 5-FU are a subject for future studies that will enable a rational dosing strategy.

In summary, our studies provide preliminary evidence that treatment using bCD mutant D314A for suicide gene/5-FC prodrug therapy is a promising approach for treatment of human colon carcinoma. Further studies on delivery systems, doses and protocols would be worthwhile to optimize this approach.

ACKNOWLEDGMENTS

The authors thank Professor Jian-Ming Li for the kind gift of pLJM1.

COMMENTS

Background

Suicide gene therapy is an appealing alternative to conventional therapies for colorectal cancer. Bacterial cytosine deaminase (bCD) can convert the non-toxic prodrug 5-fluorocytosine (5-FC) to its toxic form, 5-fluorouracil (5-FU). However, 5-FC is a poor substrate for bCD compared with its native substrate, cytosine, and its antitumor effect is limited.

Research frontiers

The bCD mutant, D314A, has been shown to prefer 5-FC over cytosine by nearly 20-fold, and has been demonstrated to enhance therapeutic efficacy against human glioma and human pancreatic tumors. However, there have been only a few studies of bCD mutants in colorectal cancer.

Innovations and breakthroughs

Recently, the authors have constructed the bCD mutant, D314A, using site-directed mutagenesis, and have demonstrated that D314A has significantly increased cytotoxicity on human colon cancer cell line LoVo compared with wild-type bCD (bCDwt). The present study indicated that mutant D314A was able to significantly enhance antitumor efficacy in human colon cancer xenograft models compared with bCDwt.

Applications

Collectively, these studies provide preliminary evidence that treatment using bCD mutant D314A for suicide gene/5-FC prodrug therapy provides a promising approach for human colon carcinoma.

Terminology

Suicide gene therapy is a form of gene-directed enzyme-prodrug therapy. When the suicide gene is delivered to cancer cells and a non-toxic prodrug is administered, the enzyme converts the non-toxic prodrug into its active and lethal form, which results in cancer cell death.

Peer review

The authors investigated whether bCD mutant D314A suicide gene and 5-FC prodrug therapy increased therapeutic efficacy in a nude mouse model of human colon cancer, using lentiviral vectors. It revealed for the first time that D314A significantly enhanced antitumor activity in human colon cancer xenograft models. The results are useful and may provide a new strategy to treat colorectal cancer.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2965

World J Gastroenterol 2011 June 28; 17(24): 2965-2971
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

CD133$^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity

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Supported by Grant from the National Natural Science Foundation of China, No. 30772172

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Received: October 25, 2010 Revised: November 28, 2010
Accepted: December 5, 2010
Published online: June 28, 2011

Abstract

AIM: To identify cancer stem cells (CSCs) in human gallbladder carcinomas (GBCs).

METHODS: Primary GBC cells were cultured under serum-free conditions to produce floating spheres. The stem-cell properties of the sphere-forming cells, including self-renewal, differentiation potential, chemoresistance and tumorigenicity, were determined *in vitro* or *in vivo*. Cell surface expression of CD133 was investigated in primary tumors and in spheroid cells using flow cytometry. The sphere-colony-formation ability and tumorigenicity of CD133$^+$ cells were assayed.

RESULTS: *In vitro* culture experiments revealed that floating spheroids were generated from primary GBC cells, and these sphere-forming cells could generate new progeny spheroids in serum-free media. Spheroid cells were differentiated under serum-containing conditions with downregulation of the stem cell markers Oct-4, Nanog, and nestin ($P < 0.05$). The differentiated cells showed lower spheroid-colony-formation ability than the original spheroid cells ($P < 0.05$). Spheroid cells were more resistant to chemotherapeutic reagents than the congenetic adherent cells ($P < 0.05$). Flow cytometry showed enriched CD133$^+$ population in sphere-forming cells ($P < 0.05$). CD133$^+$ cells possessed high colony-formation ability than the CD133$^-$ population ($P < 0.01$). CD133$^+$ cells injected into nude mice revealed higher tumorigenicity than their antigen-negative counterparts ($P < 0.05$).

CONCLUSION: CD133 may be a cell surface marker for CSCs in GBC.

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Key words: Gallbladder carcinoma; Cancer stem cell; Non-adherent spheres; CD133 protein; Self-renewal; Tumorigenicity

Peer reviewer: Giedrius Barauskas, Professor, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas, LT-50009, Lithuania

Shi CJ, Gao J, Wang M, Wang X, Tian R, Zhu F, Shen M, Qin RY. CD133$^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity. *World J Gastroenterol* 2011; 17(24): 2965-2971 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2965.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2965

INTRODUCTION

Gallbladder carcinoma (GBC) is the most common

malignant neoplasm of the biliary tract and the seventh most common gastrointestinal cancer[1]. Its clinical presentation is nonspecific and may include abdominal pain, weight loss, fever, and jaundice. Current evidence suggests that radical surgery is the only curative treatment for GBC. However, despite development in surgery, the 5-year survival rate in patients with advanced stage GBC is still only around 10%[2,3]. Pooling of carcinogens under conditions causing biliary stasis, or malignant degeneration of metaplastic changes after chronic inflammation have been suggested as possible factors**,** but the precise pathogenetic mechanisms of GBC remain unclear[1]. The biology of GBC therefore needs further investigation.

Emerging evidence has shown that the abilities for tumor growth and propagation reside in a small population of tumor cells, termed cancer stem cells (CSCs) or tumor-initiating cells. These cells possess properties of self-renewal, differentiation potential, resistance to chemotherapy, and high tumorigenicity[4-8]. Based on this hypothesis, CSCs were initially isolated from human acute myeloid leukemia[9]. Regarding solid tumors, the existence of CSCs in breast cancer was reported in 2003, when as few as 200 CD44$^+$CD24$^{-/low}$ ESA$^+$ breast cancer cells were shown to be adequate to produce new tumors in nonobese diabetic/severe combined immunodeficient mice, whereas a significantly higher number of other cell populations failed to form tumor xenografts[10]. Tumor-initiating cells with distinct cell surface markers have recently been identified in various solid tumors, such as brain[11], prostate[12], pancreatic[13], and ovarian cancer[14], and in Ewing's sarcoma[15]. It is generally considered that the identification of the CSCs could have a significant impact on the understanding of tumor biology and therapy.

Several different methods have previously been used to identify CSCs[16,17], including the culture of cancer cells under non-adherent conditions in serum-free media containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The growth of spherical colonies is considered to reflect the self-renewal ability and phenotype of CSCs. In the present study, we cultured primary GBC cells to generate spherical colonies and estimated their differentiation potential in the serum medium. We compared the chemoresistance of spheroid cells and differentiated cells *in vitro*. We also examined the expression of the CSC surface marker CD133, and investigated its use as a candidate marker to further identify the CSC phenotype in GBC, including comparing the *in vitro* spheroid-colony-formation and *in vivo* tumorigenicity of CD133$^+$ and CD133$^-$ cells. The results of this study may clarify the phenotype of CSCs in GBC, thus contributing to the development of more effective therapeutic approaches.

MATERIALS AND METHODS

Preparation of single cancer cells

Two samples of human GBC were obtained after surgical excision in accordance with Institutional Review Board-approved guidelines. Tumor tissue specimens were dissociated using scissors and scalpels, mixed with collagenase Ⅳ (Invitrogen, USA) in medium199 (collagenase 200 U/mL, Invitrogen), and incubated at 37℃ for 2.5-3 h. At the end of the incubation, cells were filtered through a 40-μm nylon mesh and washed twice with phosphate-buffered saline (PBS)/10% fetal bovine serum (FBS, Gibco, USA).

Tumor cell cultures

The single tumor cells were suspended in serum-free DMEM/F12 (1:1 volume, Gibco) consisting of 20 ng/mL human recombinant EGF (PeproTech, USA), 20 ng/mL bFGF (PeproTech), 5 μg/mL insulin (Sigma, USA), and cultured in 24-well culture plates at a density of 1×10^4/well. Fresh serum-free DMEM/F12 (described above) was added into the wells at 0.05 mL/well every day. Spheroids were collected and dissociated 2 wk after primary culture. The resulting single cells were placed into stem cell culture medium to generate progeny spheres. Images of the spheroid colonies were recorded using an inverted microscope (Nikon, Type 108) equipped with a Nikon 2000-S Inverted Photomicroscope and Nikon NIS-Elements F2.30 software.

Differentiation assay

To assess their differentiation potential, spheres were collected and placed into DMEM/F12 supplemented with 10% FBS without growth factors, as described previously, and cell morphology was observed. After 14 d of culture in differentiating medium, tumor cells were collected and suspended in serum-free DMEM/F12 (described above), and cultured in 96-well culture plates at a density of 10 cells per well. Fresh serum-free DMEM/F12 was added into the wells at 0.025 mL per well every day. After 2 wk, each well was examined under light microscope and the total number of spheroid colonies in the 96-well plates was counted.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from spheroid cells or adherent cells using RNeasy Mini kit (Qiagen), and was reverse transcribed into cDNA using M-MLV reverse transcriptase enzyme (Sigma). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems), according to the manufacturer's instructions. The relative mRNA expression levels of the tested genes were normalized to the level of endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase.

Chemoresistance experiments

Cells were seeded in 96-well plates at 3000 cells per well. Each well was supplied with DMEM medium containing 10% FBS, together with either gemcitabine (1 μg/mL, Sigma) or 5-fluorouracil (0.1 μg/mL, Sigma), or no drug as control. The culture medium was changed 3 d after

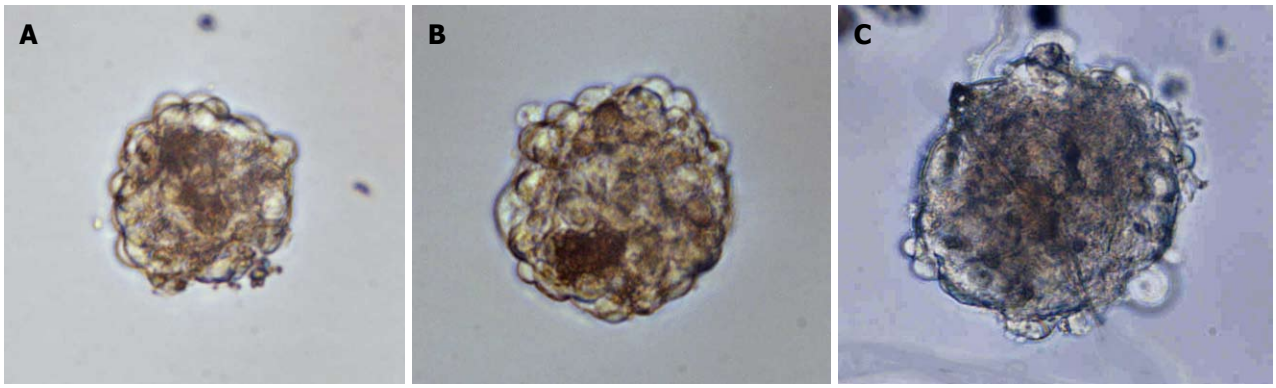

Figure 1 Culture of floating spheres. Single primary gallbladder carcinoma cells were cultured in serum-free medium containing human epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL). A: After culture for 1 wk, non-adherent spheres were observable (original magnification × 200); B: The spheres were dissociated and were plated into the same stem-cell-selective medium; similar progeny spheres emerged after 2 wk (original magnification × 200); C: The second progeny spheres were derived from the first progeny sphere-forming cells (original magnification × 200).

initial treatment and the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, 20 μL of MTT (5 mg/mL in PBS, Sigma) was added to the medium for 4 h. Medium and MTT were removed, dimethylsulfoxide (Sigma) was added, and the absorbance was measured at 490 nm using a plate reader Multiskan EX (Thermo Fisher Scientific Inc., Waltham, MA).

Detection of CD133 expression using flow cytometry

Cells derived from primary tumors or spheres were separately resuspended in PBS with 2% FBS at a concentration of 10^6/100 μL. Anti-CD133/1-phycoerythrin (eBioscience, USA) was added to the samples, and incubated on ice for 30 min. After incubation, the samples were washed twice with 2% FBS/PBS and resuspended in 2% FBS/PBS. Flow cytometric analysis was performed using a FACSAria (BD Immunocytometry Systems, Franklin Lakes, NJ, USA).

Spheroid-colony-formation assay of CD133$^+$ GBC cells

CD133$^+$ and CD133$^-$ populations were sorted from sphere-forming cells using fluorescence-activated cell sorting (FACS). For FACS, cells were collected and stained, and sorted using a FACSAria. The sorted tumor cells were suspended in serum-free DMEM/F12, and cultured in 96-well culture plates at a density of 10/well. After 2 wk, the total number of spheroid colonies in the 96-well plate was counted, as described above.

Tumorigenicity of CD133$^+$ GBC cells in vivo

Female nude mice (BALB/C), 4-6 wk old, were purchased from Hunan Slack King of Laboratory Animal Co., Ltd. (Changsha, China). CD133$^+$ and CD133$^-$ populations were sorted from two primary tumors (tumor 3 and tumor 4), and from sphere-forming cells using FACS. Cells were routinely sorted twice, and reanalyzed for a purity, which was typically > 90%. Sorted cells were resuspended in PBS/Matrigel mixture (1:1 volume). The mice were anesthetized using ethyl ether and 10000 tumor cells were injected subcutaneously into the abdominal region, using a gauge needle. The mice were maintained under standard conditions according to the institutional guidelines for animal care. Tumor appearance was inspected weekly by visual observation and palpation. Animal experiments were terminated 3 mo after cell injection.

Hematoxylin and eosin staining and immunohistochemistry

Primary tumor tissues and mouse xenografts were fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (HE) to assess tumor type. The sections were incubated with anti-human CA19-9 antibody (Abcam, UK) and secondary antibodies using an ImmunoPure ABC Staining kit (Santa Cruz, USA), according to standard immunohistochemical procedures. Negative controls containing no primary antibody were prepared. All microscopic images were captured as above.

Statistical analysis

Data were expressed as mean ± SD and Student's *t* test was used to compare the differences between groups. Values of $P < 0.05$ were considered significant.

RESULTS

Spheroid formation

Previous studies indicated that CSCs could produce floating three-dimensional tumor spheroids under stem-cell-selective conditions[18-21]. Based on these studies, we cultured primary human GBC cells in serum-free DMEM/F12 in an attempt to expand human GBC CSCs. Non-adherent spheres derived from human GBCs were observable after *in vitro* culture for 1 wk (Figure 1A), and these continued to expand for 2-3 wk in serum-free media. The spheres were dissociated and the resulting single cells were plated in the same stem-cell-selective medium; similar progeny spheres emerged after 2 wk (Figure 1B and C). This demonstrated that tumor sphere cells had self-renewing characteristics.

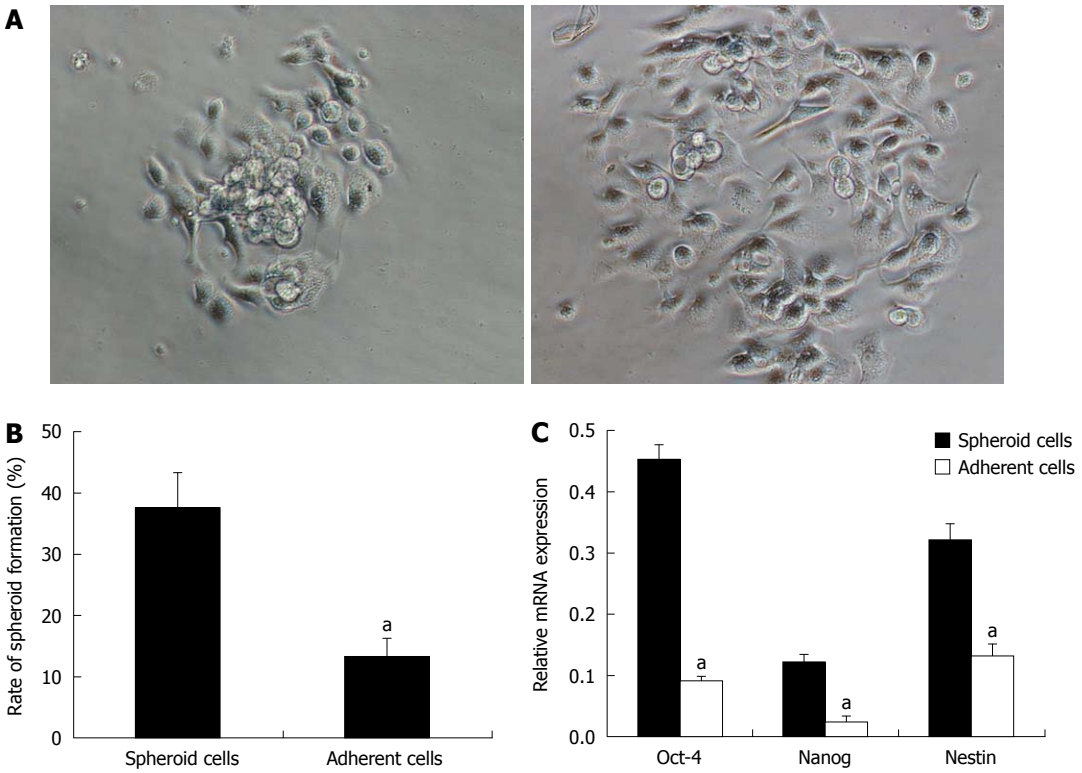

Figure 2 Differentiation of spheroid cells. Non-adherent spheres were collected and placed into DMEM/F12 supplemented with 10% fetal bovine serum. A: After 6 h, cells migrated from the spheres and became adherent and the sphere volume was significantly reduced (original magnification × 200); B: The spheroid-colony-forming ability of spheroid cells decreased under differentiating conditions. $^aP < 0.05$ *vs* spheroid cells under stem-cell-selective conditions; C: The expression levels of Oct-4, Nanog and nestin were examined using real-time quantitative reverse transcription-polymerase chain reaction. The stem cell markers were downregulated in the adherent cells. $^aP < 0.05$ *vs* spheroid cells under stem-cell-selective conditions.

Sphere-forming cells displayed differentiation potential

Spheres were cultivated under differentiating conditions to determine the differentiation potential of the tumor sphere cells. After 6 h of culture, the floating tumor spheres attached to the bottom of the culture plates and cells migrated from the spheres and became adherent (Figure 2A). After 14 d of culture in differentiating conditions, the sphere-formation ability of the adherent cells was assayed. The spheroid-colony-forming ability decreased, compared with that of the original sphere-forming cells ($P < 0.05$, Figure 2B). The expression of stem cell markers, including Oct-4, Nanog and nestin, were examined using real-time RT-PCR. These markers indicate an undifferentiated stem cell phenotype[22,23]. Spheroid cells showed higher expression of these markers than adherent cells ($P < 0.05$, Figure 2C), strongly supporting the idea that spheroid cells were differentiated in serum-containing medium.

Sphere-forming cells displayed high chemoresistance in vitro

Previous studies suggested that CSCs in several solid tumors possessed higher chemoresistance than non-CSCs[24-26]. To examine if our spheroid cells also possessed a CSC chemoresistant phenotype, the chemosensitivities of these cells were assessed under stem-cell-selective *vs* differentiating conditions. Spheroid cells under stem-cell-selective conditions displayed a greater resistance to gemcitabine and 5-fluorouracil than those under differentiating conditions ($P < 0.05$, Figure 3).

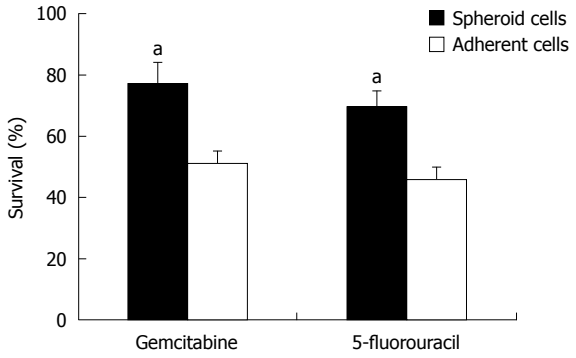

Figure 3 Chemoresistance assays of spheroid cells. Sphere-forming cells and differentiated cells were seeded in 96-well plates at 3000 cells/well. Chemotherapeutic reagents gemcitabine (1 μg/mL) and 5-fluorouracil (0.1 μg/mL) were added and cell survival was estimated by 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide assay. $^aP < 0.05$ *vs* differentiated cells.

CD133$^+$ cells were enriched in tumor spheres

The expression pattern of a possible candidate cell surface marker for CSCs was examined in primary human GBC and in sphere-forming cells, using flow cytometry. CD133 was selected as a potential marker, based on the results of previous studies of CSCs in solid tumors. Flow cytometric analysis revealed that CD133$^+$ cells were pres-

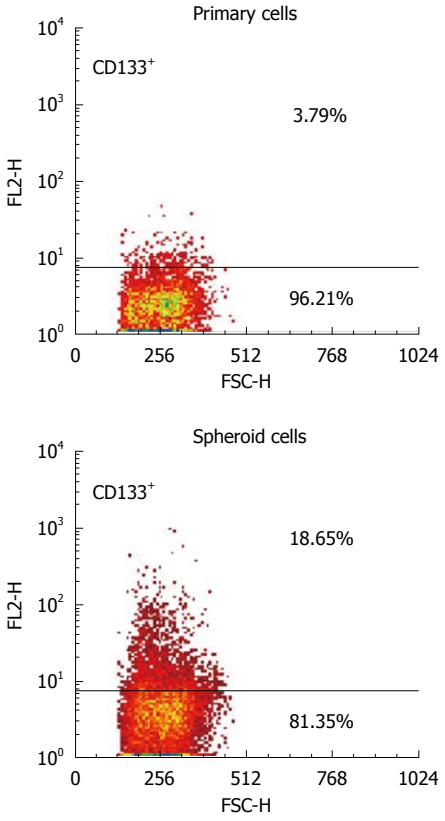

Figure 4 CD133 expression in gallbladder carcinoma spheroid cells. The single cells dissociated from primary tumors or spheres were incubated with anti-CD133/1-phycoerythrin and flow cytometric analysis was performed. The percentage of CD133$^+$ cells was higher in the spheroid cells. $P < 0.05$ *vs* primary gallbladder carcinoma cells.

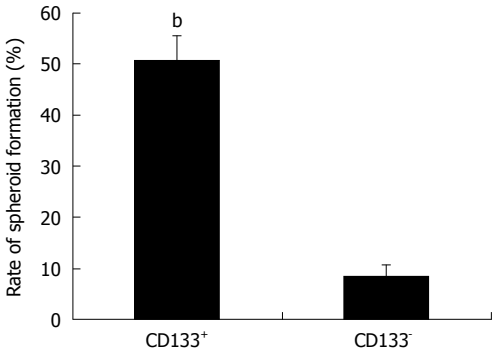

Figure 5 Spheroid-colony-formation assay of CD133$^+$ cells. Sphere-forming cells were isolated by fluorescence-activated cell sorting for marker CD133 and cultured in 96-well plates in serum-free medium (10 cells per well) containing human epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL). After 2 wk, the total number of spheroid colonies containing more than 20 cells was counted. CD133$^+$ cells generated more spheroid colonies. $^bP < 0.01$.

Table 1 Tumorigenicity of CD133$^+$ and CD133$^-$ cells sorted from primary gallbladder carcinomas or spheroids

Cell type	Fraction	1st mo	2nd mo	3rd mo[a]
Spheroid cells	CD133$^+$	1/6	3/6	6/6
	CD133$^-$	0/6	0/6	1/6
Tumor 3	CD133$^+$	0/3	1/3	3/3
	CD133$^-$	0/3	0/3	1/3
Tumor 4	CD133$^+$	1/3	2/3	3/3
	CD133$^-$	0/3	0/3	0/3

Freshly-sorted cells were injected subcutaneously into the abdominal regions of nude mice at a dose of 10 000 cells. $^aP < 0.05$.

ent at relatively low percentages in samples from both primary tumors (3.79% in tumor 1 and 3.15% in tumor 2). The CD133$^+$ populations, however, were significantly increased to 18.65% (tumor 1) and 21.54% (tumor 2) in the tumor spheres ($P < 0.05$, Figure 4). These results suggest that CD133 could be a candidate cellular surface marker for GBC progenitors.

CD133$^+$ GBC cells showed higher spheroid-colony-forming ability in vitro

The growth of spherical colonies is considered to reflect the self-renewal ability and phenotype of CSCs[16]. CD133$^+$ cells were isolated from spheres and placed into stem-cell-selective conditions. After *in vitro* culture for 2 wk, the total number of spheroid colonies containing more than 20 cells was counted, and CD133$^+$ cells generated more spheroid colonies than the CD133$^-$ fractions ($P < 0.01$, Figure 5). These results suggest that the CD133$^+$ subset plays a dominant role in the spheroids.

CD133$^+$ GBC cells showed higher tumorigenicity in vivo

To authenticate the *in vitro* findings, sorted GBC cells were transplanted into nude mice. An apparent difference in tumorigenicity was observed between the cell populations ($P < 0.05$, Table 1, Figure 6A). It was found that 10^4 CD133$^+$ GBC cells were able to generate tumors in six out of six or three out of three nude mice after 9-12 wk, while the same number of CD133$^-$ cells induced tumors in only one out of six or one out of three nude mice, with smaller mass and longer latency. HE staining and immunohistochemistry demonstrated that the xenografts in the immunodeficient mice were generated from the injected human GBC cells. The xenograft tumors revealed similar histologic characteristics and expression of CA19-9 to those of the primary GBC (Figure 6B). Taken together, these results indicate that CD133$^+$ GBC cells exhibited cancer stem-cell-like characteristics, strongly supporting the existence of tumor-initiating cells in this population.

DISCUSSION

A number of studies have demonstrated the presence of CSCs in solid tumors. These cells possess the abilities of self-renewal and differentiation, high tumorigenicity, and resistance to current treatments[10-15]. In this study, we described the characterization of CSCs in human GBC. Previous studies showed that tumor spheres could be generated from tumor cells in serum-free medium and that the constituent cells exhibited the properties of CSCs, including self-renewal, differentiation potential, chemotherapy resistance, and high tumorigenicity[11,14].

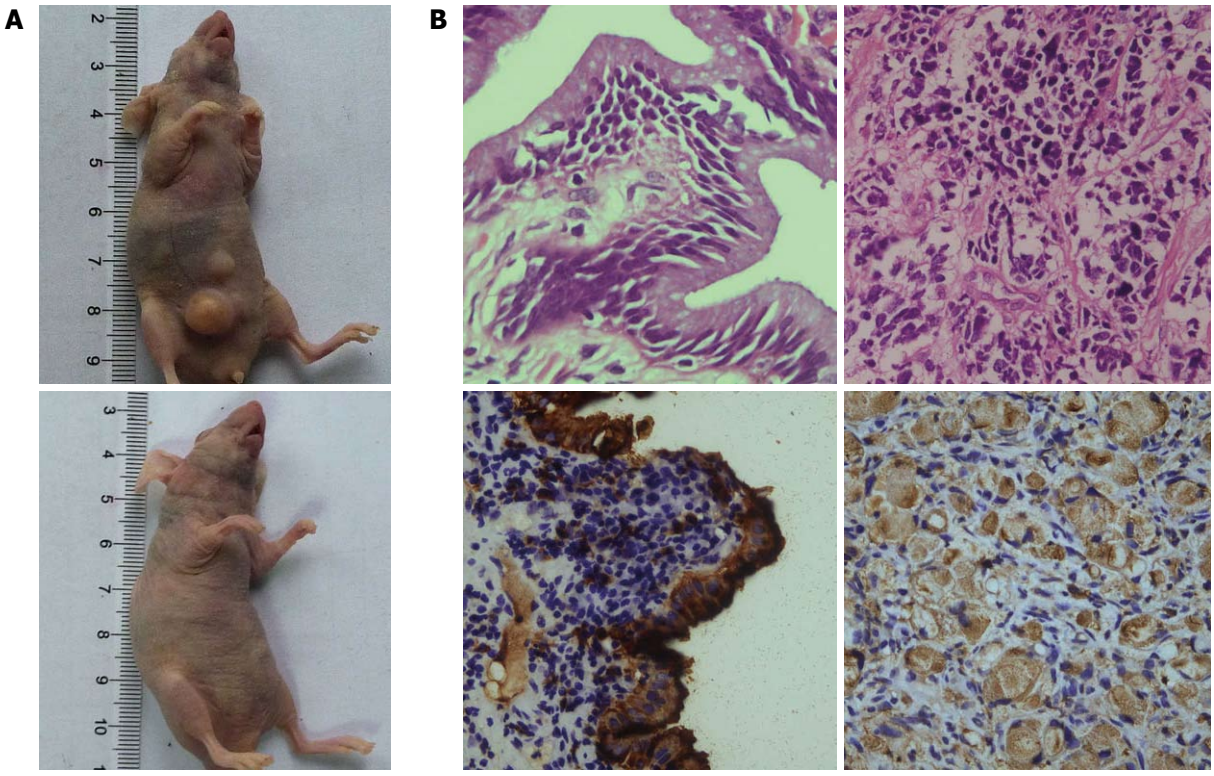

Figure 6 Xenograft formation of CD133$^+$ gallbladder carcinoma cells. Sorted cells were injected subcutaneously into the abdominal regions of nude mice at a dose of 10 000 cells. A: CD133$^+$ cells produced palpable xenograft tumors at the injection site, whereas CD133$^-$ cells generated no tumors; B: Hematoxylin and eosin staining of xenograft and expression of CA19-9 (original magnification × 200).

In our experiments, primary GBC cells formed tumor spheres when cultivated under stem-cell-selective conditions similar to those reported previously. The self-renewal and differentiation potentials, proliferation ability and chemosensitivity of the sphere-forming cells were assessed. These cells displayed CSC properties by regenerating new tumor spheres in serum-free medium, overexpressing stem cell markers and showing a higher resistance to chemotherapeutic reagents, while these features were diminished under differentiating conditions. These results indicate that CSCs were enriched in these floating GBC spheres.

Cell surface markers of CSCs can help distinguish, isolate and purify these tumor-initiating cells for further biological investigation. The protein CD133 is cell surface marker for CSCs in brain tumor[11], Ewing's sarcoma[15] and liver cancers[26]. The development and differentiation of human bile ducts and liver are closely related; both start from hepatic endodermal cells and hepatoblasts just after liver primordium formation. We therefore selected CD133 as a potential CSC marker in the current study, and detected its expression in primary GBC and in sphere-forming cells. CD133$^+$ cells comprised a small fraction of the total tumor population in all three samples studied, but represented an increased percentage of the sphere-forming cells. This suggests that CD133 could act as a cell surface marker for CSCs in GBC. We also investigated the use of this cell surface protein as a candidate marker to further identify the CSC phenotype in GBC. The self-renewal ability of CD133$^+$ cells was tested using spheroid-forming assays in serum-free medium. CD133$^+$ cells possessed higher clonogenicity than their antigen-negative counterparts. Subsequent *in vivo* tumorigenesis experiments demonstrated that CD133$^+$ cells possessed higher tumorigenicity than the CD133$^-$ subpopulation. Furthermore, the tumors generated in nude mice displayed the same phenotype as the primary GBC tissue. Taken together, these results firmly suggest that CD133$^+$ cells possess the potentials for self-renewal and high tumorigenicity, exhibiting cancer stem-cell-like characteristics in human GBC.

The internal relationship between the expression of CD133 and the characteristics of CSCs remains unclear. Previous studies suggested that CD133 expression was associated with cell motility in melanoma[27] and colorectal cancer cells[28], and a high level of CD133 was also associated with increased resistance to staurosporine-inducing apoptosis[28]. These associations may be due to the interaction between CD133 and the canonical Wnt pathway[27]. However, the role of CD133 in these biological activities remains to be further clarified.

In summary, the results of this study demonstrate that CSCs are enriched in non-adherent spheres derived from GBC cells, and that CD133 protein may represent a cell surface marker for this cell population.

ACKNOWLEDGMENTS

We thank Ying Xiang from Organ Transplantation Institute of Tongji Hospital, Tongji Medical College, Hua-

zhong University of Science and Technology, Wuhan, China, for her technical assistance.

COMMENTS

Background

Gallbladder carcinoma (GBC) is the most common malignant neoplasm of the biliary tract and the seventh most common gastrointestinal cancer. Emerging evidence has shown that the abilities for tumor growth and propagation reside in a small population of tumor cells, termed cancer stem cells (CSCs) or tumor-initiating cells.

Research frontiers

Tumor-initiating cells with distinct cell surface markers have recently been identified in various solid tumors. In this study, the authors demonstrate that primary human GBC cells also contain tumor-initiating cells and that CD133 protein may be a cell surface marker for this cell population.

Innovations and breakthroughs

Previous studies have suggested that CD133 expression is associated with cell motility and is a cell surface marker for tumor-initiating cells in some tumors. This is the first study to report upregulation of CD133 in spheroids derived from primary human GBC cells. Furthermore, the *in vitro* and *in vivo* studies suggest that CD133 protein may represent a cell surface marker for CSCs in GBC.

Applications

This study may provide a novel approach to the diagnosis and treatment of GBC.

Peer review

The presented manuscript deals with extremely thrilling issue of cancer development. It should be of great interest for the readers reflecting progress in our comprehension of carcinogenesis.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2972

World J Gastroenterol 2011 June 28; 17(24): 2972-2975
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report

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Received: December 4, 2010 Revised: February 9, 2011
Accepted: February 16, 2011
Published online: June 28, 2011

Abstract

Chronic intestinal pseudo-obstruction (CIPO) is a rare disease due to a severe gastrointestinal motility disorder which may mimic, on both clinical and radiological grounds, mechanical obstruction. We report a case of a 26-year-old woman who presented to our institution for plain abdominal radiography for referred long-lasting constipation with recurrent episodes of abdominal pain and distension. At X-ray, performed both in the upright and supine position, an isolated air-fluid level was depicted in the left flank, together with a number of radiological signs suggestive of pneumoperitoneum. First, subphrenic radiolucency could be observed in the upright film. Second, the intestinal wall of some jejunal loops appeared to be outlined in the right flank. Third, the inferior cardiac border was clearly depicted in the upright film. The patient however had no evidence of peritoneal signs but only hypoactive bowel movements. Unenhanced multi-detector computed tomography (MDCT) of the abdomen and pelvis was therefore performed. MDCT revealed abnormal air-driven distension of the small and large bowel, without evidence of extra-luminal air. All radiological signs of pneumoperitoneum turned out to be false-positive results. The patient was submitted to pan-colonoscopy and to anorectal manometry to rule out Hirshprung's disease, and was finally discharged with a diagnosis of CIPO.

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Key words: Pseudopneumoperitoneum; Abdominal radiography; Multi-detector computed tomography; Motility disorders; Chronic intestinal pseudo-obstruction

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Camera L, Calabrese M, Sarnelli G, Longobardi M, Rocco A, Cuomo R, Salvatore M. Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report. *World J Gastroenterol* 2011; 17(24): 2972-2975 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2972.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2972

INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is a rare motility disorder of the gastrointestinal (GI) tract that is usually observed in a number of different neuropathies, mesenchymopathies or myopathies, but it can also be idiopathic[1]. It is characterized by failure of the GI tract to propel its content and may result in a clinical picture mimicking mechanical obstruction, with patients complaining of recurrent episodes of abdominal distension,

with or without abdominal pain, nausea and vomiting[2].

The diagnosis of CIPO is mainly clinical and is usually postulated after exclusion of any organic lesion occluding the gut lumen at endoscopy, while it can be supported by radiographic documentation of dilated small and/or large bowel loops, without evidence of a definite transition zone. This, in adults, is now best accomplished by multi-detector computed tomography (MDCT), which can obviate the need for unnecessary laparotomy[3]. However, plain abdominal films are one of the most important examinations in the diagnosis of CIPO in which abnormal air-driven distension of both small and large bowel is usually depicted[4].

Here, we report a case of a 26-year-old woman who presented to our institution for plain abdominal radiography as part of clinical and radiological work-up for referred long-lasting constipation with recurrent episodes of abdominal pain and distension. At X-ray, an isolated air-fluid level could be observed in the left flank region, together with a number of radiological signs suggestive of pneumoperitoneum. These could be appreciated both in the upright and supine films, but turned out to be false-positive results, as an unenhanced MDCT scan failed to show evidence of extra-luminal air in the peritoneal cavity.

CASE REPORT

A 26-year-old woman with a history of recurrent episodes of abdominal pain and distension came to our institution to undergo plain abdominal radiography. Abdominal plain films were obtained both in the upright (Figure 1A) and supine (Figure 1B) position. The upright film showed an isolated air-fluid level in the left flank region, consistent with an obstruction of the descending colon, together with a number of X-ray findings suggestive of pneumoperitoneum. First, subphrenic radiolucencies were clearly depicted on both sides in the upright film. Second, the inferior cardiac border could be observed from the cardiac apex to the inferior vena cava. Third, the intestinal wall of some jejunal loops appeared to be outlined in the right flank (Figure 1A). This latter finding was also evident in the supine film, configuring the so-called bas-relief or Rigler's sign together with the lack of normal hepatic shadow in the right subphrenic space, the so-called hyperlucent liver sign (Figure 1B). Based on these X-ray findings, the on-call radiologist immediately contacted the referring physician to communicate a diagnosis of pneumoperitoneum.

At physical examination, however, only a large abdominal distension with hypoactive bowel could be observed, but no peritoneal signs. It was therefore agreed upon to perform MDCT (Aquilion 4; Toshiba, Japan). This was performed with a detector configuration of 4 × 5 mm, table speed 30 mm/s, rotation time = 0.5 s, beam pitch = 0.75, 120 kVp, 250 mA. Only unenhanced acquisition was performed. The MDCT scan showed marked dilation of the large bowel, with both hepatic and splenic flexures displaced underneath the diaphragm (Figure 2A). Air-driven distension involved both small (Figure 2B) and large bowel (Figure 2C) loops. There was no evidence of any obstructive lesion and both the sigmoid colon and rectum were normally filled with feces (Figure 2D). No evidence of free air in the peritoneal cavity was found, therefore, a diagnosis of pneumoperitoneum could not be confirmed.

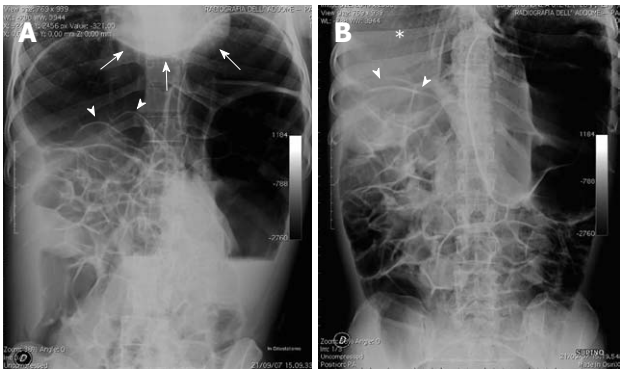

Figure 1 Upright (A) and supine (B) films in a female patient complaining of long-lasting constipation, with recurrent episodes of abdominal pain and distension. A: An isolated air-fluid level is depicted in the left flank, suggesting mechanical obstruction at the level of the descending colon. Subphrenic radiolucency is evident on both sides, together with the outlining of the intestinal wall of some small bowel loops in the right flank (arrowheads) and that of the inferior cardiac border (arrows); B: Outline of the intestinal wall of small bowel loops appears to be depicted in the right flank (arrowheads), configuring the so-called bas-relief or Rigler's sign. In addition, hyperlucency is depicted in the right subphrenic space in place of the normal hepatic shadow, configuring the bright or hyperlucent liver sign (*).

Further clinical, biochemical and instrumental investigation involving pan-colonoscopy and anorectal manometry revealed no underlying causes, and the patient was finally discharged with a diagnosis of CIPO.

DISCUSSION

Pneumoperitoneum is usually referred to as the presence of free air within the peritoneal cavity. Its radiological diagnosis usually relies on the evidence of typical subphrenic radiolucency in the posterior-anterior projection taken in the upright position[5]. As a result, the term pseudopneumoperitoneum is used when the subphrenic radiolucency does not correspond to free intraperitoneal air[6], but can be traced to either subphrenic fat pad[7] or basal lung atelectasis[8]. In these cases, the clarifying role of CT has been acknowledged[9].

To the best of our knowledge, whereas the possible occurrence of pneumoperitoneum in CIPO has been described[10], the occurrence of pseudopneumoperitoneum in CIPO has not been reported to date. In our case, subphrenic radiolucency depicted in the upright film (Figure 1A) was due to hepatic and splenic colonic flexures abnormally dilated and displaced below the diaphragmatic shadows, as shown by CT (Figure 2A).

In the emergency setting, prompt recognition of radiological signs of pneumoperitoneum in the anterior-

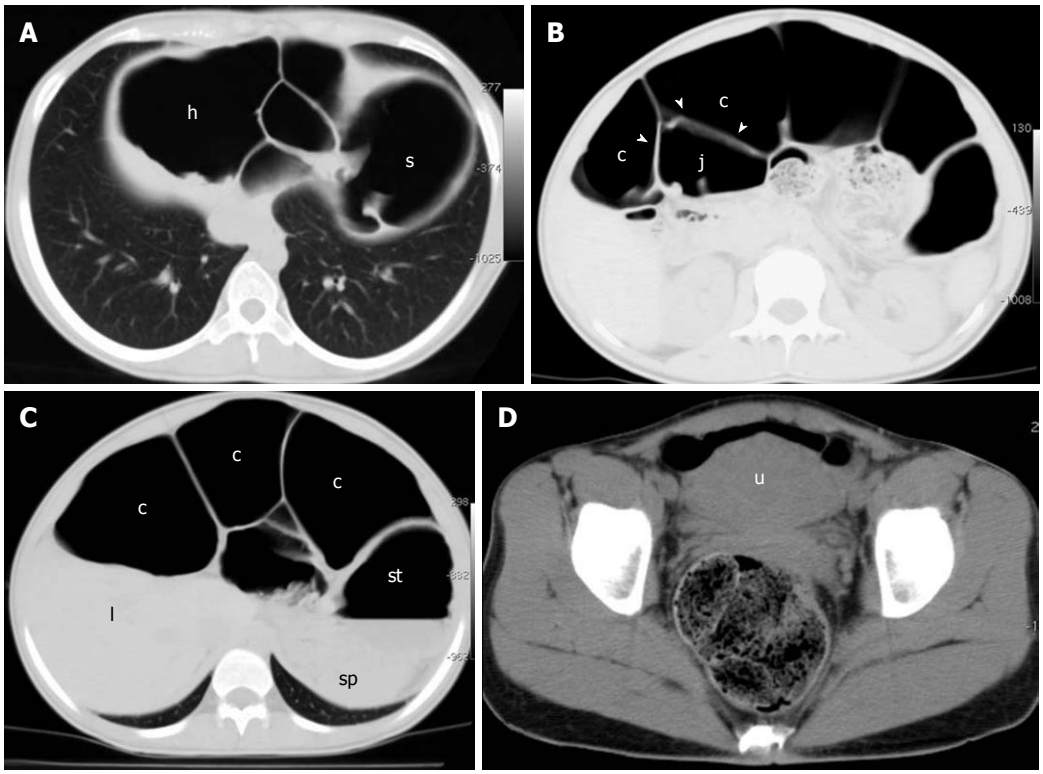

Figure 2 Unenhanced four-row MDCT. Images are displayed with lung (A-C) and soft tissue (D) windows. A: Hepatic (h) and splenic (s) flexures, air-filled and abnormally dilated, are displaced under the diaphragm, accounting for the subphrenic radiolucency depicted in the upright film; B: Intraluminal air in the large bowel (c) appears to outline the intestinal wall (arrowheads) of a jejunal loop (j) that is also mildly dilated and air-filled, accounting for the Rigler's sign depicted on the upright and supine films; C: Abnormally dilated colonic segment situated between the liver (l) and anterior abdominal wall, simulating the hyperlucent (bright) liver sign depicted in the supine film; st: Stomach; sp: Spleen; D: Rectum is normally filled with feces. u: Uterus.

posterior projection taken in the supine position may be of diagnostic value because it allows a confident diagnosis of pneumoperitoneum whenever an L-L projection is not available or cannot be performed[11].

These radiological signs, which can be mostly traced to either the intestinal wall[12] or various peritoneal folds[13-15], outlined by the presence of free air within the peritoneal cavity, have been recently revised and classified into four major categories as bowel-related signs, right upper quadrant signs, peritoneal ligament signs, and other signs[16].

For at least one of these signs, namely the Rigler's sign, the possible occurrence of false-positive cases is well acknowledged. Rigler's sign can be simulated by two contiguous, moderately dilated and air-filled loops of bowel, whereby intraluminal air in one loop of bowel may appear to outline the wall of the adjacent loop[17]. These false-positives cases, however, have only been reported in the supine film where the sign itself was originally described[12]. To the best of our knowledge, the occurrence of a false-positive Rigler's sign in the upright position has never been described. As shown by MDCT, the intestinal wall of some jejunal loops was in our case outlined by intraluminal air in the large bowel, which was abnormally dilated (Figure 2B). The anomalous dislocation of jejunal loops in the right flank was not further investigated, although it was likely due to concomitant malrotation.

Possible occurrence of false-positive results for the inferior cardiac border sign were also postulated in the original description. The inferior border of the heart becomes visible on supine radiography whenever it is outlined by air; either free air in the peritoneal cavity, such as in pneumoperitoneum, or air within the pleural and/or pericardial sac. Occasionally, gas-filled loops collected beneath the left hemi-diaphragm may simulate this sign[18]. As with Rigler's sign, these false-positive signs are also expected to occur in the supine position and not in the upright. In our case, however, the sign could not be detected in the supine film because of suboptimal technical positioning (Figure 1B).

Finally, the hyperlucent liver sign which could be observed on the supine film (Figure 1B) also turned out to be a false-positive result because it was clearly due to the interposition of dilated, air-filled large bowel loops between the liver and the anterior abdominal wall, as shown by MDCT (Figure 2C). To the best of our knowledge, false-positive cases of hyperlucent liver sign have never been reported.

In the present case, MDCT not only ruled out the radiological diagnosis of pneumoperitoneum but it also excluded mechanical obstruction, and showed that the sigmoid colon and rectum were normally filled with feces, without evidence of a transition zone (Figure 2D). As this latter CT finding is rather atypical in patients with Hirschsprung's disease[19], this was also excluded by anorectal manometry, which revealed a normal inhibitory

rectoanal reflex[20]. The patient was therefore discharged with a diagnosis of CIPO.

We have described a case of pseudopneumoperitoneum in a patient with CIPO. All radiological findings of pneumoperitoneum that were clearly depicted in the upright and supine films turned out to be false-positive results, as shown by MDCT. To the best of our knowledge, such a case has not been reported to date.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2976

World J Gastroenterol 2011 June 28; 17(24): 2976
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Hepatocellular carcinoma and industrial epidemics

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Received: September 1, 2010 Revised: January 17, 2011
Accepted: January 24, 2011
Published online: June 28, 2011

Abstract

Worldwide, the burden of the non viral causes of hepatocellular carcinoma (HCC) is usually underestimated. Clearly industrial goods, tobacco, alcohol and processed foods are the agents of new epidemics in modern times which far outscore the burden of infectious agents on morbidity and mortality. Smoking, a dose-related contributing factor for HCC, receives too little attention in clinical practice. In France, tobacco, hepatitis B and C virus and alcohol are the main risk factors for HCC mortality (33%, 31% and 26%, respectively). In developing countries, where tobacco consumption is dramatically increasing, this epidemic may soon surpass hepatitis B. Obesity and diabetes are the contributing factors too. The role of industrial processed foods in the increase of the prevalence of obesity and diabetes cannot be ignored.

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Key words: Hepatocellular carcinoma; Tobacco; Alcohol; Processed foods; Industrial epidemics

Peer reviewer: Dr. Ajith TA, Associate Professor, Department of Biochemistry, Amala Institute of Medical Sciences, Amala Nagar, Thrisssur, Kerala-680 555, Pakistan

Braillon A, Dubois G. Hepatocellular carcinoma and industrial epidemics. *World J Gastroenterol* 2011; 17(24): 2976 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2976.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2976

TO THE EDITOR

Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death. The Journal must be acknowledged for the publication of Blonski *et al*[1] review who wisely underlined the burden of non viral causes of HCC which are usually underestimated.

Blonski *et al*[1] rightly stressed the role of smoking, a dose related contributing factor for HCC, and this is important because some are still ignoring it[2]. In France, tobacco, hepatitis B and C virus and alcohol are the main risk factors for HCC mortality (33%, 31% and 26%, respectively)[3,4].

Blonski *et al*[1] also listed obesity and diabetes as contributing factors for HCC. The role of industrial processed foods in the increase of the prevalence of obesity and diabetes cannot be ignored.

Clearly industrial goods, tobacco, alcohol and processed foods are the agents of new epidemics in modern times which far outscore the burden of infectious agents on morbidity and mortality.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 June 28; 17(24): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 June 28; 17(24): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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, Venise, 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 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 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 J Gastroenterol 2011 June 28; 17(24): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Name of journal

World Journal of Gastroenterology

ISSN and EISSN
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, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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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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